Supporting Information

Synthesis and Preliminary Evaluation of Octreotate Conjugates of Bioactive Synthetic Amatoxins for Targeting Somatostatin Receptor (Sstr2) Expressing Cells.

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Materials and Methods

General Information. Reactions were performed in flame-dried borosilicate round-bottom flasks fitted with a rubber septum under a positive pressure of Ar unless otherwise noted. Air/moisture sensitive liquids and solutions were transferred via syringe under positive Ar pressure. Controlled temperature reactions were performed using a mineral oil bath and a temperature-controlled hot plate (Corning, PC 420D). Analytical thin layer chromatography (TLC) was performed using pre-coated Merck aluminum-backed silica gel plates (Silica gel 60 F254). Visualization was achieved using ultraviolet light (254 nm) and chemical staining with silica gel impregnated with iodine, *p*-anisaldehyde, potassium permanganate, bromocresol green, and ninhydrin as appropriate. Flash column chromatography purification was performed using silica gel 60 (230-400 mesh, Silicycle, Quebec, Canada and 230-400 mesh, high-purity grade (9385), Sigma Aldrich, Germany). Prior to use, silica gel was washed with four volumes of ammonium hydroxide/dichloromethane/ethanol (0.5:4:4.5) solution, filtered, and baked to neutralize small amounts of acid on silica gel. Solvents were dried according to standard methods.[1] Reagents and solvents were purchased from Sigma-Aldrich, Novabiochem, Alfa Aesar, Acros Organics, AK Scientific Inc., Oakwood Chemical, TCI America and used without further purification unless noted otherwise. Pyridine was stirred with 4-toluenesulfonyl chloride (~2 g for 50 mL of pyridine) for 20 hours to quench any amine impurities, vacuum distilled, and stored under Ar(g). Sep-Pak C18 cartridges were purchased from Waters (Sep-Pak C18 35 cc Vac Cartridge, 10 g, 55-105 μm; Sep-Pak C18 12 cc Vac Cartridge, 2 g, 55-105 μm).

Instrumentation. ^{1}H, and ^{13}C NMR spectra were recorded on Bruker Avance 300 (300 MHz), Bruker Avance 400dir (400 MHz), and Bruker Avance 400inv (400 MHz) spectrometers. Chemical shifts (δ) are reported in ppm and referenced to the appropriate residual solvent peaks (acetone-D_6, CD_3CN, CD_2Cl_2, or $CDCl_3$). Low-resolution mass spectrometry (LRMS) in Electrospray Ionization (ESI) mode was obtained using Waters ZQ mass spectrometer equipped with ESCI ion source and Waters 2695 HPLC. High-resolution mass spectrometry (HRMS) in ESI mode was obtained using Water/Micromass LCT-TOF mass spectrometer equipped with an ESI ion source. MALDI-TOF was obtained on Bruker Autoflex MALDI-TOF I either linear or reflectron mode. UV-Vis spectroscopy was performed using Cary5000 Spectrophotometer. Tryptathionine containing compounds were quantified using tryptathionine as a chromophore with the extinction coefficient of 10,000 $M^{-1}cm^{-1}$ at λ_{max} (in MeOH or H_2O). Typically, quantities >5 nmol are accurately quantified by UV-Vis spectroscopy using a 0.5-mL cuvette. All HPLC chromatograms were generated on an Agilent 1100 system equipped with an auto-injector and a diode array detector. Analytical injections were performed using an Agilent Eclipse XDB C-18, 5 μm (4.6 x 250 mm) column with a flow rate of 2 mL/min.

List of HPLC Methods

HPLC **gradient A**: 2ml/min; solv. A = 0.1 % FA H_2O and solv. B = 0.1% FA ACN.

Time (min)	% B (0.1 % FA ACN)
0	5
18	50
21	100
26	100
31	5

HPLC **gradient B**: 2ml/min; solv. A = 0.1 % FA H_2O and solv. B = 0.1% FA ACN.

Time (min)	% B (0.1 % FA ACN)
0	32
3	45
17	60
21	70
22	100
26	100
28	32
32	32

HPLC **gradient C**: 2ml/min; solv. A = 0.1 % FA H_2O and solv. B = 0.1% FA ACN.

Time (min)	% B (0.1 % FA ACN)
0	40
18	60
22	100
25	100
27	40
31	40

HPLC **gradient D**: 2ml/min; solv. A = 0.1 % TFA H_2O and solv. B = 0.1% TFA ACN.

Time (min)	% B (0.1 % TFA ACN)
0	5
18	50

21	100
26	100
31	5
36	5

HPLC **gradient E**: 2ml/min; solv. A = 0.1 % FA H_2O and solv. B = 0.1% FA ACN.

Time (min)	% B (0.1 % FA ACN)
0	35
17	55
21	100
26	100
33	5

HPLC **gradient F**: 2ml/min; solv. A = 0.1 % FA H_2O and solv. B = 0.1% FA ACN.

Time (min)	% B (0.1 % FA ACN)
0	18
21	40
26	100
30	100
32	5

HPLC **gradient G**: 2ml/min; solv. A = 0.1 % FA H_2O and solv. B = 0.1% FA ACN.

Time (min)	%B (0.1% FA ACN)
0	8
18	40
22	42
24	100
28	100
31	8
35	8

HPLC **gradient H**: 2ml/min; solv. A = 0.1 % FA H_2O and solv. B = 0.1% FA ACN.

Time (min)	% B (0.1 % FA ACN)
0	10
18	70
21	100
26	100
29	10

HPLC **gradient I**: 2ml/min; solv. A = 0.1 % TFA H_2O and solv. B = 0.1% TFA ACN.

Time (min)	% B (0.1 % TFA ACN)
0	18
21	40
26	100
30	100
32	5

HPLC **gradient J**: 2ml/min; solv. A = 0.1 % TFA H_2O and solv. B = 0.1% TFA ACN.

Time (min)	% B (0.1 % TFA ACN)
0	25
21	34
26	100
30	100
32	5

HPLC **gradient K**: 2ml/min; solv. A = 0.1 % FA H_2O and solv. B = 0.1% FA ACN.

Time (min)	% B (0.1 % FA ACN)
0	8
21	40
23	50
25	100
30	100
32	8
35	8

HPLC **gradient L**: 2ml/min; solv. A = 5 mM phosphate buffer and solv. B = ACN.

Time (min)	% B (ACN)
0	5
15	32
19	35
22	5
25	5

Cell culture

Materials and methods: MEM_{α} and F-12K cell culture media, fetal bovine serum (FBS), 0.25% trypsin (with 1.3 mM EDTA), 0.85% Trypan blue, and the antibiotic mixture Pen/Strep (10K U/mL penicillin, 10K mg/mL streptomycin) were purchased from Gibco. Cell culture plasticware and poly-D-lysine coated glass-bottom dishes were obtained from Corning and Falcon. Cells were cultured at 37 °C in a humidified chamber with 5% CO_2. CellTiter 96® AQ_{ueous} One Solution cell proliferation assay (MTS) reagent was purchased from Promega. Absorbance measurements of the 96-well plates were obtained using a Beckman-Coulter DTX 880 multimode detector, equipped with an excitation filter of 490 nm. DMSO was purified by filtration through a 0.2 mm filter. All experiments are carried out in a laminar flow culture cabinet unless otherwise noted.

Rat pancreatic carcinoma Ar42J cells (ATCC CRL-1492) were cultured in F-12K medium supplemented with 20% (v/v) fetal bovine serum, 1 mM glutamine, 100 U/mL penicillin and 10 mg/mL streptomycin in a T-25 flask at 37 °C and 5% CO_2. A sub-cultivation ratio of 1:3 was used to split cells at the point of 70-80% confluency.

Chinese hamster ovary (CHO) cells (ATCC CCL-61) were cultured in MEM_{α} medium supplemented with 10% (v/v) fetal bovine serum and 100 U/mL penicillin and 10 mg/mL streptomycin in a T-25 flask at 37 °C and 5% CO_2. A sub-cultivation ratio of 1:5 was used to split cells at the point of 70-80% confluency.

In vitro cell proliferation assay

To assay cell viability, Ar42J or CHO cells were trypsinized and seeded onto 96-well plates (5,000 cells/well for Ar42J and 3,000 cells/well for CHO). The cells were allowed to form monolayers for 24 hours. The compound analyzed (α-amanitin, amanitin-Prg, 5'-OH-amanitin, amanitin-NH_2, TATE-PEG-Ama, TATE-SS-Ama, TATE-N_3 or TATE-VCit-Ama) was then added using 0.25% DMSO in F-12K Medium. Media control and 0.25% DMSO with media control were run in parallel and compound readings were normalized according to these controls. The cells were incubated at 37°C for 72 hours in a humidified, 5% CO_2 atmosphere. Following a 72-h period, 20 µL of MTS reagent were added to each well and the cells were incubated for another 3 hours at 37°C in a humidified, 5% CO_2 atmosphere. The absorbance reading was recorded at 490 nm using a 96-well plate reader. IC_{50} and EC_{50} values were calculated using GraphPad Prism 7 software with non-linear 3-parameter regression fit to the following equation: Y=Bottom + (Top-Bottom)/(1+10^((X-LogIC50))) with Hill slope of -1.0.

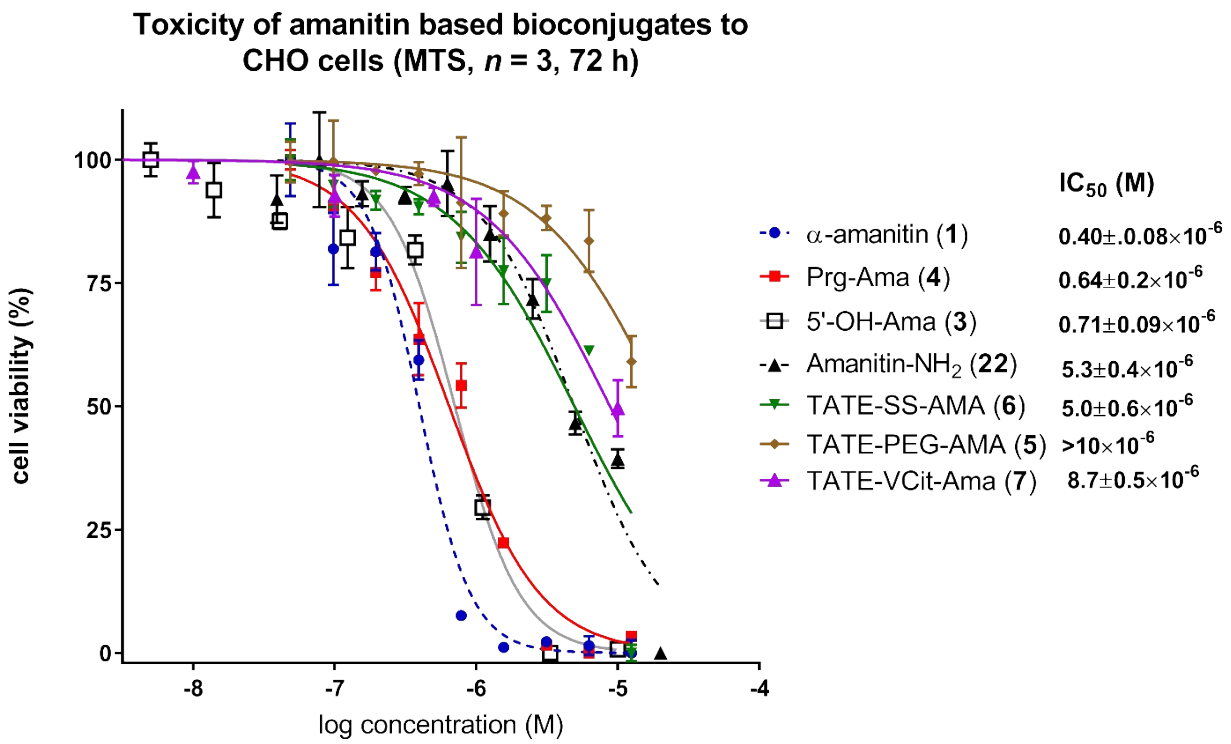

Figure S1. MTS viability assay of octreotate-amanitin bioconjugates on sstr2-negative CHO cell line.

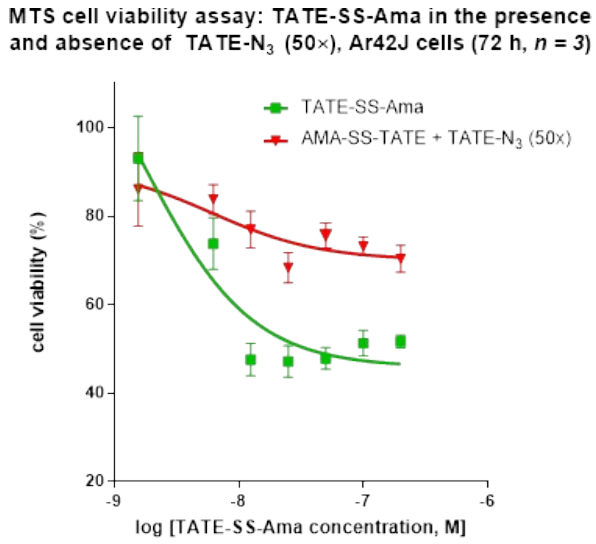

Figure S2. Blocking assay: MTS viability assay of octreotate-amanitin bioconjugates in the presence and absence of TATE-N3 on sstr2-positive Ar42J cell line.

Cell death kinetics of bioconjugate TATE-SS-Ama **6** and α-amanitin **1**

To study the cell death kinetics of bioconjugate TATE-SS-Ama **6** and α-amanitin **1**, Ar42J cells were trypsinized and seeded onto 96-well plates (10,000 cells/well for Ar42J). The cells were allowed to form monolayers for 24 hours. TATE-SS-Ama and α-amanitin were dissolved in 0.5% DMSO F-12K medium and added to the wells to achieve 62 nM and 10 μM concentrations, respectively. Media control and 0.5% DMSO media control were run in parallel and compound readings were normalized according to these controls. The cells were incubated at 37°C for 24 and 48 hours in a humidified, 5% CO_2 atmosphere. Following a 24-h and a 48-h period, 20 μL of MTS reagent were added to each well and the cells were incubated for another 3 hours at 37°C in a humidified, 5% CO_2 atmosphere. The absorbance reading was recorded at 490 nm using a 96-well plate reader and the experiment was run in triplicate (n = 3).

Fluorescence microscopy studies

Blocking Experiment

Ar42J cells were trypsinized and seeded onto poly-D-lysine coated glass-bottom dishes (20,000 cells/plate). The cells were allowed to form monolayers for 24 hours. TATE-SS-Ama was then added to half of the plates using 0.25% DMSO in F-12K medium to a final concentration of 250 nM and the cells were incubated at 37°C for 30 mins in a humidified, 5% CO_2 atmosphere. Following this, TATE-SS-Rhod was added to all plates to a final concentration of 5 nM. The cells were incubated at 37°C for 30 mins in a humidified, 5% CO_2 atmosphere and then washed three times with pH 7.4 PBS buffer. The fluorescent images were obtained using Olympus IX70 Inverted Fluorescence Microscope equipped with Olympus DP80 dual CCD 12.7 megapixel colour camera and 1.4 megapixel cooled monochrome camera. The microscope fitted with U-MNG2 fluorescence filter cube (excitation: 510-550 nm, emission: 590 nm, dichromatic mirror: 570 nm). Images were obtained at 20× magnification. Cells, where TATE-SS-Ama was used as a blocking agent (Figure S3, panel b), were only 5% as fluorescent as the cells stained with TATE-SS-Rhodamine as quantified with FIJI software.

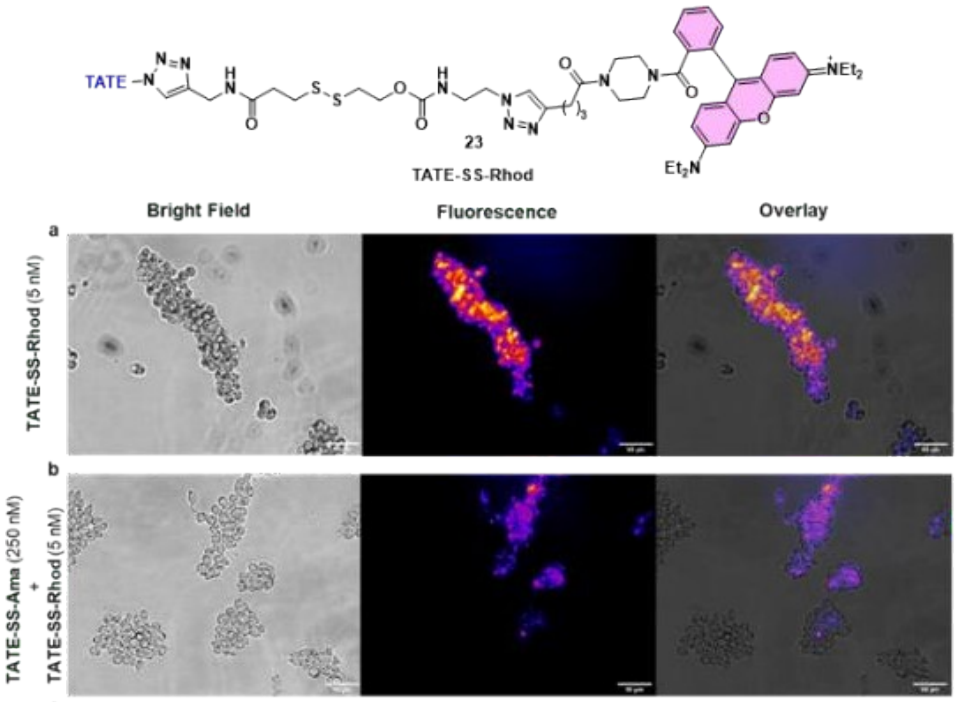

Figure S3. Fluorescence imaging of Ar42J cancer cells (sstr2-positive) using TATE-SS-Rhod **23**; a) cells were incubated with 5 nM TATE-SS-Rhod **23** for 30 mins - control; b) blocking - cells were pre-treated with 250 nM TATE-SS-Ama **6** (30 min) followed by incubation with 5 nM TATE-SS-Rhod **23** (30 min); quantification of fluorescence: cells, where TATE-SS-Ama was used as a blocking agent (panel b), were only 5% as fluorescent as the cells stained with TATE-SS-Rhodamine as quantified with FIJI software.

Microscope Settings used to acquire fluorescence images:

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Origin (Y)=0 µm
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Subarray=No
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Averaged Frames=No
Multi Channel Shift Correction=Off
Pseudo Color Mode=Off

Microscope Settings used to acquire bright field images:

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Subarray=No
Hot Pixel Correction=Yes
Color/Grayscale Centering=No
Photobleaching Correction=Off
Averaged Frames=No
Multi Channel Shift Correction=Off
Pseudo Color Mode=Off

Flow Cytometry analysis

Ar42J cells were trypsinized and seeded onto 12-well plates (100,000 cells/well). The cells were allowed to form a monolayers for 24 hours. TATE-SS-Ama was dissolved in 0.5% DMSO F-12K medium and added to the wells to achieve 30 and 60 nM concentration. The cells were incubated for 48, 72, 96 and 120 hours in a humidified, 5% CO_2 atmosphere at 37°C. Following a the specified time period,the cells were subjected to analysis by flow cytometry using anti-sstr2 TATE-PEG_2-FITC as a flurescent reporter. Both supernatant and cells were collected and pooled for each time point. Cells were trypsinized for 2 minutes to harvest and dissociate clumps. Cells were washed using ice cold buffer (PBS with 2%FBS and 0.05% Sodium Azide). Cells were incubated with 6 nM TATE-PEG_2-FITC (**S7**) on ice for one hour. Viability dye, 7AAD (Ebioscience, excitation 488 nm, emission 630 nm), was added in the last 10 minutes of the one hour incubation period. Cells were washed twice with buffer and then analyzed with a FACSCalibur (BD Biosciences) using FlowJo Software. Only 7AAD-negative cell population was analyzed for TATE-PEG_2-FITC binding (excitation 488 nm, emission 530 nm).

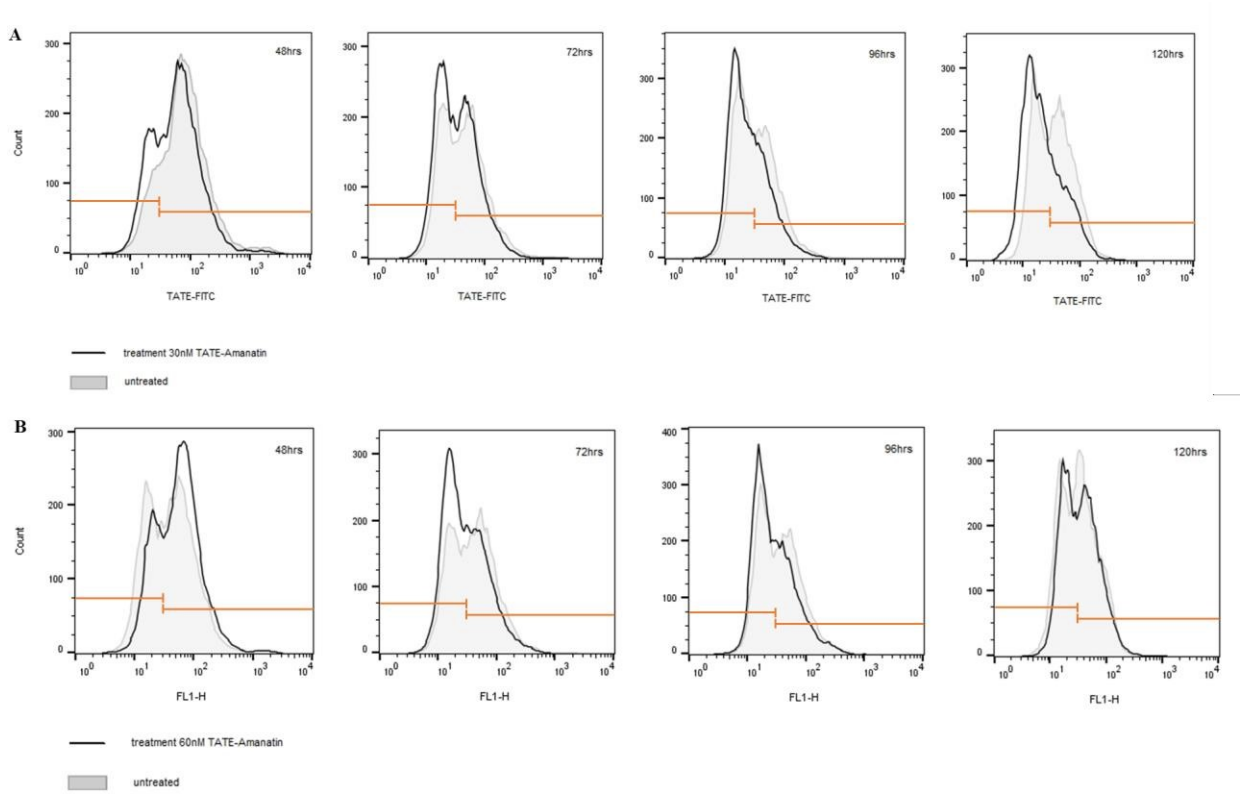
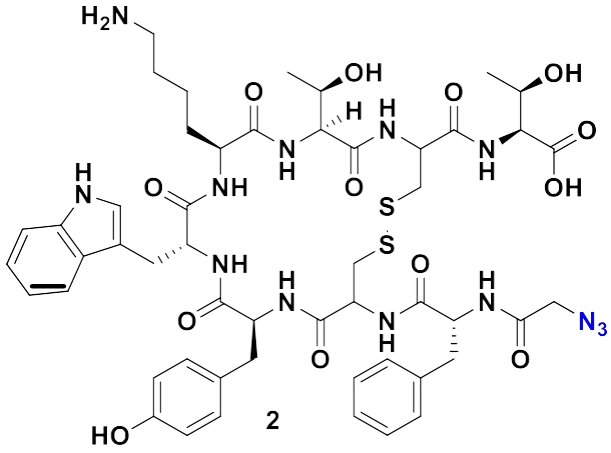
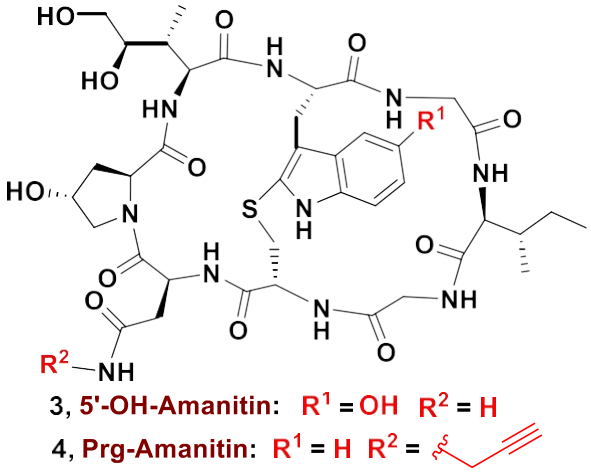


Figure S4. FACS scan of Ar42J cells that were untreated (gray) show a time-dependent increase in an sstr2-negative population from 48-120 h. Cells treated with (A) 30 nM and (B) 60 nM TATE-SS-Ama **6** show a similar effect (black).

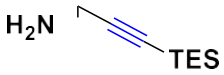
List of compound synthesized according to reported procedures accompanied by corresponding references



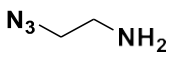
Compound **2** was synthesized according to the reported procedure.[2]



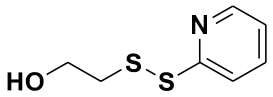
Compounds **3** and **4** were synthesized according to the reported procedures.[3-4]



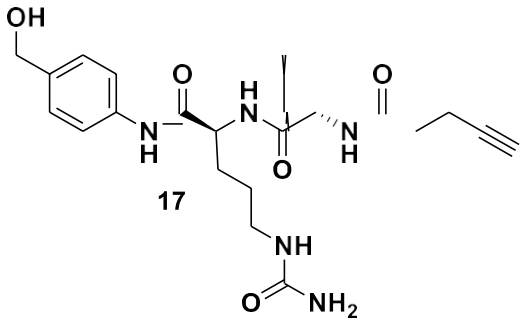
3-(Triethylsilyl)prop-2-yn-1-amine (**8**) was synthesized according to the reported procedure.[5]



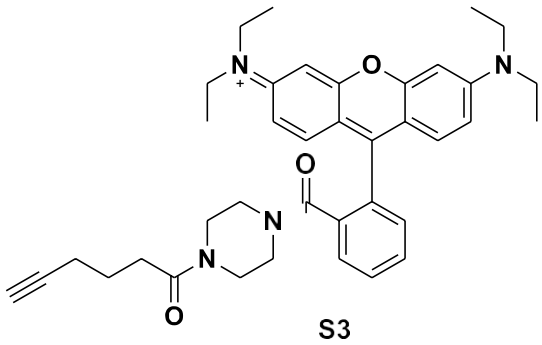
2-Azidoethylamine (**11**) was synthesized according to the reported procedure.[6]



2-(Pyridin-2-yldisulfaneyl)ethan-1-ol (**13**) was synthesized according to the reported procedure.[7]

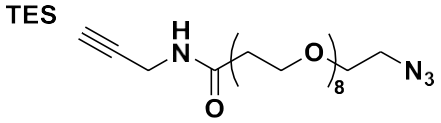


Compound **17** was synthesized according to the reported procedure.[8]



Compound **S3** was synthesized according to the reported procedure.[9]

Synthesis and characterization of compound **10**



Chemical Formula: $C_{28}H_{54}N_4O_9Si$
Exact Mass: 618.37
Molecular Weight: 618.84

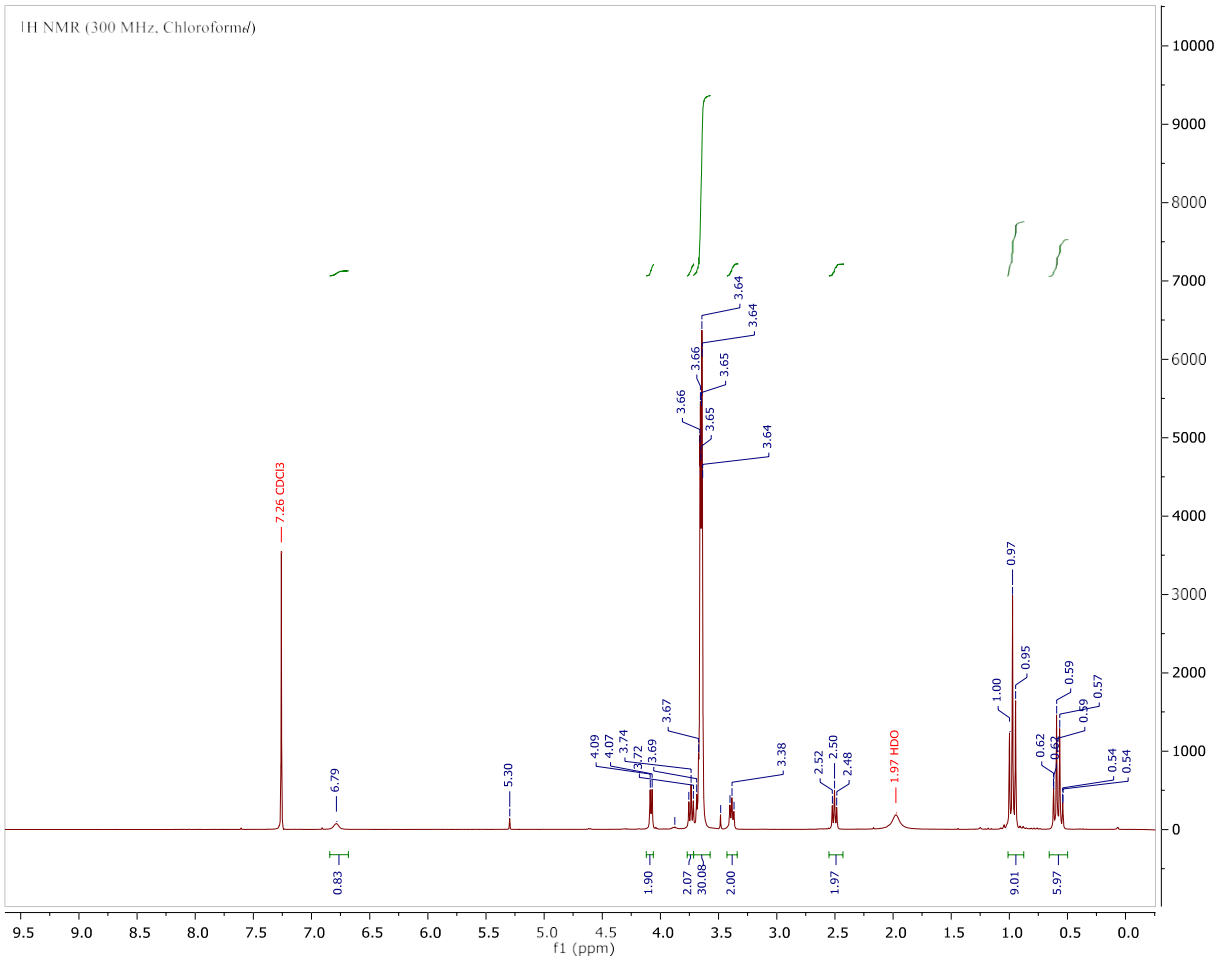
24-((l1-Oxidaneyl)methyl)-1-azido-N-(3-(triethylsilyl)prop-2-yn-1-yl)-3,6,9,12,15,18,21-heptaoxapentacosan-25-amide (10). Azido-PEG_8-NHS (1 eq., 5.0 mg, 8.86 µmol) was placed into a 1.5-mL Eppendorf tube charged with a stir-bar. TES-protected propargyl amine (5 eq., 7.50 mg, 44.3 µmol) dissolved in 130 µL of DCM was then added to the vessel followed by DIPEA (2.5 eq., 3.86 µL, 22.1 µmol). The reaction was run for 24 h at 21 °C and the progress was monitored by TLC and mass spectrometry. Upon completion, the clear and colourless reaction mixture was concentrated under reduced pressure. The concentrate was purified using silica gel flash column chromatography (gradient elution, dichloromethane to 6:94 methanol:dichloromethane) to obtain **10** (4.0 mg, 6.464 µmol) as a colourless clear oil in 73% isolated yield.

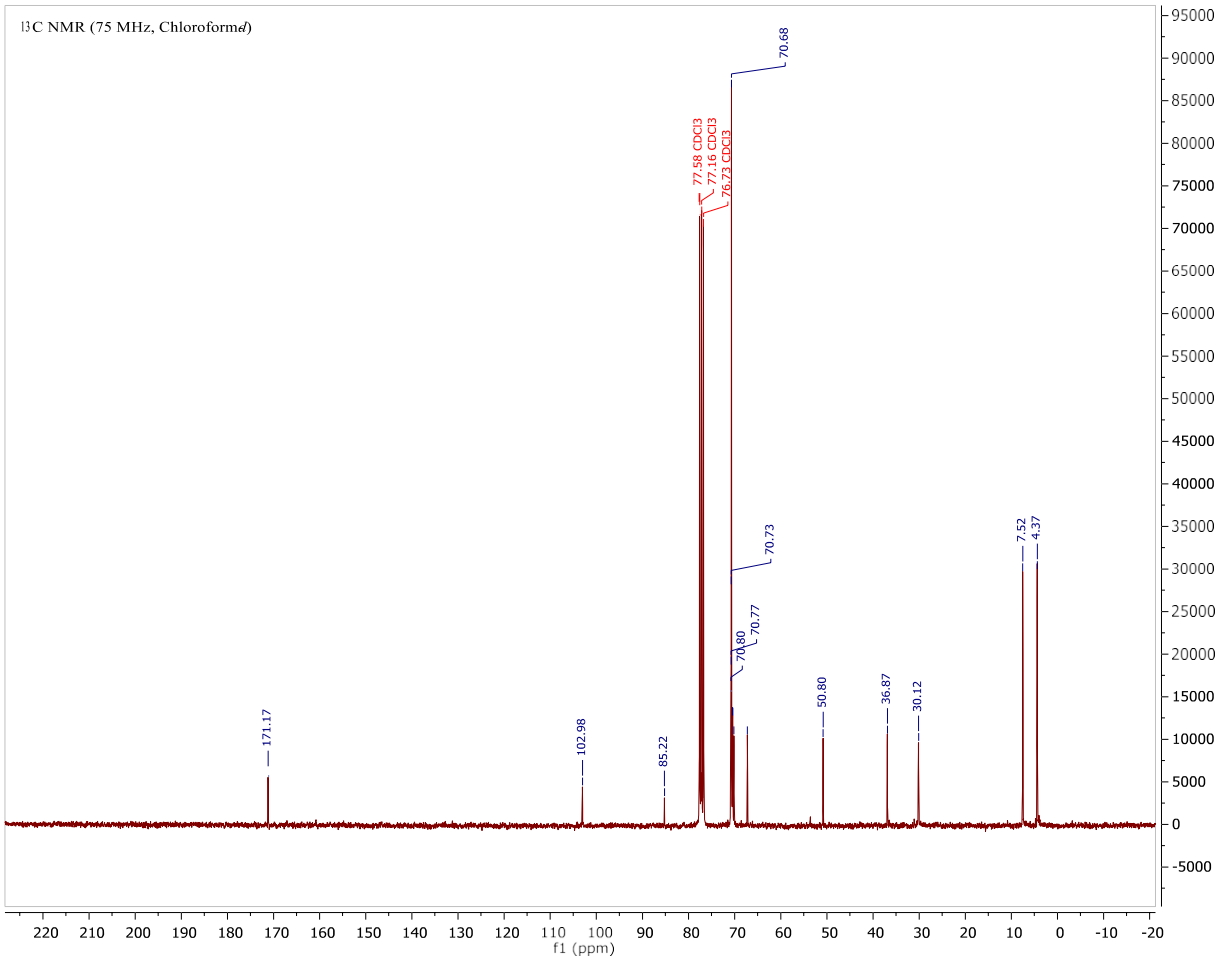
^{1}H NMR (300 MHz, Chloroform-*d*) δ 6.79 (s, 1H), 4.08 (d, *J* = 5.1 Hz, 2H), 3.74 (t, *J* = 5.7 Hz, 2H), 3.72 – 3.57 (m, 30H), 3.38 (t, *J* = 5.1 Hz, 2H), 2.50 (t, *J* = 5.7 Hz, 2H), 0.97 (t, *J* = 7.9 Hz, 9H), 0.66 – 0.50 (m, 6H).

^{13}C NMR (75 MHz, Chloroform-*d*) δ 4.37, 7.52, 30.12, 36.87, 50.80, 67.23, 70.14, 70.39, 70.45, 70.68, 70.73, 70.77, 70.80, 85.22, 102.98, 171.17.

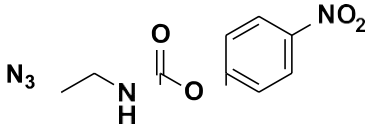
HRMS (ESI-TOF, m/z): $[M+H]^+$ found 619.3746; calc. 619.3738 for $C_{28}H_{55}N_4O_9Si$.

TLC (dichloromethane:methanol 90:10 v/v): R_f = 0.35 ($KMnO_4$, vanillin).


1H NMR (300 MHz, Chloroform-d)
7.26 CDCl3
6.79
5.30
4.09
4.07
3.74
3.72
3.69
3.67
3.66
3.65
3.64
3.38
2.52
2.50
2.48
1.97 HDO
1.00
0.97
0.95
0.62
0.59
0.57
0.54
0.83
1.90
2.07
30.08
2.00
1.97
9.01
5.97
f1 (ppm)


13C NMR (75 MHz, Chloroform-d)
70.68
77.58 CDCl3
77.16 CDCl3
76.73 CDCl3
70.73
70.80
70.77
171.17
102.98
85.22
50.80
36.87
30.12
7.52
4.37
f1 (ppm)

Synthesis and characterization of compound **12**



Chemical Formula: $C_9H_9N_5O_4$
Exact Mass: 251.07
Molecular Weight: 251.20

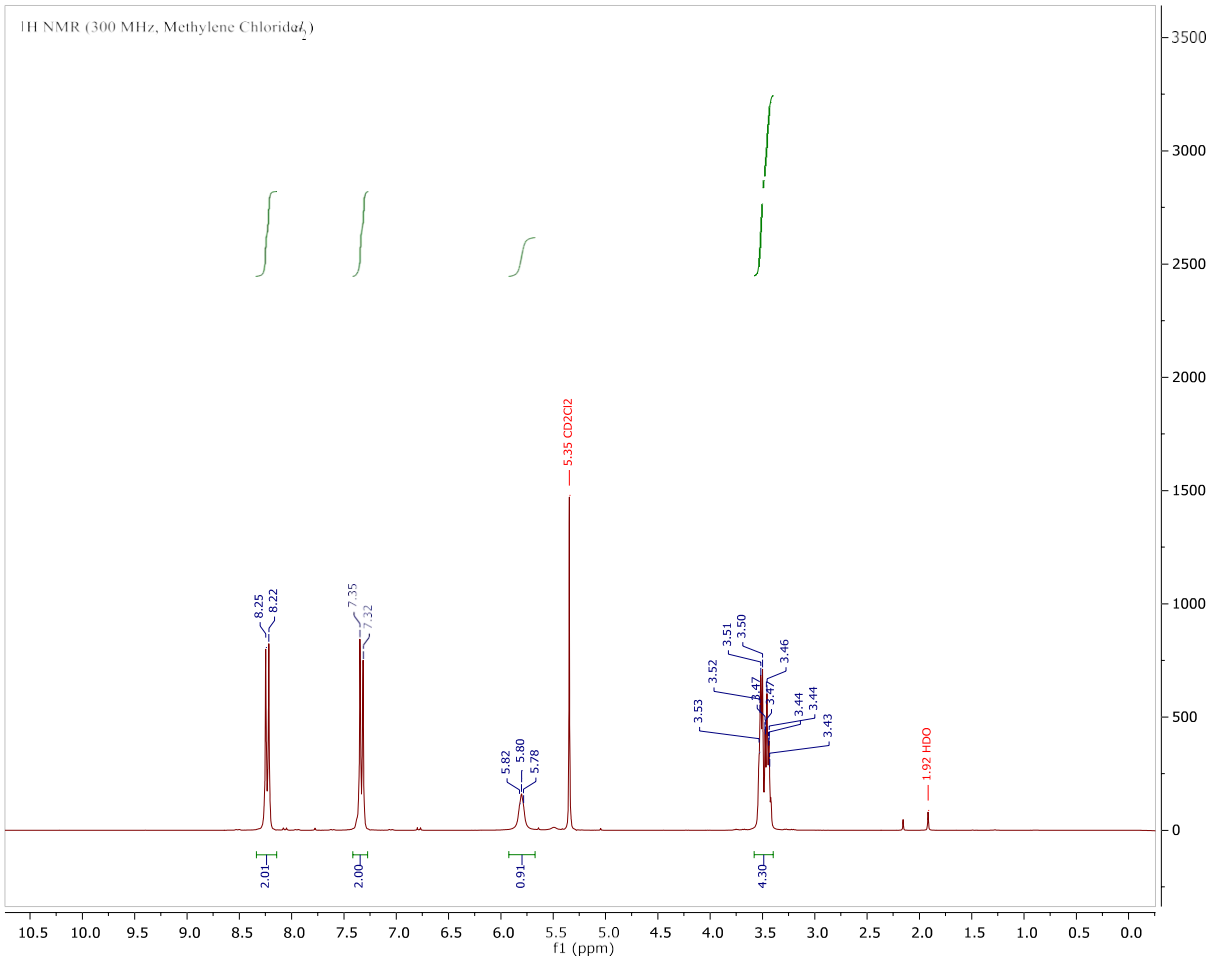
4-Nitrophenyl (2-azidoethyl)carbamate (12). A flame-dried round bottom flask under positive Ar atmosphere was charged with *p*-nitrophenyl chloroformate (1.05 eq., 3.323 g, 16.48 mmol) dissolved in DCM (35 mL) and cooled to 0 °C with an ice bath. Azidoethylamine (**11**) (1eq., 1.347g, 15.64 mmol) was then added to the reaction mixture followed by DMAP (1 eq., 1.910 g, 15.64 mmol). The reaction was run for 3 h at 0 °C and the progress was monitored by TLC. Upon completion, the clear yellow reaction mixture was concentrated under reduced pressure. The concentrate was purified using silica gel flash column chromatography (dry sample loading, gradient elution, 97:3 to 85:15 ethyl acetate:dichloromethane) to obtain 3.26 g (12.98 mmol) of **12** as a beige crystalline solid in 83% isolated yield.

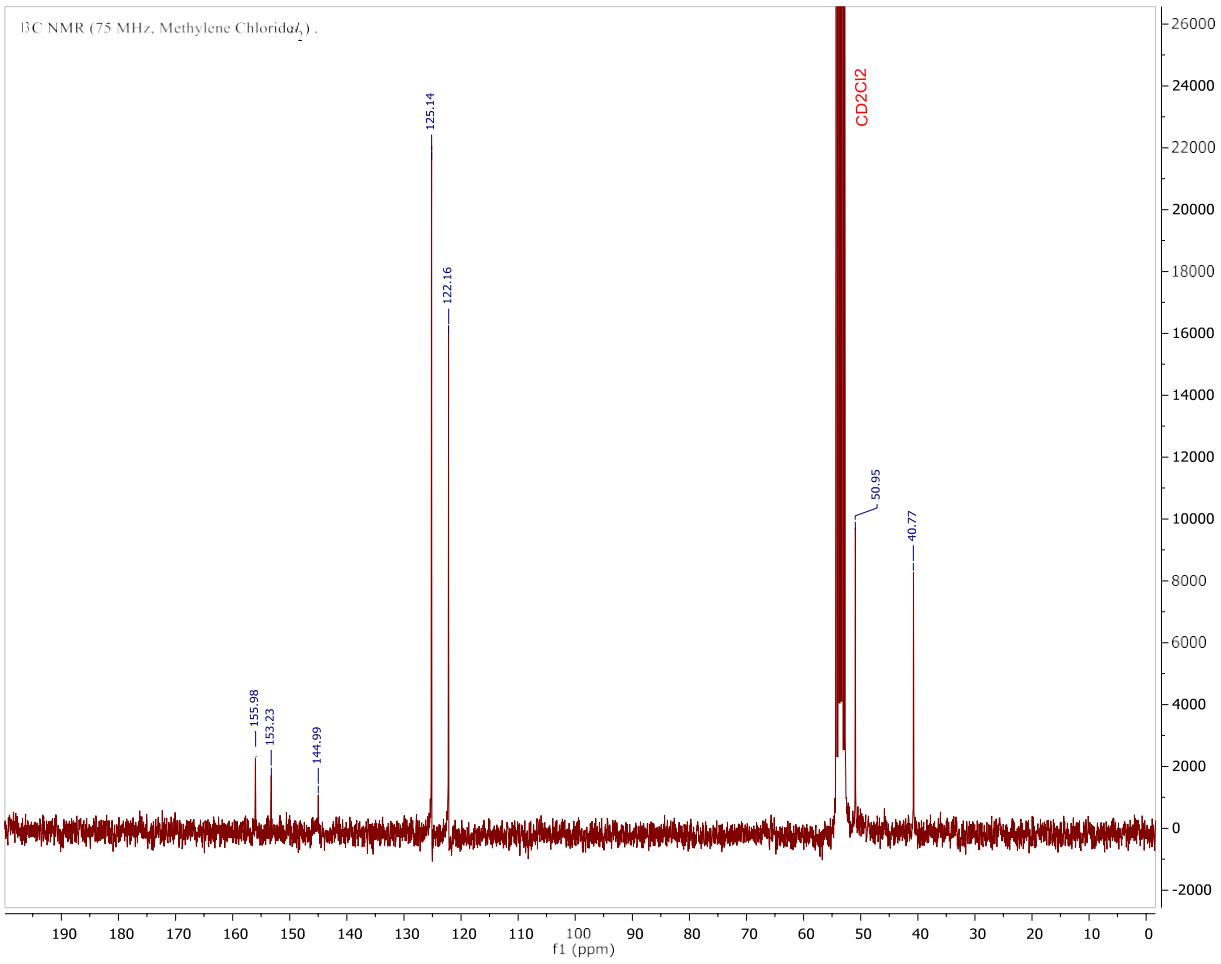
^{1}H NMR (300 MHz, Methylene Chloride-d_2) δ 8.23 (d, J = 9.1 Hz, 2H), 7.33 (d, J = 9.2 Hz, 2H), 5.80 (t, J = 5.8 Hz, 1H), 3.58 – 3.40 (m, 4H).

^{13}C NMR (75 MHz, Methylene Chloride-d_2) δ 40.77, 50.95, 122.16, 125.14, 144.99, 153.23, 155.98.

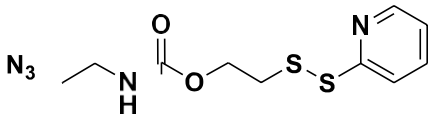
HRMS (ESI-TOF, m/z): $[M+Cl]^-$ found 286.0346; calc. 286.0343 for $C_9H_9N_5O_4Cl$.

TLC (EtOAc:hexane 60:40 v/v): R_f = 0.55 (UV, I_2).


1H NMR (300 MHz, Methylene Chloride-d2)
8.25
8.22
7.35
7.32
5.82
5.80
5.78
5.35 CD2Cl2
3.53
3.52
3.51
3.50
3.47
3.46
3.44
3.44
3.43
1.92 HDO
2.01
2.00
0.91
4.30
f1 (ppm)


13C NMR (75 MHz, Methylene Chloride-d2)
125.14
122.16
CD2Cl2
50.95
40.77
155.98
153.23
144.99
f1 (ppm)

Synthesis and characterization of compound **14**



Chemical Formula: $C_{10}H_{13}N_5O_2S_2$
Exact Mass: 299.05
Molecular Weight: 299.37

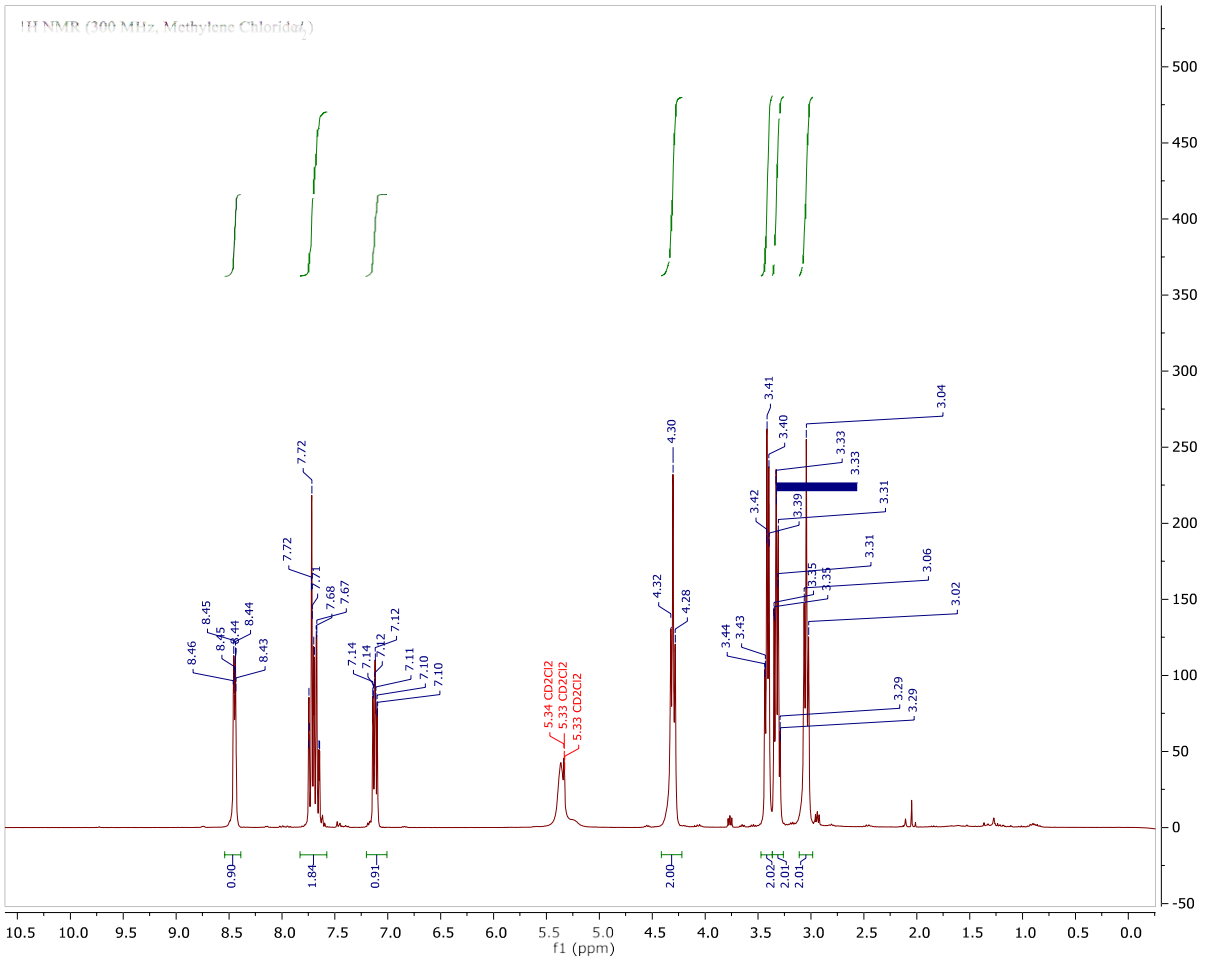
2-(pyridin-2-yldisulfaneyl)ethyl (2-azidoethyl)carbamate (14). A flame-dried round bottom flask under positive Ar atmosphere was charged with *p*-nitrophenyl-activated carbamate ester **12** (1 eq., 2.293 g, 9.004 mmol) in anhydrous ACN (20 mL). To this clear light beige solution, added mixed disulfide 2-(pyridine-2-yl-disulfanyl) ethanol **13** (1 eq., 1.686g, 9.130 mmol) in ACN (10 mL) followed by DMAP (2 eq., 2.235 g, 18.28 mmol) in anhydrous ACN (20.0 mL). The reaction was run for 20 h at 60 °C and the progress was monitored by TLC. Upon completion, the clear yellow reaction mixture was concentrated and triturated with EtOAc. The reaction mixture was filtered and the precipitate was discarded. The filtrate was concentrated under reduced pressure and purified using 230-400 mesh, high-purity grade (9385) silica gel flash column chromatography (dry sample loading, gradient elution, 95:5 to 65:35 hexanes:ethyl acetate) to obtain 1.985 g (6.630 mmol) of **14** as a white solid in 74% isolated yield.

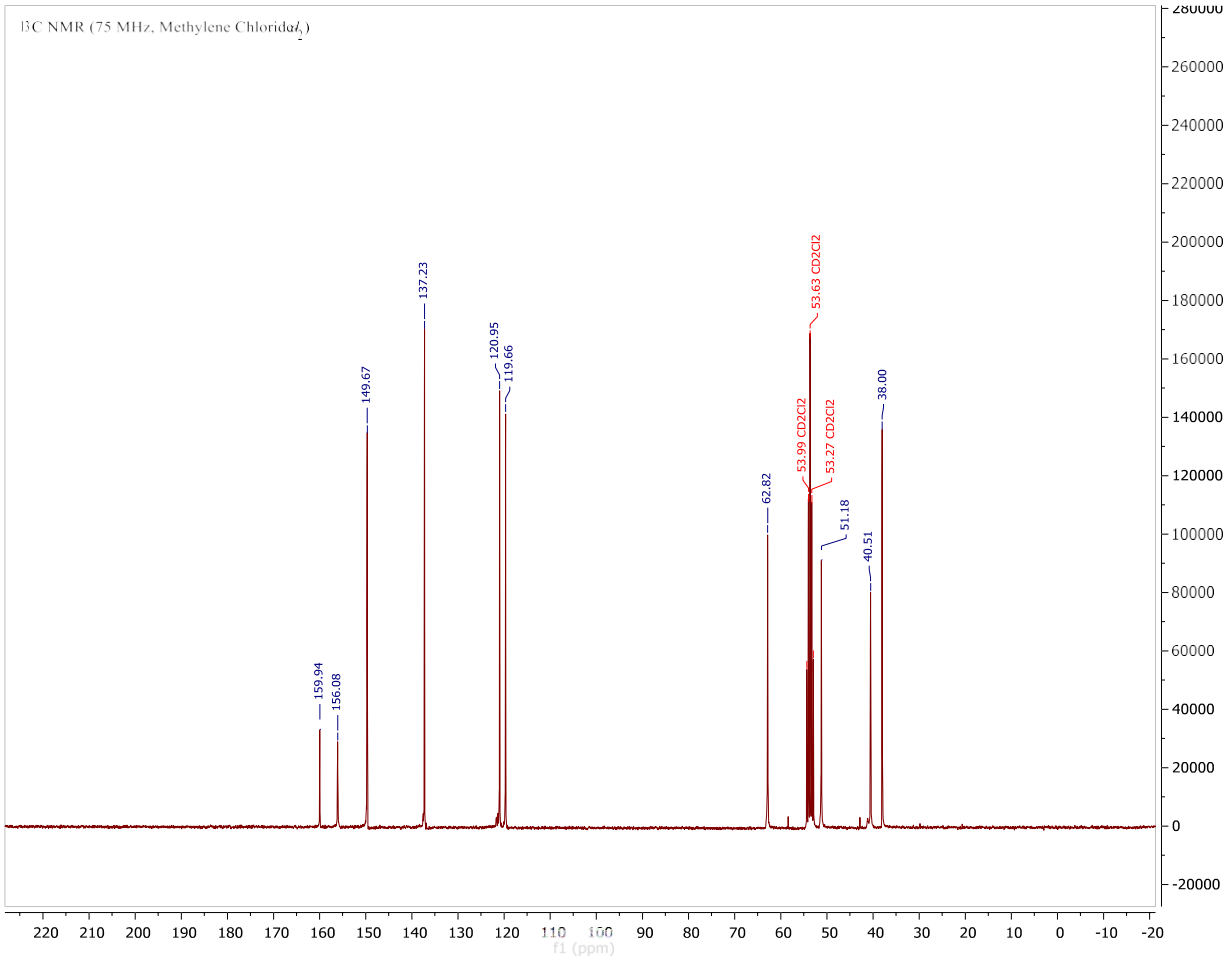
^{1}H NMR (300 MHz, Methylene Chloride-d_2) δ 8.45 (dt, J = 4.7, 1.3 Hz, 1H), 7.83 – 7.57 (m, 2H), 7.12 (ddd, J = 6.8, 4.8, 1.4 Hz, 1H), 4.30 (t, J = 6.3 Hz, 2H), 3.47 – 3.36 (m, 2H), 3.36 – 3.26 (m, 2H), 3.04 (t, J = 6.3 Hz, 2H).

^{13}C NMR (75 MHz, Methylene Chloride-d_2) δ 38.00, 40.51, 51.18, 62.82, 119.66, 120.95, 137.23, 149.67, 156.08, 159.94.

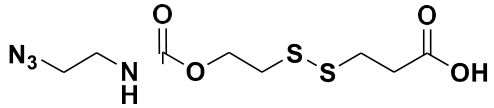
HRMS (ESI-TOF, m/z): $[M+H]^+$ found 300.0592; calc. 300.0589 for $C_{10}H_{14}N_5O_2S_2$.

TLC (EtOAc:hexane 60:40 v/v): R_f = 0.45 (UV, I_2).


1H NMR (300 MHz, Methylene Chloride-d2)
8.46
8.45
8.45
8.44
8.44
8.43
7.72
7.72
7.71
7.68
7.67
7.14
7.14
7.12
7.12
7.11
7.10
7.10
5.34 CD2Cl2
5.33 CD2Cl2
5.33 CD2Cl2
4.32
4.30
4.28
3.44
3.43
3.42
3.41
3.40
3.39
3.35
3.33
3.33
3.31
3.31
3.29
3.29
3.06
3.04
3.02
0.90
1.84
0.91
2.00
2.02
2.01
2.01
f1 (ppm)


13C NMR (75 MHz, Methylene Chloride-d2)
159.94
156.08
149.67
137.23
120.95
119.66
62.82
53.99 CD2Cl2
53.63 CD2Cl2
53.27 CD2Cl2
51.18
40.51
38.00
f1 (ppm)

Synthesis and characterization of compound **15**



Chemical Formula: $C_8H_{14}N_4O_4S_2$
Exact Mass: 294.05
Molecular Weight: 294.34

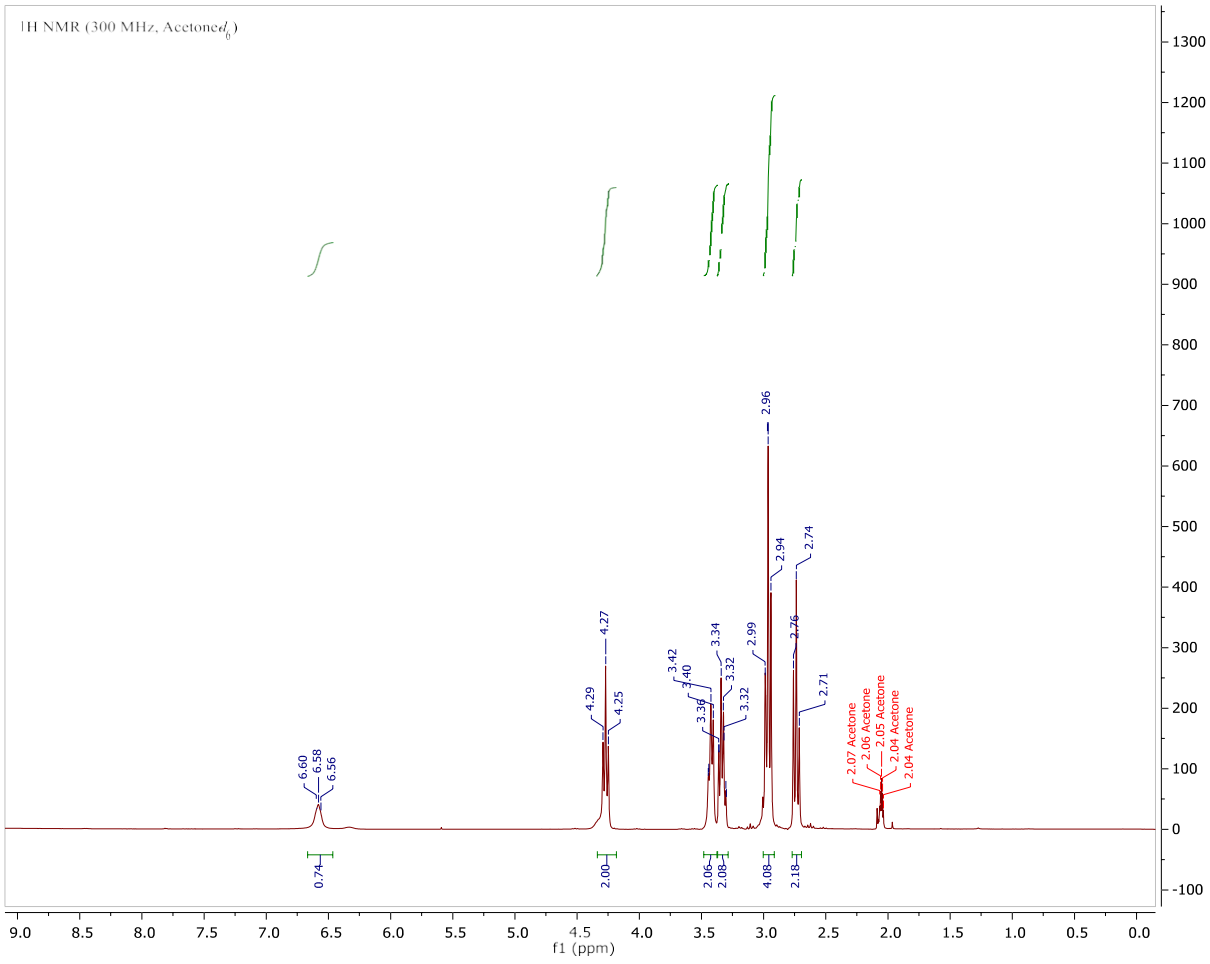
3-((2-(((2-azidoethyl)carbamoyl)oxy)ethyl)disulfaneyl)propanoic acid (15). A flame-dried round bottom flask under positive Ar atmosphere was charged with activated mixed disulfide-bridged 2-(pyridine-2-yl-disulfanyl) ethanol **14** (1 eq., 1.279 g, 4.287 mmol) dissolved in DMF (8.0 mL). The reaction was maintained under a steady stream of argon bubbling into the reaction mixture. Next, mercaptopropionic acid (2.2 eq., 1.000 g, 0.82 mL, 9.432 mmol) was added in one portion. The reaction mixture changed colour from light beige to yellow indicating the release of mercaptopyridine. The pH of the solution was slowly adjusted to pH ~5-6 with a saturated aqueous solution of $NaHCO_3$. The reaction was run for 15 minutes at 21 °C and the progress was monitored by TLC. Upon completion, the reaction mixture was diluted with 120 mL of water and acidified to pH 1 with 0.5 M HCl (aq). The reaction mixture was then extracted with EtOAc (5x40 mL), and washed with by 10% LiCl(aq) followed by brine (1x60 mL). The organic layer was dried with Na_2SO_4 and concentrated in vacuo. The residue was purified using silica gel flash column chromatography (isocratic elution, 70:29.5:0.5 dichloromethane:ethyl acetate:acetic acid) to obtain 1.035 g (3.515 mmol) of **15** as a white solid in 82% isolated yield.

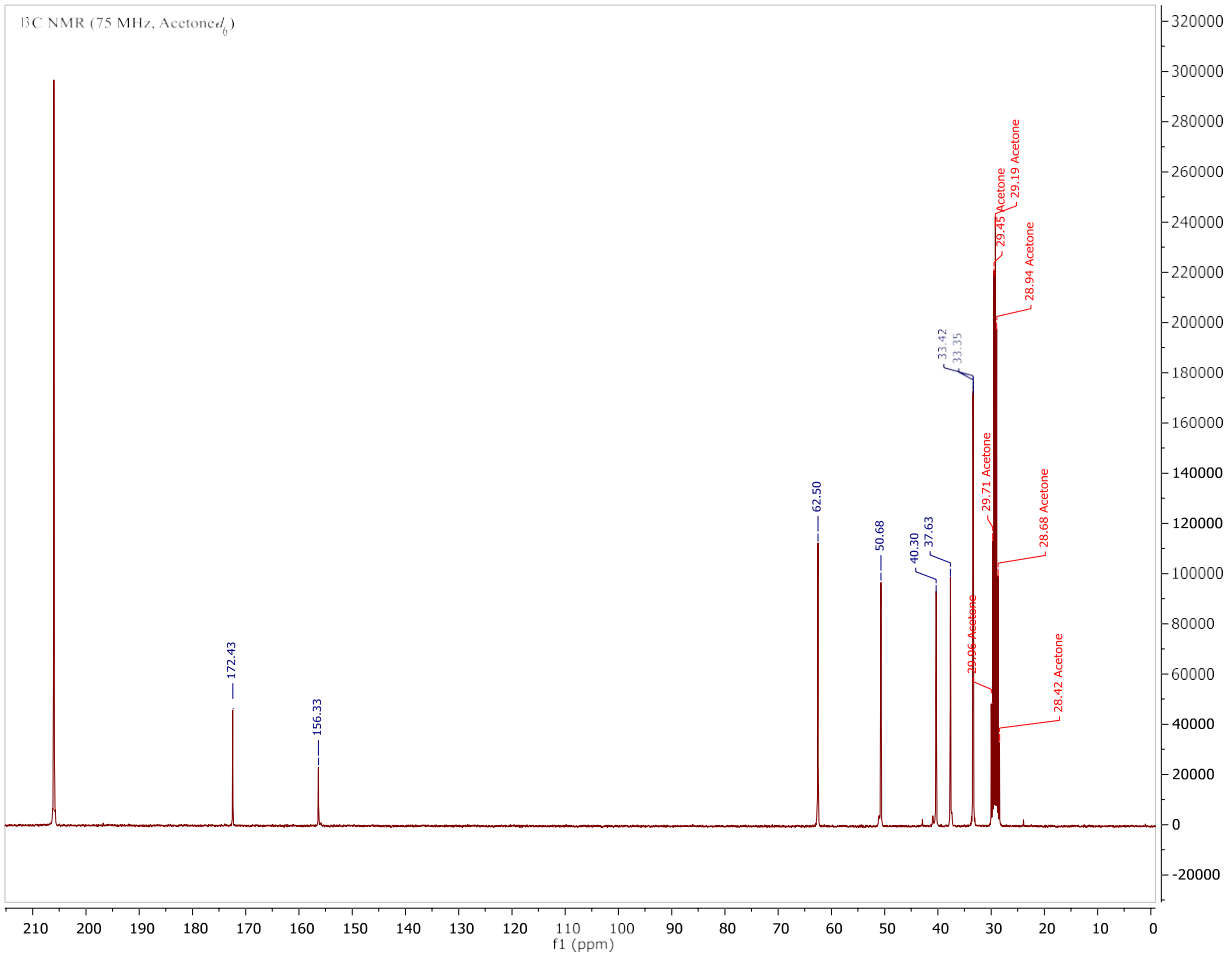
^{1}H NMR (300 MHz, Acetone-d_6) δ 6.67 – 6.46 (br t, 1H), 4.27 (t, J = 6.4 Hz, 2H), 3.42 (t, J = 6.1 Hz, 2H), 3.37 – 3.28 (m, 2H), 2.96 (t, J = 7.0 Hz, 4H), 2.74 (t, J = 7.0 Hz, 2H).

^{13}C NMR (75 MHz, Acetone-d_6) δ 33.35, 33.42, 37.63, 40.30, 50.68, 62.50, 156.33, 172.43.

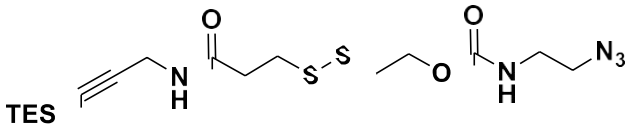
HRMS (ESI-TOF, m/z): $[M+Na]^+$ found 317.0355; calc. 317.0354 for $C_8H_{14}N_4O_4S_2Na$.

TLC (DCM:EtOAc:acetic acid 50:49:1 v/v/v): R_f = 0.4 (I_2, bromocresol green).


1H NMR (300 MHz, Acetone-d6)
6.60
6.58
6.56
4.29
4.27
4.25
3.42
3.40
3.36
3.34
3.32
3.32
2.99
2.96
2.94
2.76
2.74
2.71
2.07 Acetone
2.06 Acetone
2.05 Acetone
2.04 Acetone
2.04 Acetone
0.74
2.00
2.06
2.08
4.08
2.18
f1 (ppm)


13C NMR (75 MHz, Acetone-d_6)
172.43
156.33
62.50
50.68
40.30
37.63
33.42
33.35
30.06 Acetone
29.71 Acetone
29.45 Acetone
29.19 Acetone
28.94 Acetone
28.68 Acetone
28.42 Acetone
f1 (ppm)

Synthesis and characterization of compound **16**



Chemical Formula: $C_{17}H_{31}N_5O_3S_2Si$
Exact Mass: 445.16
Molecular Weight: 445.67

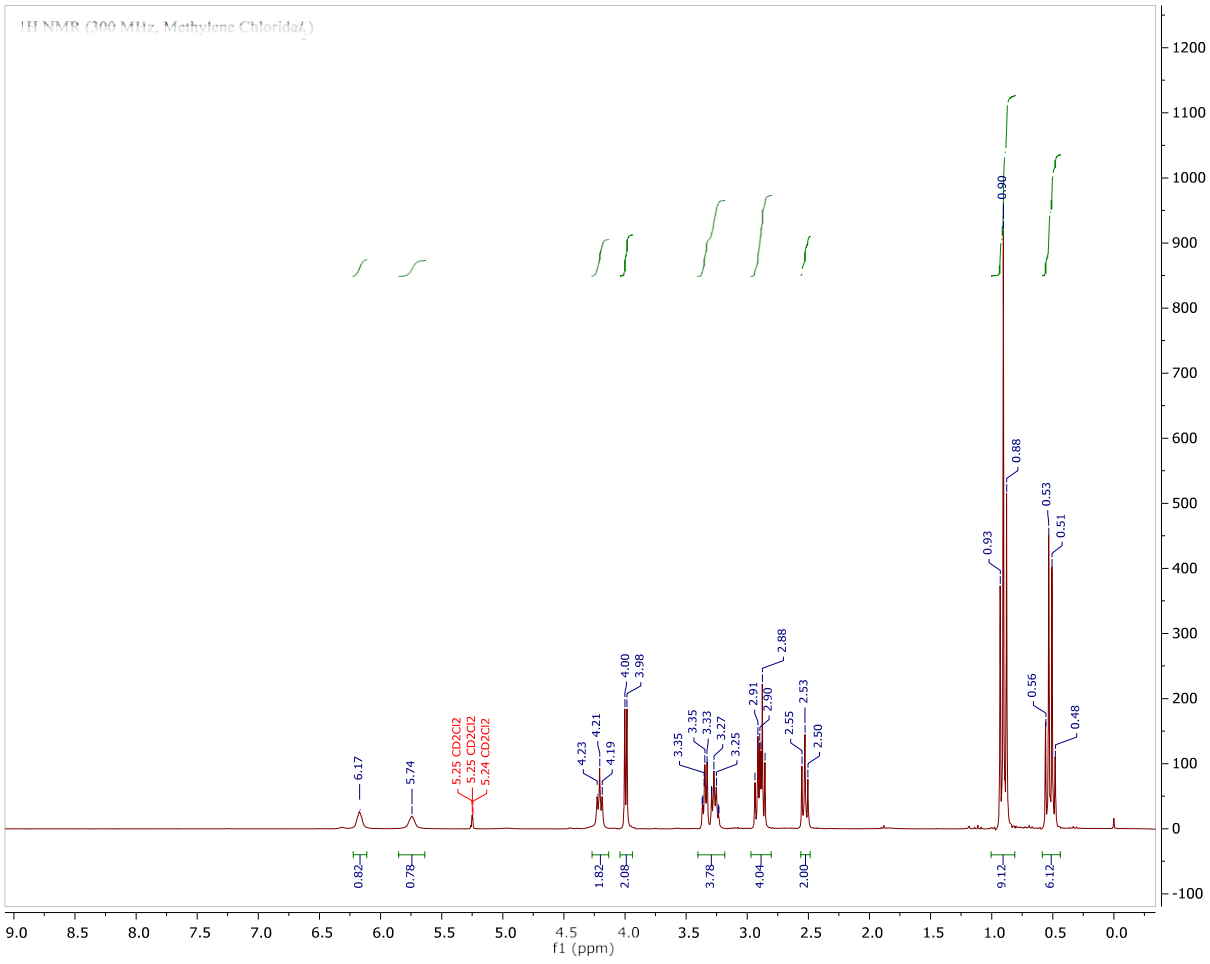
2-((3-oxo-3-((3-(triethylsilyl)prop-2-yn-1-yl)amino)propyl)disulfaneyl)ethyl (2-azidoethyl)carbamate (16). A flame-dried round bottom flask under positive Ar atmosphere was charged with mixed disulfide carboxylic acid **15** (1 eq., 0.217 g, 0.736 mmol) dissolved in DCM (10 mL) followed by EDC-HCl (3.5 eq., 0.496 g, 2.577 mmol), HOBt (3.5 eq., 0.350g, 2.577 mmol), TES-protected propargylamine **8** (1.2 eq., 0.202 g, 0.915 mmol) and DIPEA (5 eq., 0.64 mL, 3.681 mmol). The reaction was run for 6 h at 21 °C and the progress was monitored by TLC. Upon completion, the reaction mixture was concentrated in vacuo. The residue was purified using silica gel flash column chromatography (gradient elution, 85:15 to 55:45 petroleum ether:ethyl acetate) to obtain 0.259 g (0.580 mmol) of **16** as a beige solid in 79% isolated yield.

^{1}H NMR (300 MHz, Methylene Chloride-d_2) δ 6.17 (br s, 1H), 5.74 (br s, 1H), 4.21 (t, J = 6.2 Hz, 2H), 3.99 (d, J = 5.2 Hz, 2H), 3.40 – 3.18 (m, 4H), 2.97 – 2.80 (m, 4H), 2.53 (t, J = 7.4 Hz, 2H), 0.90 (t, J = 7.9 Hz, 9H), 0.52 (q, J = 8.4, 7.8 Hz, 6H).

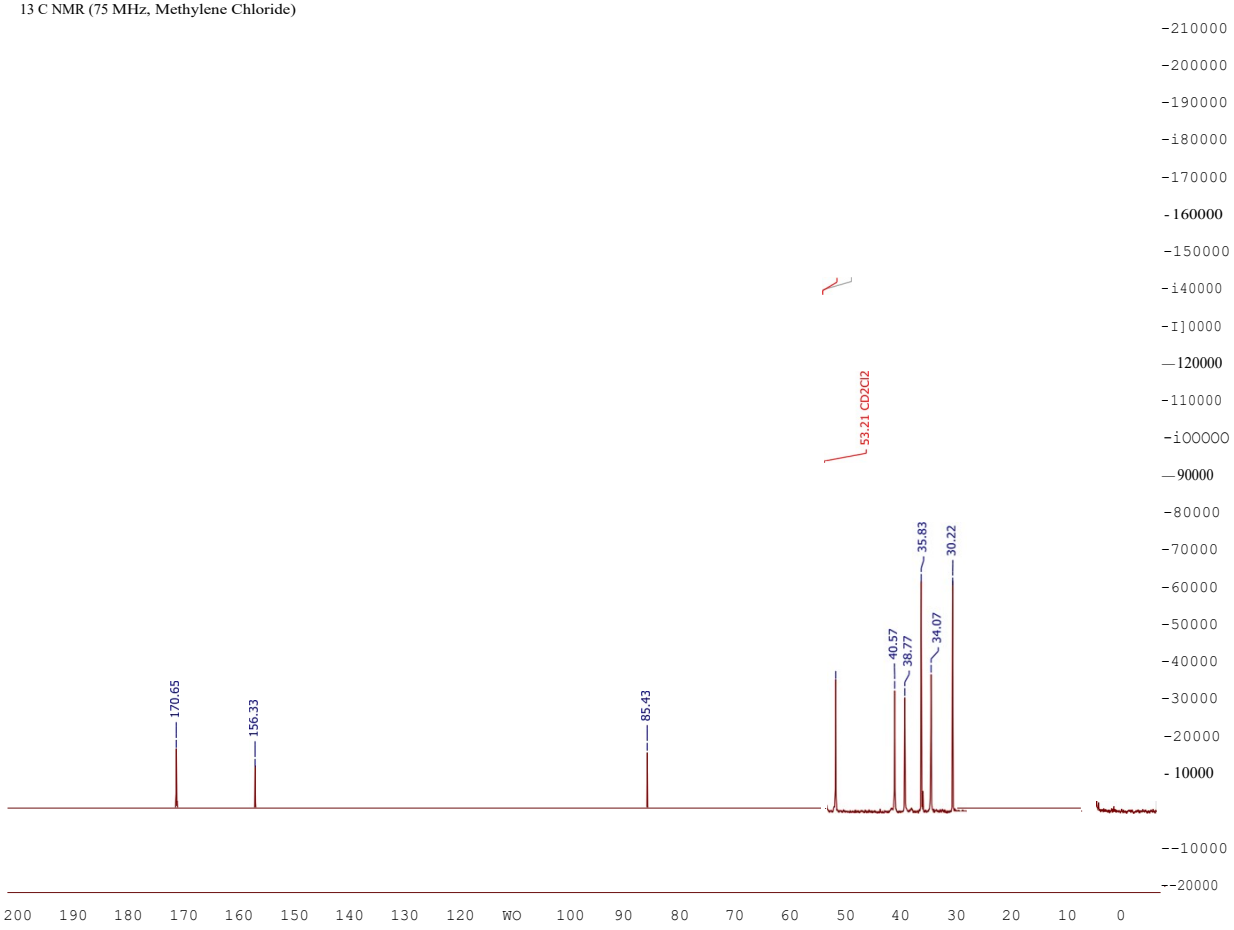
^{13}C NMR (75 MHz, Methylene Chloride-d_2) δ 4.30, 7.27, 30.22, 34.07, 35.83, 38.77, 40.57, 51.11, 62.84, 85.43, 102.54, 156.33, 170.65.

HRMS (ESI-TOF, m/z): $[M+H]^+$ found 446.1714; calc. 446.1716 for $C_{17}H_{32}N_5O_3S_2Si$.

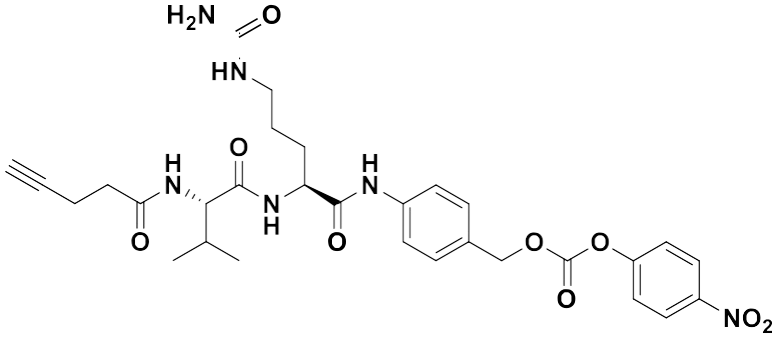
TLC (petroleum ether:EtOAc 55:45 v/v): R_f= 0.4 ($KMnO_4$, I_2).


1H NMR (300 MHz, Methylene Chloride-d2)
6.17
5.74
5.25 CD2Cl2
5.25 CD2Cl2
5.24 CD2Cl2
4.23
4.21
4.19
4.00
3.98
3.35
3.35
3.33
3.27
3.25
2.91
2.90
2.88
2.55
2.53
2.50
0.93
0.90
0.88
0.56
0.53
0.51
0.48
0.82
0.78
1.82
2.08
3.78
4.04
2.00
9.12
6.12
f1 (ppm)

13 C NMR (75 MHz, Methylene Chloride)



Synthesis and characterization of compound **18**



Chemical Formula: $C_{30}H_{36}N_6O_9$
Exact Mass: 624.25
Molecular Weight: 624.65

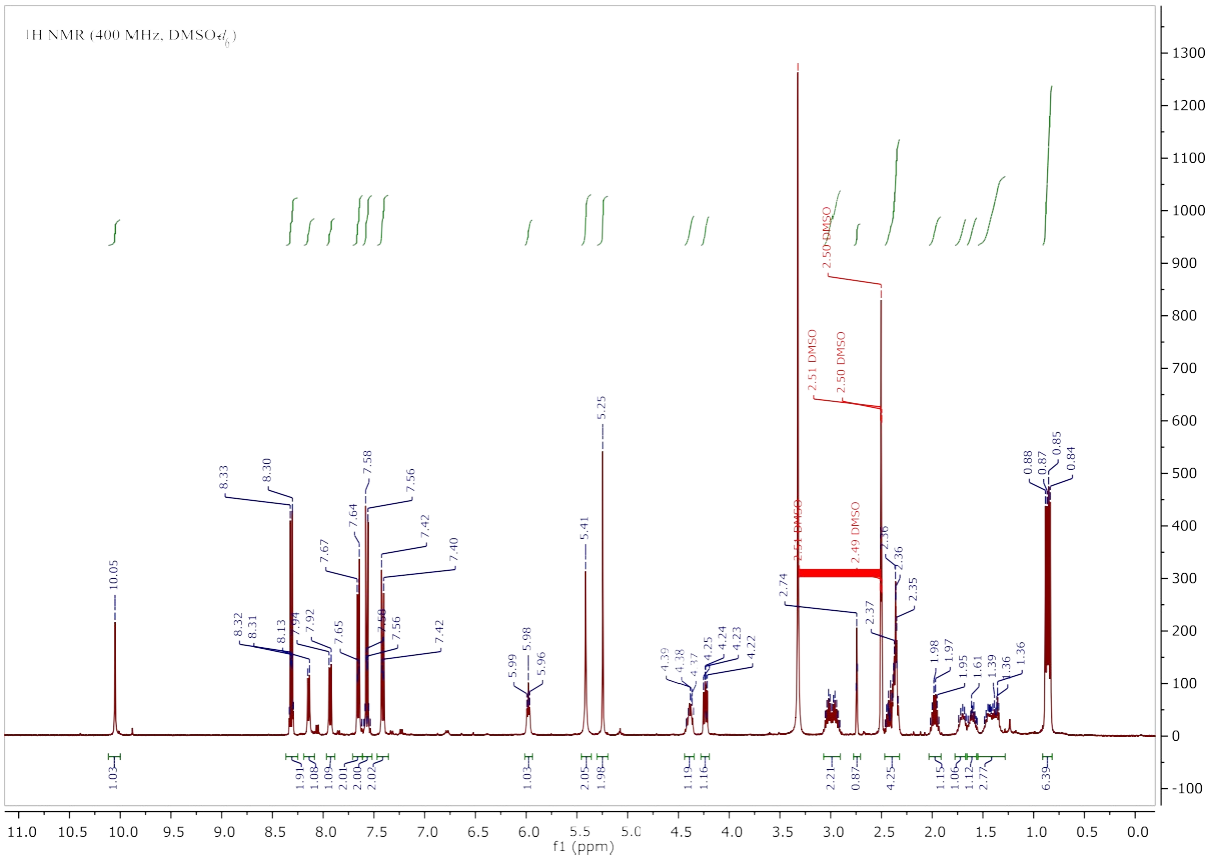
4-((S)-2-((S)-3-methyl-2-(pent-4-ynamido)butanamido)-5-ureidopentanamido)benzyl (4-nitrophenyl) carbonate (18). A 1.5-mL Eppendorf tube was charged with compound **17** (1 eq., 8.0 mg, 17.4 µmol) dissolved in DMF (0.5 mL) followed by bis-PNP carbonate (5.25 eq., 28 mg, 91.3 µmol), and DIPEA (3.1eq., 9.5 µL, 54.4 µmol). The reaction was stirred for 1.5 h at 21 °C and the progress was monitored by TLC. Upon completion, the reaction mixture was triturated with ACN (3x10 mL) to obtain 8 mg (12.9 µmol) of **18** as beige solid in 72% isolated yield.

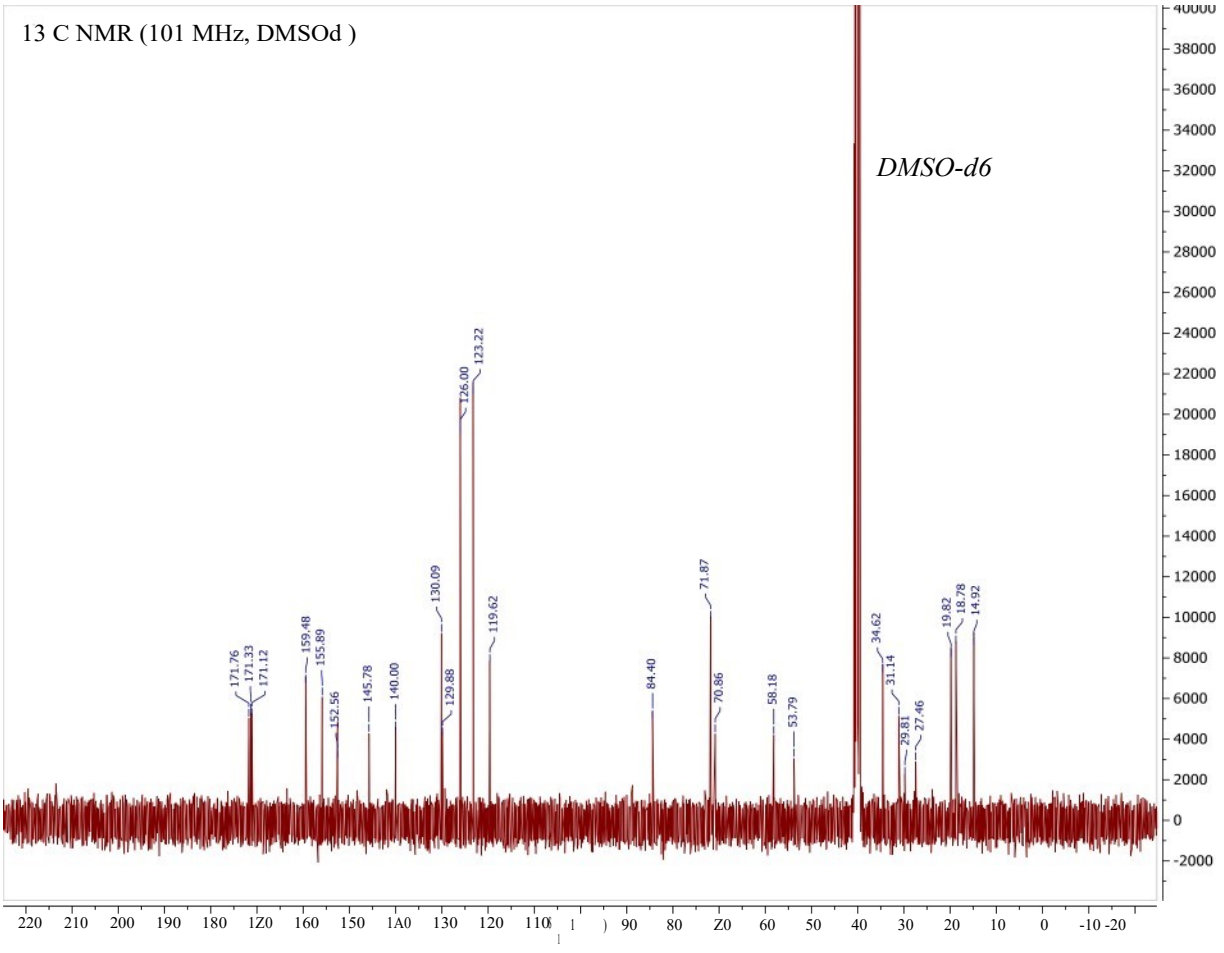
^{1}H NMR (400 MHz, DMSO-d_6) δ 10.05 (s, 1H), 8.36 – 8.27 (m, 2H), 8.14 (d, J = 7.4 Hz, 1H), 7.93 (d, J = 8.6 Hz, 1H), 7.69 – 7.59 (m, 2H), 7.62 – 7.51 (m, 2H), 7.45 – 7.38 (m, 2H), 5.98 (t, J = 5.9 Hz, 1H), 5.41 (s, 2H), 5.25 (s, 2H), 4.39 (t, J = 7.0 Hz, 1H), 4.23 (dd, J = 8.6, 6.7 Hz, 1H), 2.98 (dh, J = 25.9, 6.4 Hz, 2H), 2.74 (t, J = 2.3 Hz, 1H), 2.49 – 2.36 (m, 1H), 2.36 (tt, J = 6.2, 3.6 Hz, 3H), 1.97 (h, J = 6.8 Hz, 1H), 1.71 (q, J = 7.6 Hz, 1H), 1.64 – 1.55 (m, 1H), 1.51 – 1.32 (m, 1H), 1.37 (s, 2H), 0.86 (dd, J = 12.7, 6.8 Hz, 6H).

^{13}C NMR (101 MHz, DMSO-d_6) δ 14.92, 18.78, 19.82, 27.46, 29.81, 31.14, 34.62, 53.79, 58.18, 70.86, 71.87, 84.40, 119.62, 123.22, 126.00, 129.88, 130.09, 140.00, 145.78, 152.56, 155.89, 159.48, 171.12, 171.33, 171.76.

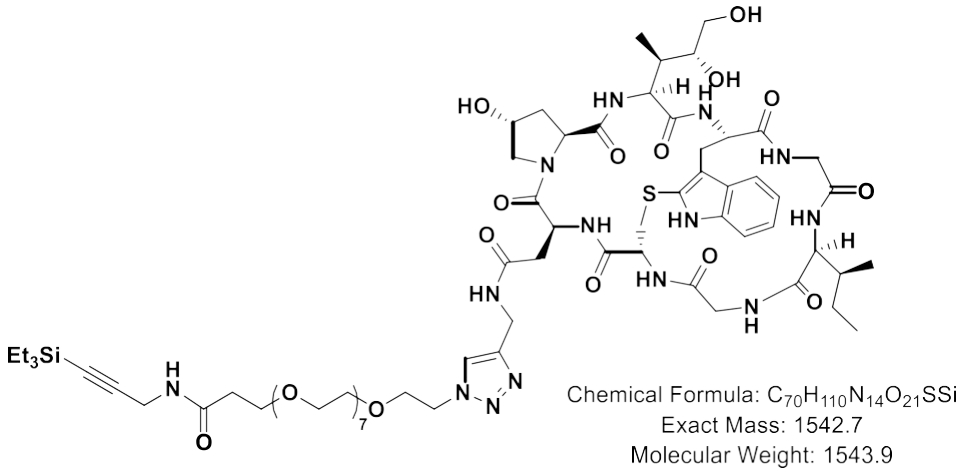
HRMS (ESI-TOF, m/z): $[M+Na]^+$ found 647.2438; calc. 647.2441 for $C_{30}H_{36}N_6O_9Na$.

TLC (dichloromethane/methanol 90:10 v/v): R_f = 0.3 (UV, I_2).


1H NMR (400 MHz, DMSO-d6)
f1 (ppm)


13 C NMR (101 MHz, DMSOd)
DMSO-d6
171.76
171.33
171.12
159.48
155.89
152.56
145.78
140.00
130.09
129.88
126.00
123.22
119.62
84.40
71.87
70.86
58.18
53.79
34.62
31.14
29.81
27.46
19.82
18.78
14.92

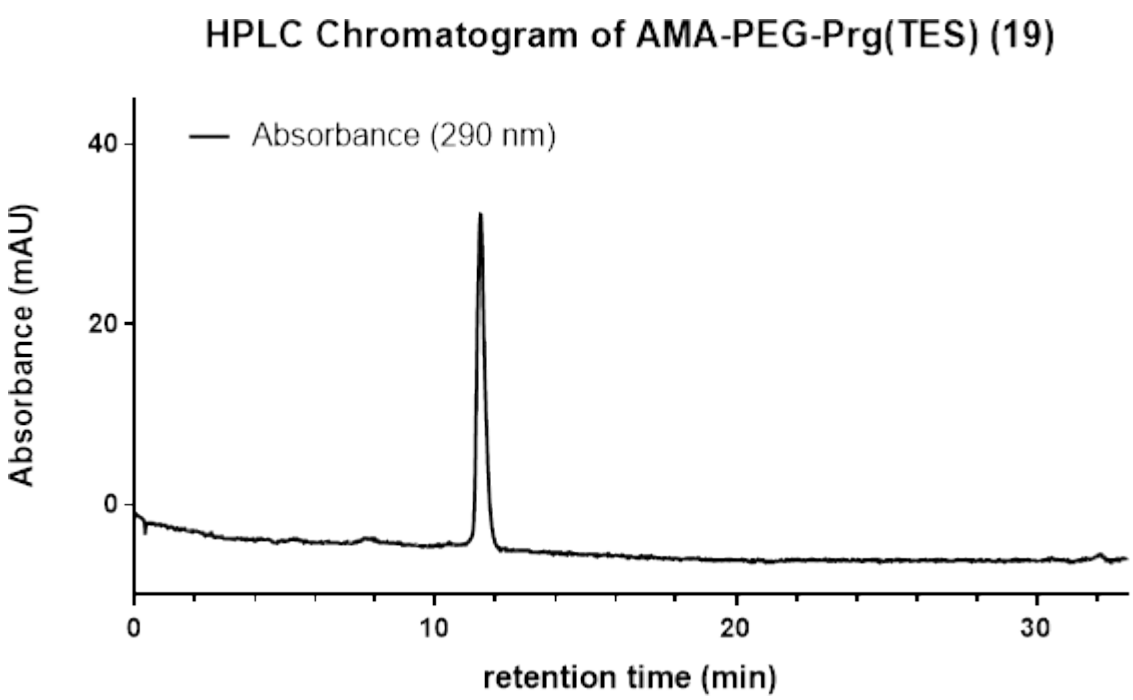
Synthesis and characterization of compound **19**

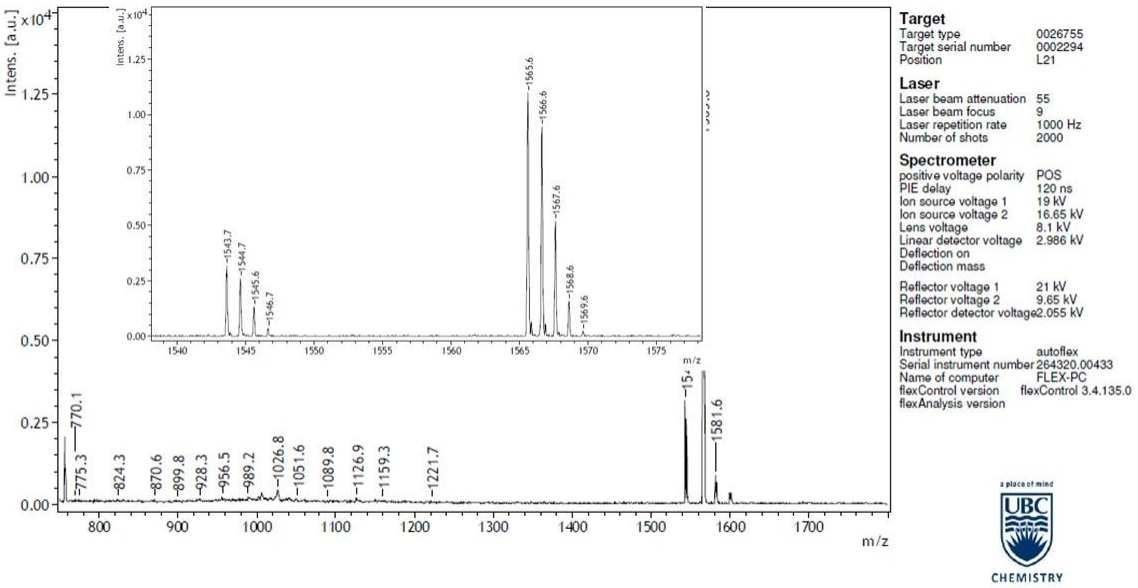


Amanitin-PEG-Prg(TES) (19). $CuSO_4$/TBTA/Na-ascorbate solution was prepared first in the following manner: 1.5 µL of 400 mM aqueous $CuSO_4$ (1 eq., 95 µg, 600 nmol) were combined with 3 µL of 200 mM TBTA solution in DMSO (1 eq., 318 µg, 600 nmol) and allowed to rest for 5 mins at 21 °C. The blue clear $CuSO_4$/TBTA solution was then mixed with 2.3 µL of DMSO and 2.3 µL of 800 mM aqueous sodium ascorbate (3 eq., 357 µg, 1.8 µmol) to give a light beige and clear solution. A 15-mL Falcon tube was charged with amanitin-Prg **4** followed by Azido-PEG_8-Prg(TES) **10** (3 eq., 1.1 mg, 1.8 µmol) dissolved in 1.368 mL of *t*BuOH, 420 µL of H_2O, and 70 µL of pH 7.4 10 mM PBS buffer. $CuSO_4$/TBTA/Na-ascorbate mixture was then added to the reaction flask and the reaction contents were stirred for 1 hour at 45 °C. The reaction contents were concentrated under reduced pressure, re-dissolved in 0.1% formic acid ACN/H_2O, and purified using C18 SEP-PAK (gradient elution, 5:95 ACN:H_2O to 70:30 ACN/H_2O in the presence of 0.1% formic acid). The fractions were analyzed by HPLC using gradient **E**. Product-containing fractions were lyophilized to obtain 675 µg (438 nmol) of **19** as a white solid in 73% isolated yield. Quantification was done using extinction coefficient ε = 10,000 $mol^{-1}Lcm^{-1}$ in methanol at 290 nm.

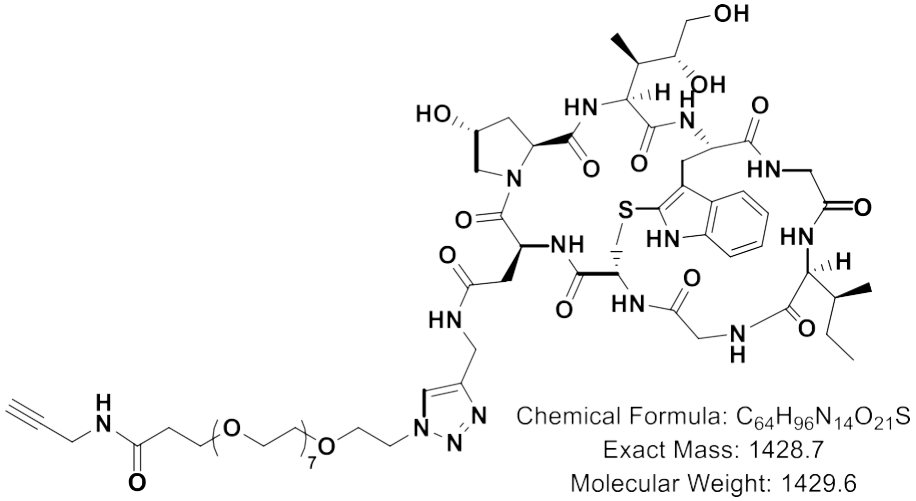
MALDI-TOF (m/z): $[M+Na]^+$ found 1565. 6; calc. 1565.7 for $C_{70}H_{110}N_{14}O_{21}SSiNa$.

HPLC (gradient **E**) $\mathbf{t_R}$ = 11.5 min; λ_{max} = 290 nm.


HPLC Chromatogram of AMA-PEG-Prg(TES) (19)
Absorbance (290 nm)
Absorbance (mAU)
40
20
0
0
10
20
30
retention time (min)


Intens. [a.u.]
x10^4
1.25
1.00
0.75
0.50
0.25
0.00
770.1
775.3
824.3
870.6
899.8
928.3
956.5
989.2
1026.8
1051.6
1089.8
1126.9
1159.3
1221.7
1581.6
800
900
1000
1100
1200
1300
1400
1500
1600
1700
m/z
1543.7
1544.7
1545.6
1546.7
1565.6
1566.6
1567.6
1568.6
1569.6
1540
1545
1550
1555
1560
1565
1570
1575
Target
Target type 0026755
Target serial number 0002294
Position L21
Laser
Laser beam attenuation 55
Laser beam focus 9
Laser repetition rate 1000 Hz
Number of shots 2000
Spectrometer
positive voltage polarity POS
PIE delay 120 ns
Ion source voltage 1 19 kV
Ion source voltage 2 16.65 kV
Lens voltage 8.1 kV
Linear detector voltage 2.986 kV
Deflection on
Deflection mass
Reflector voltage 1 21 kV
Reflector voltage 2 9.65 kV
Reflector detector voltage 2.055 kV
Instrument
Instrument type autoflex
Serial instrument number 264320.00433
Name of computer FLEX-PC
flexControl version flexControl 3.4.135.0
flexAnalysis version
a place of mind
UBC
CHEMISTRY

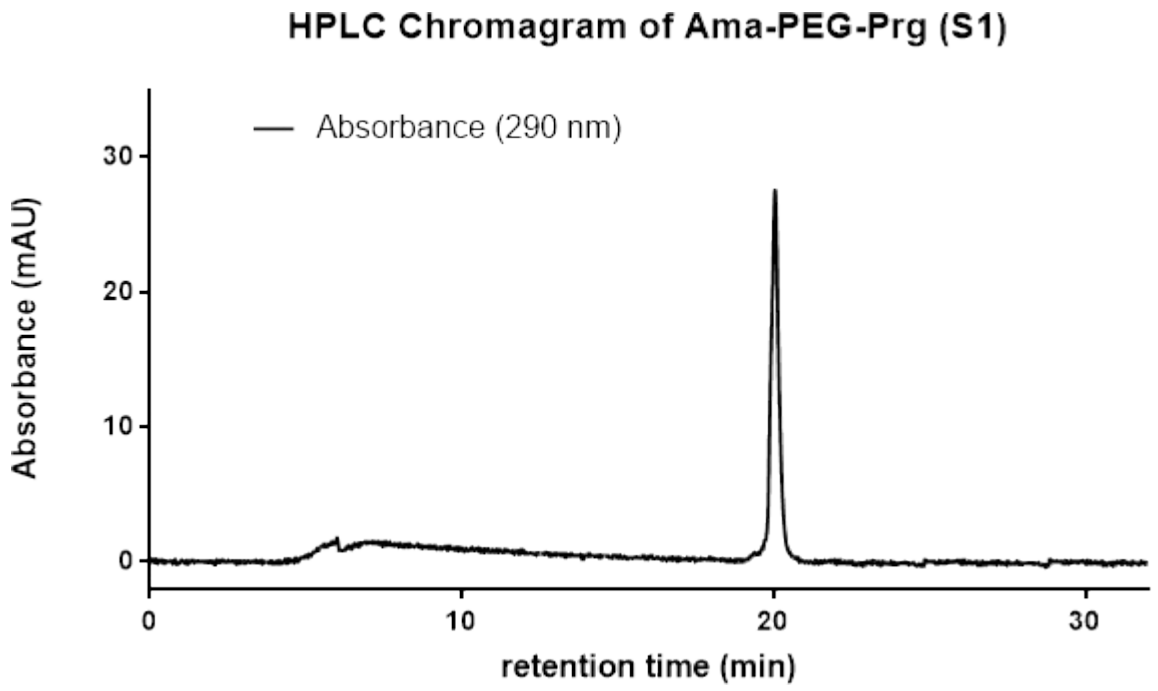
Synthesis and characterization of compound **S1**

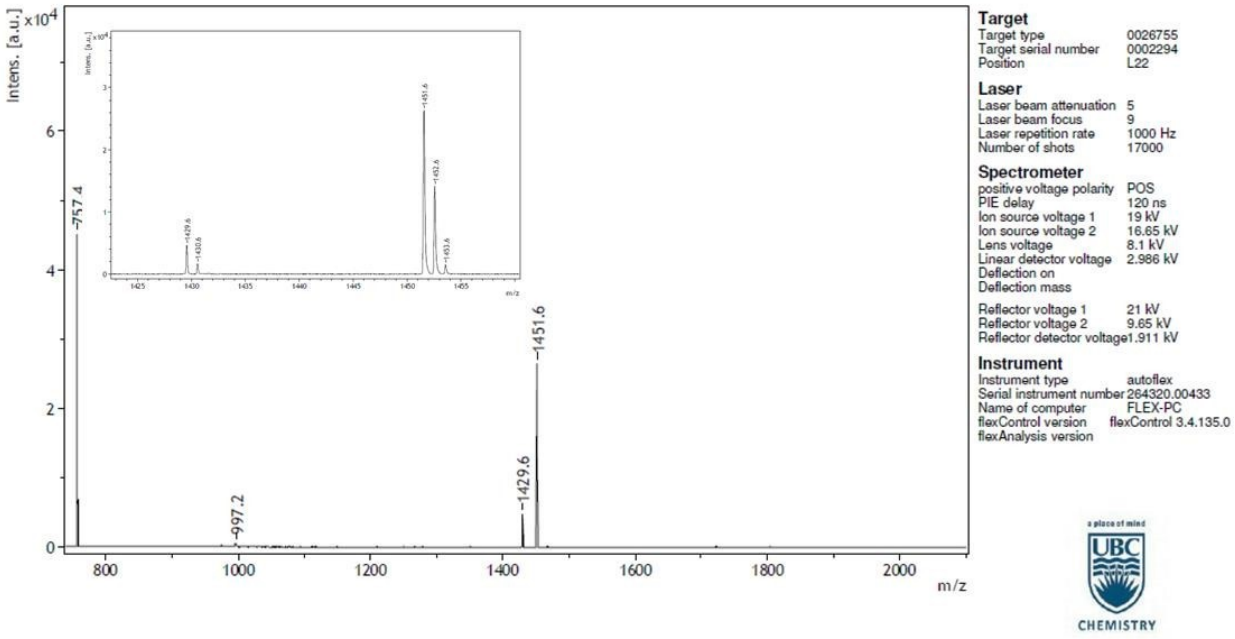


Amanitin-PEG-Prg (S1). A 1.5-mL Eppendorf tube was charged with Amanitin-PEG_8-Prg(TES) **19** (1 eq., 675 µg, 438 nmol) in 120 µL of DMSO followed by 5.2 µL of aqueous 3M KHF_2 (excess). The reaction mixture was warmed to 60 °C to give a clear colourless solution. The reaction was run for 1 hour at 60 °C. The reaction progress was analyzed by HPLC using gradient **F**. Upon completion, the reaction contents were concentrated under reduced pressure, re-dissolved in 0.1% formic acid ACN/H_2O, and purified using C18 SEP-PAK (gradient elution, 100% H_2O to 20:70 ACN/H_2O in the presence of 0.1% formic acid). The product-containing fractions were lyophilized to obtain 538 µg (377 nmol) of **S1** as a white powder in 86% isolated yield. Quantification was done using extinction coefficient ε = 10,000 $mol^{-1}Lcm^{-1}$ in methanol at 290 nm.

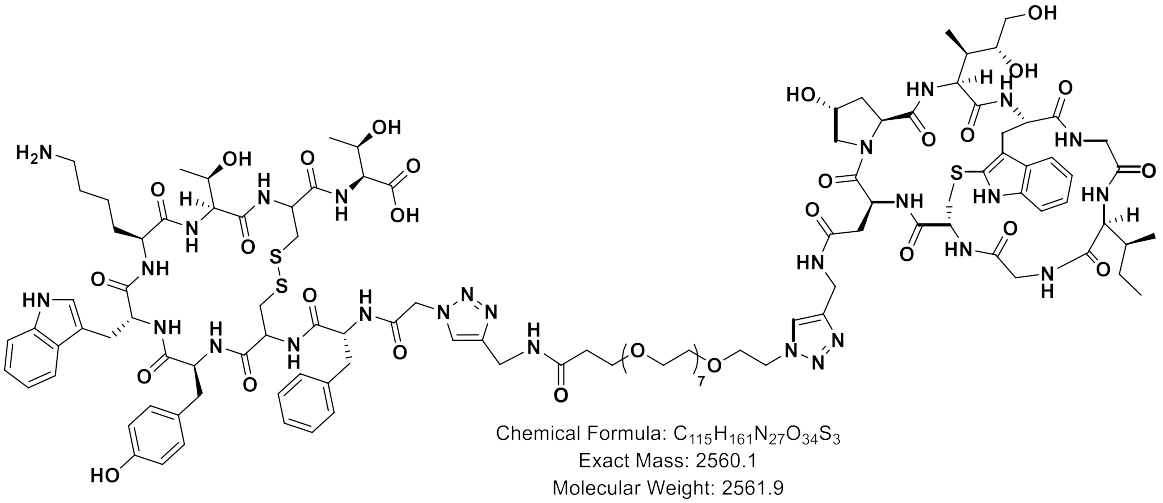
MALDI-TOF (m/z): $[M+Na]^+$ found 1451. 6; calc. 1451.6 for $C_{64}H_{96}N_{14}O_{21}SNa$.

HPLC (gradient **F**) $\mathbf{t_R}$ = 20.1 min; λ_{max} = 290 nm.


HPLC Chromagram of Ama-PEG-Prg (S1)
Absorbance (290 nm)
Absorbance (mAU)
30
20
10
0
0
10
20
30
retention time (min)


Intens. [a.u.]
x10^4
6
4
2
0
757.4
997.2
1429.6
1451.6
800
1000
1200
1400
1600
1800
2000
m/z
Target
Target type 0026755
Target serial number 0002294
Position L22
Laser
Laser beam attenuation 5
Laser beam focus 9
Laser repetition rate 1000 Hz
Number of shots 17000
Spectrometer
positive voltage polarity POS
PIE delay 120 ns
Ion source voltage 1 19 kV
Ion source voltage 2 16.65 kV
Lens voltage 8.1 kV
Linear detector voltage 2.986 kV
Deflection on
Deflection mass
Reflector voltage 1 21 kV
Reflector voltage 2 9.65 kV
Reflector detector voltage 1.911 kV
Instrument
Instrument type autoflex
Serial instrument number 264320.00433
Name of computer FLEX-PC
flexControl version flexControl 3.4.135.0
flexAnalysis version
UBC
CHEMISTRY

Synthesis and characterization of compound **5**

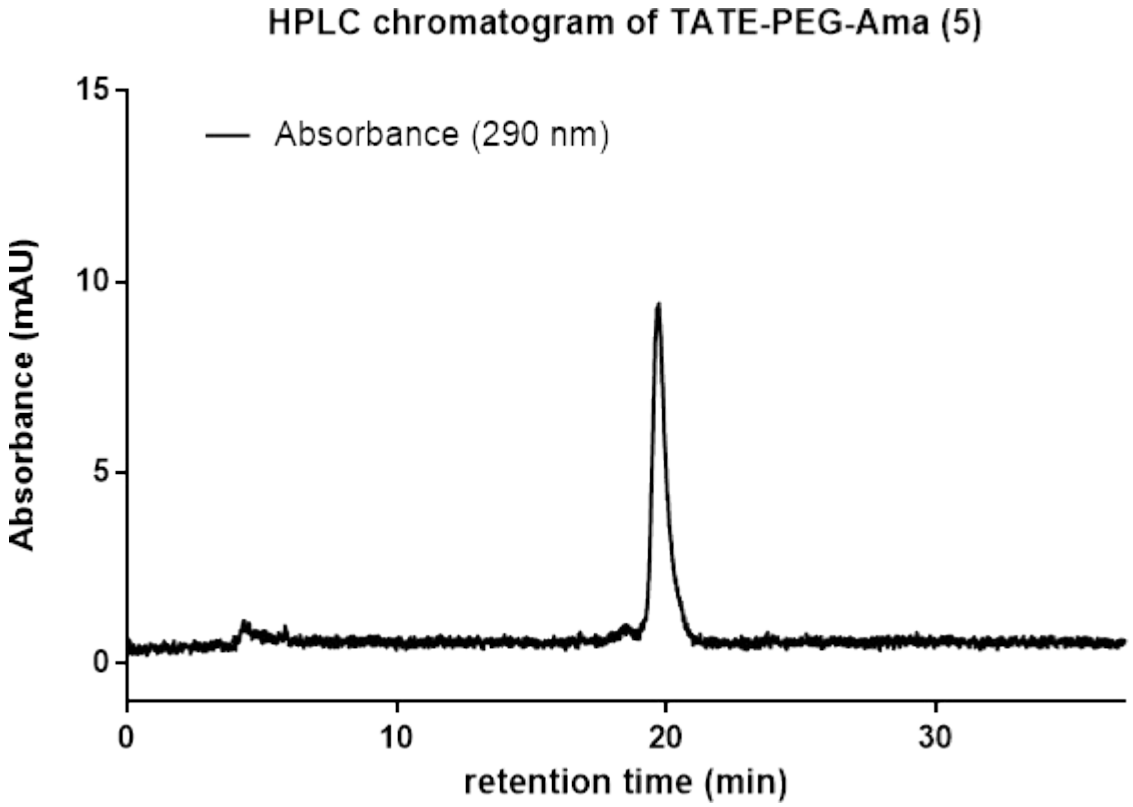


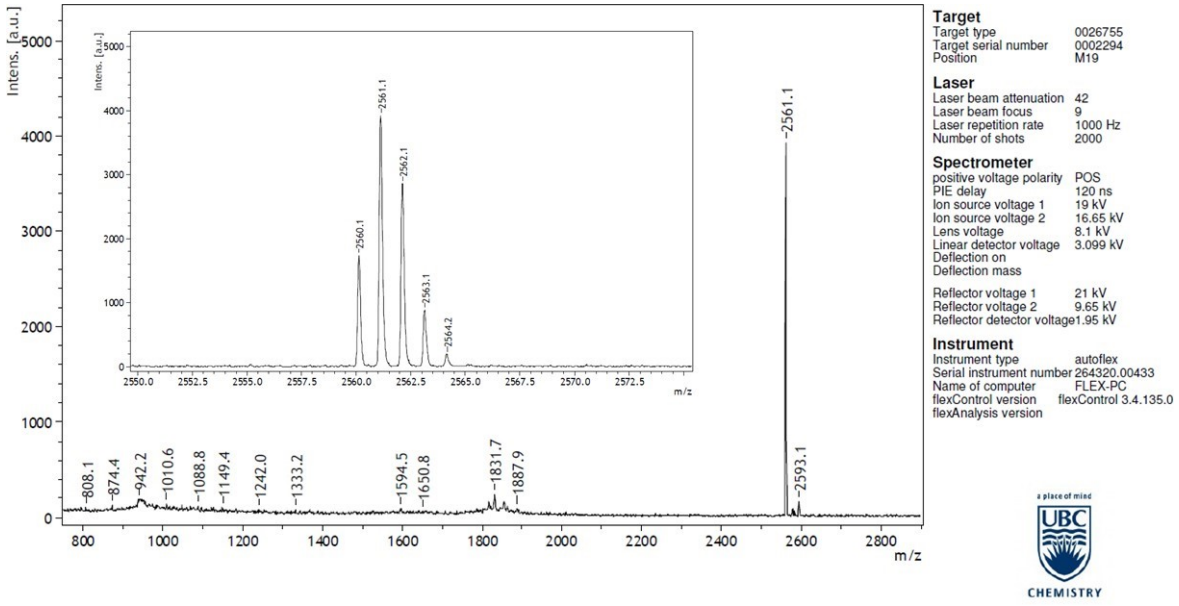
TATE-PEG-Amanitin (5). CuAAC conjugation of compound **S1** (1 eq., 538 µg, 377 nmol) to TATE-N_3 **2** (1.5 eq., 640 µg, 566 nmol) was carried out following the protocol described for the synthesis of compound **19**. Upon completion, the reaction contents were concentrated under reduced pressure, re-dissolved in 0.1% formic acid ACN/H_2O, and purified using C18 SEP-PAK (gradient elution, 5:95 ACN:H_2O to 80:30 ACN/H_2O in the presence of 0.1% trifluoroacetic acid). The fractions were analyzed by HPLC using gradient **I**. The product-containing fractions were lyophilized to obtain 704 µg (275 nmol) of **5** as a white solid in 73% isolated yield. Quantification was done using extinction coefficient ε = 13,308 $mol^{-1}Lcm^{-1}$ in methanol at 290 nm.

Note: 0.1% TFA has to be used as an additive during the HPLC analysis; use of formic acid as an additive results in poor separation.

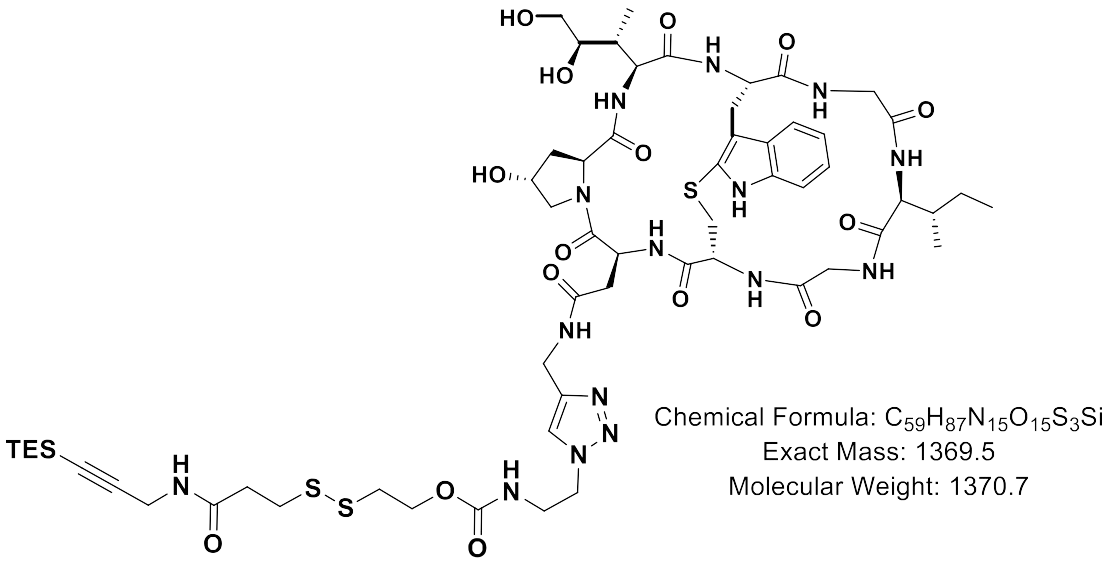
MALDI-TOF (m/z): $[M+H]^+$ found 2561.1; calc. 2561.1 for $C_{115}H_{162}N_{27}O_{34}S_3$

HPLC (gradient **I**) $\mathbf{t_R}$ = 19.6 min; λ_{max} = 290 nm.


HPLC chromatogram of TATE-PEG-Ama (5)
Absorbance (290 nm)
Absorbance (mAU)
retention time (min)
0
5
10
15
0
10
20
30


Intens. [a.u.]
m/z
2561.1
2560.1
2562.1
2563.1
2564.2
808.1
874.4
942.2
1010.6
1088.8
1149.4
1242.0
1333.2
1594.5
1650.8
1831.7
1887.9
2593.1
Target
Target type 0026755
Target serial number 0002294
Position M19
Laser
Laser beam attenuation 42
Laser beam focus 9
Laser repetition rate 1000 Hz
Number of shots 2000
Spectrometer
positive voltage polarity POS
PIE delay 120 ns
Ion source voltage 1 19 kV
Ion source voltage 2 16.65 kV
Lens voltage 8.1 kV
Linear detector voltage 3.099 kV
Deflection on
Deflection mass
Reflector voltage 1 21 kV
Reflector voltage 2 9.65 kV
Reflector detector voltage 1.95 kV
Instrument
Instrument type autoflex
Serial instrument number 264320.00433
Name of computer FLEX-PC
flexControl version flexControl 3.4.135.0
flexAnalysis version
a place of mind
UBC
CHEMISTRY

Synthesis and characterization of compound **20**

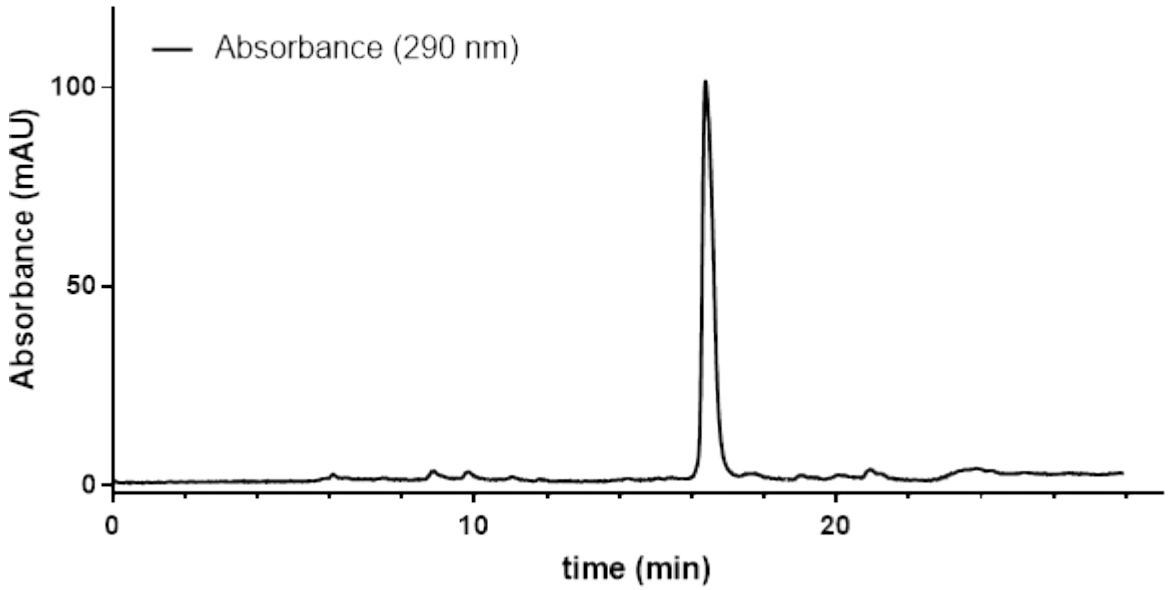


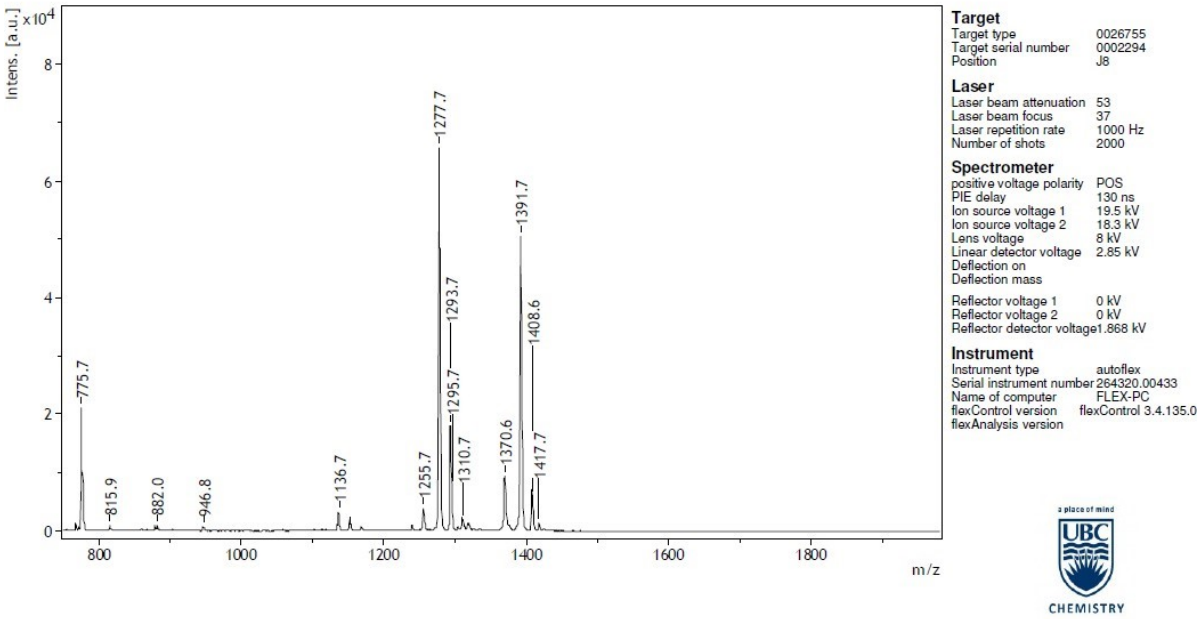
Amanitin-SS-Prg(TES) (20). CuAAC conjugation of compound **4** (1 eq., 324 µg, 350 nmol) to the bio-reducible linker **16** (3.4 eq., 0.53 mg, 1.19 µmol) was carried out following the protocol described for the synthesis of compound **19**. Upon completion, reaction contents were concentrated under reduced pressure, re-dissolved in 0.1% formic acid ACN/H_2O, and purified using C18 SEP-PAK (gradient elution, 5:95 ACN:H_2O to 70:30 ACN/H_2O in the presence of 0.1% formic acid). The fractions were analyzed by HPLC using gradient H. The product-containing fractions were lyophilized to obtain 342 µg (251 nmol) of **20** as a white solid in 72% isolated yield. Quantification was done using extinction coefficient ε = 10,000 $mol^{-1}Lcm^{-1}$ in methanol at 290 nm.

MALDI-TOF (m/z): $[M+Na]^+$ found 1391.7; calc. 1392.5 for $C_{59}H_{87}N_{15}O_{15}S_3SiNa$.

HPLC (gradient **H**) $\mathbf{t_R}$ = 16.5 min; λ_{max} = 290 nm.

HPLC chromatogram of Amanitin-SS-Prg(TES) (20)

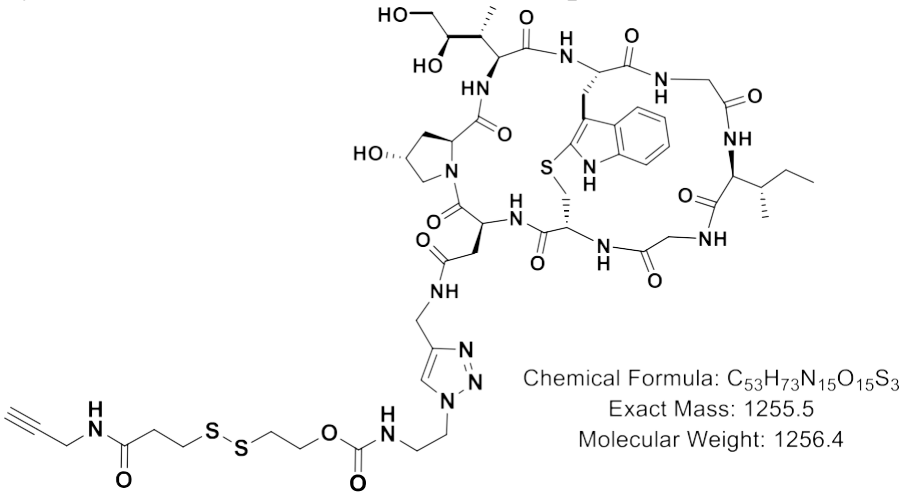




Date of Acquisition 2017-10-19T15:56:07.693-07:00
Acquisition method D:\Bruker_service\April 6 2015\Compass 1.4 April 2015\Methods\flexControlMethods\LP_700-2000_Da.par
Samples Name
File Name D:\Data\Perrin\Alla\AMANITIN-LINK-TES\EL12952\0_J8\1\1SLin

printed: 7/6/2018 4:47:01 PM

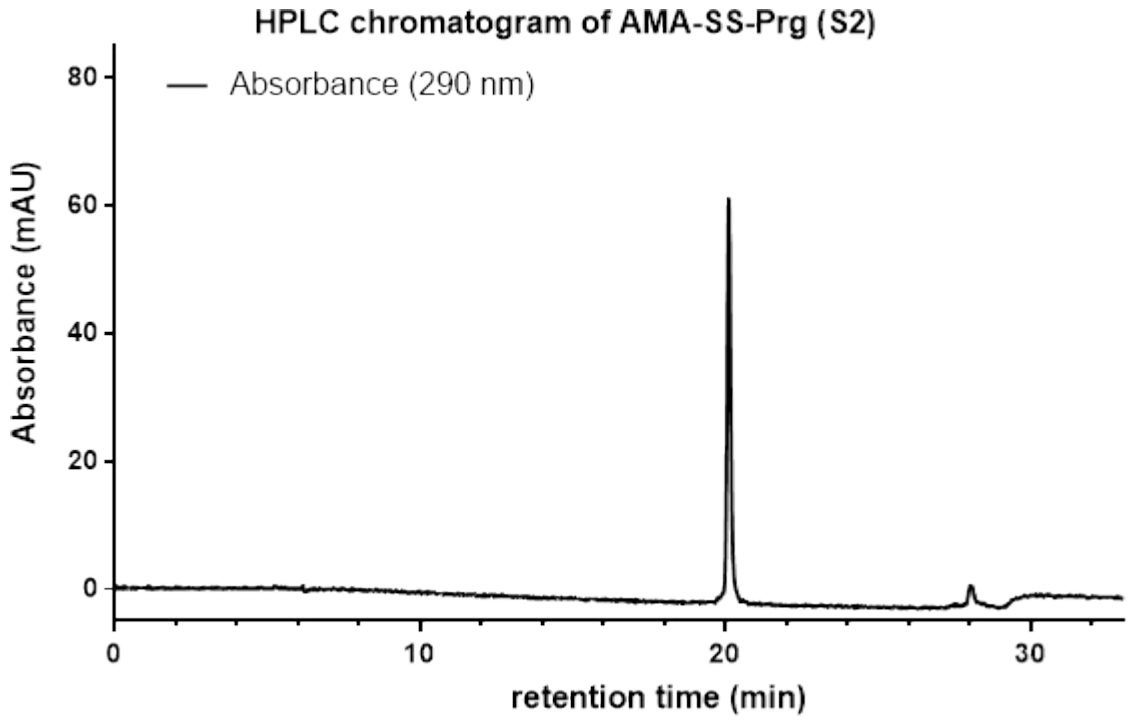
Synthesis and characterization of compound **S2**

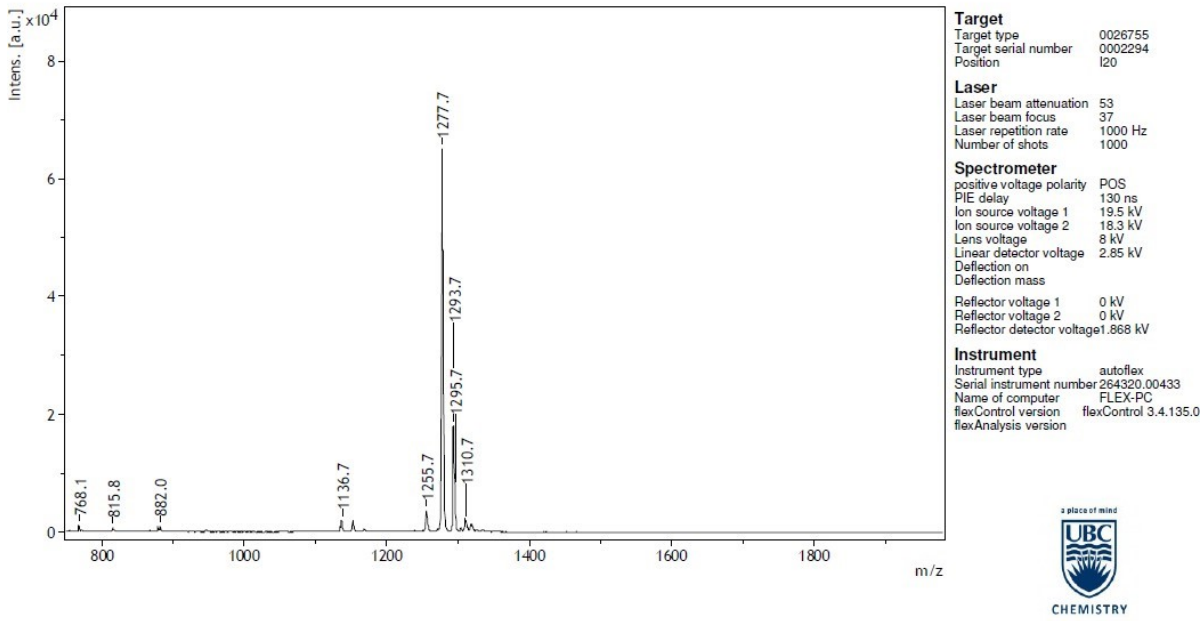


Ama-SS-Prg (**S2**). A Falcon tube was charged with lyophilized compound **20** (1 eq., 360 µg, 265 nmol) dissolved in 90 µL of DMSO followed by 10 µL of aqueous 3M KHF_2 (excess). The reaction was run at 60 °C for 1 hour using a heat block and a sand bath. The reaction progress was analyzed by HPLC using method G. Upon completion, the mixture was concentrated, re-dissolved in 300 µL of MeOH/H_2O/ACN (1:4:1) and purified using C18 SEP-PAK (gradient elution, 5:95 ACN:H_2O to 70:40 ACN/H_2O in the presence of 0.1% formic acid). The product-containing fractions were lyophilized to obtain 279 µg (222 nmol) of **S2** as a white powder in 84% isolated yield. Quantification was done using extinction coefficient ε = 10,000 $mol^{-1}Lcm^{-1}$ in methanol at 290 nm.

MALDI-TOF (m/z): $[M+Na]^+$ found 1277.7; calc. 1278.4 for $C_{53}H_{73}N_{15}O_{15}S_3Na$.

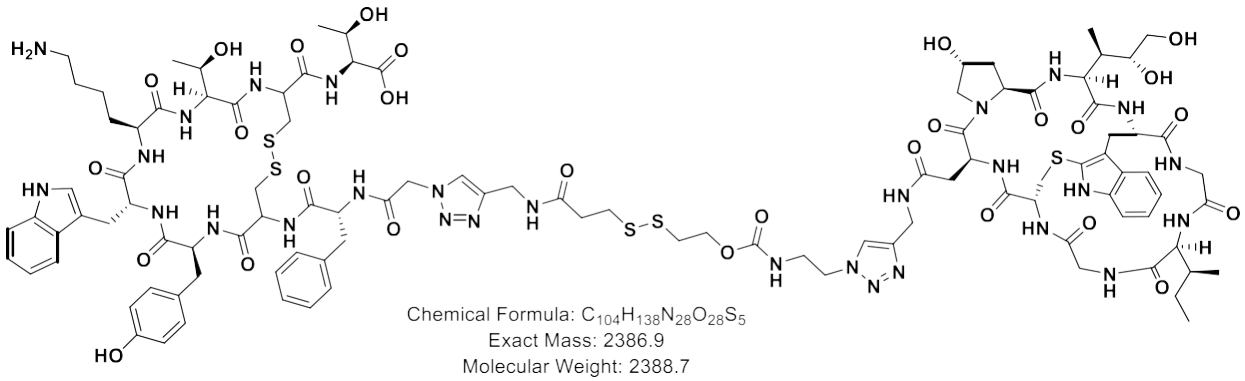
HPLC (gradient **G**) $\mathbf{t_R}$ = 20.0 min; λ_{max} = 290 nm.





Date of Acquisition 2017-10-19T15:54:27.350-07:00
Acquisition method D:\Bruker_service\April 6 2015\Compass 1.4 April 2015\Methods\flexControlMethods\LP_700-2000_Da.par
Samples Name
File Name D:\Data\Perrin\Alla\Amanitin-link-notes\EL12951\0_I20\1\1SLin
printed: 7/6/2018 4:01:11 PM

Synthesis and characterization of compound **6**

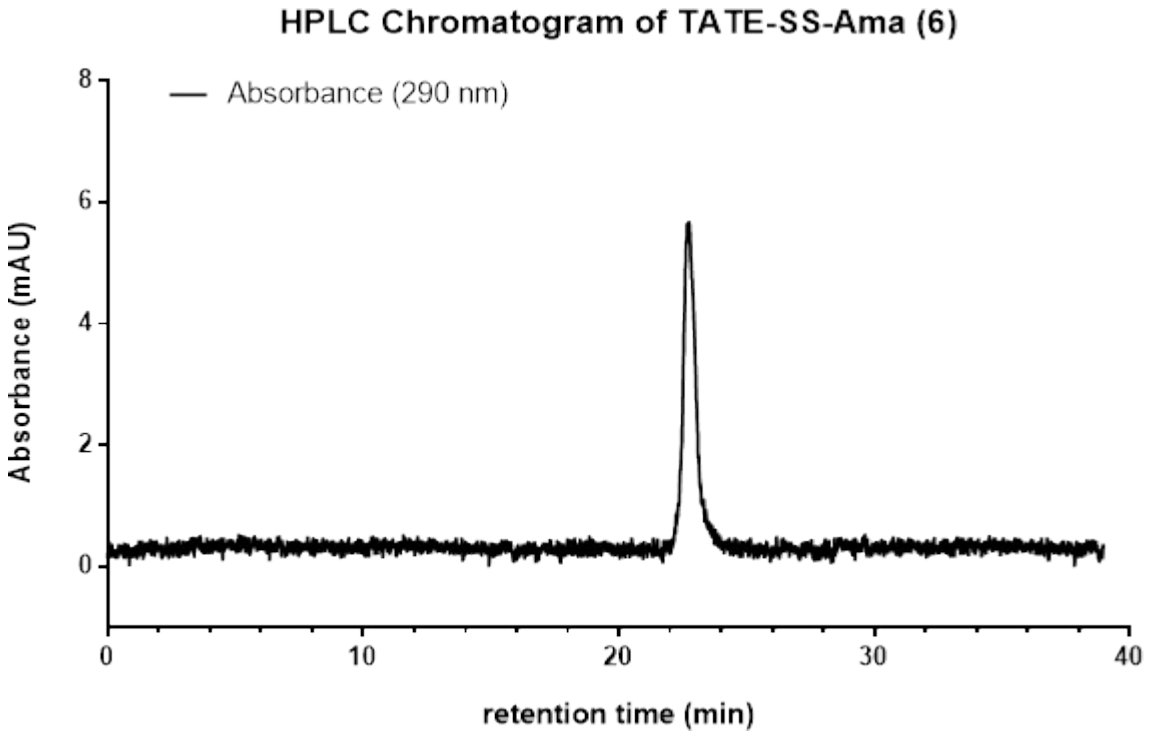


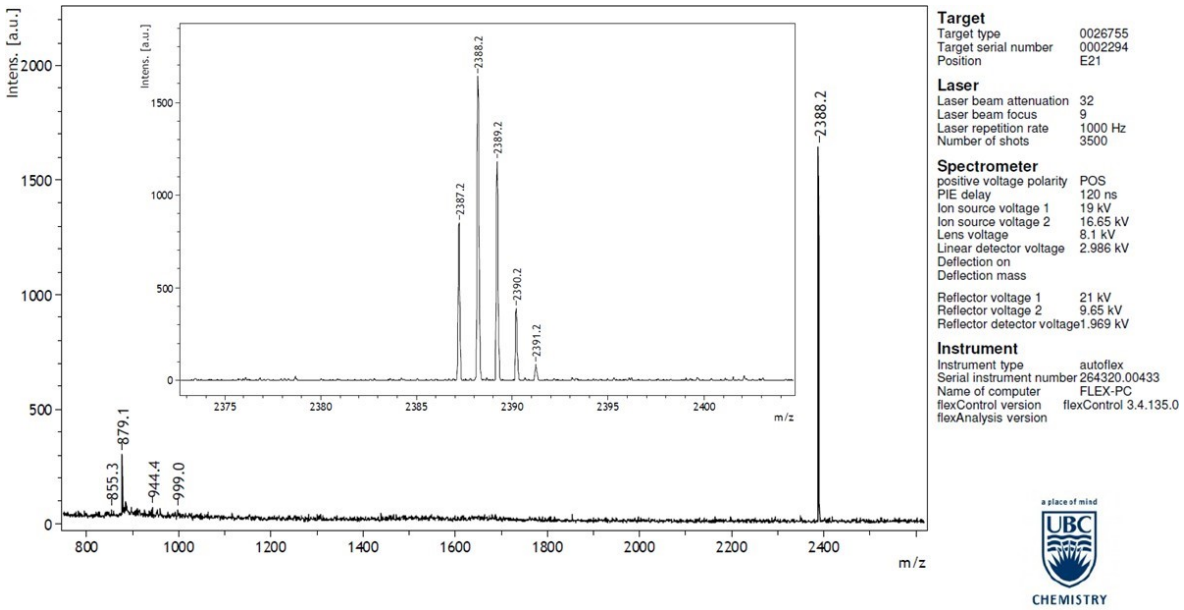
TATE-SS-Amanitin (6). CuAAC conjugation of compound **S2** (1 eq., 279 µg, 222 nmol) to TATE-N_3 **2** (1.5 eq., 380 µg, 333 nmol) was carried out following the protocol described for the synthesis of compound **19**. Upon completion, reaction contents were concentrated under reduced pressure, dissolved in 0.1% formic acid ACN/H_2O, and purified by HPLC using gradient J. Pure material was lyophilized to obtain 353 µg (148 nmol) of **6** as a white powder in 67% isolated yield. Quantification was done using extinction coefficient ε = 13,308 $mol^{-1}Lcm^{-1}$ in methanol at 290 nm.

Note: 0.1% TFA has to be used as an additive during the HPLC purification for efficient separation; use of formic acid as an additive results in poor separation.

MALDI-TOF (m/z): $[M+H]^+$ found 2388.2; calc. 2387.9 for $C_{104}H_{139}N_{28}O_{28}S_5$.

HPLC (gradient **J**) $\mathbf{t_R}$ = 22.8 min; λ_{max} = 290 nm.


HPLC Chromatogram of TATE-SS-Ama (6)
Absorbance (290 nm)
Absorbance (mAU)
retention time (min)
0
2
4
6
8
0
10
20
30
40


Intens. [a.u.]
m/z
2387.2
2388.2
2389.2
2390.2
2391.2
879.1
855.3
944.4
999.0
Target
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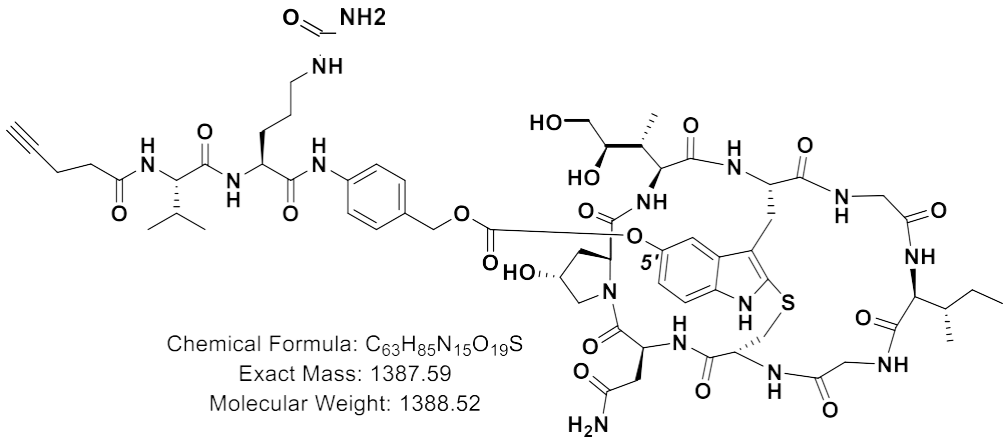
Target serial number 0002294
Position E21
Laser
Laser beam attenuation 32
Laser beam focus 9
Laser repetition rate 1000 Hz
Number of shots 3500
Spectrometer
positive voltage polarity POS
PIE delay 120 ns
Ion source voltage 1 19 kV
Ion source voltage 2 16.65 kV
Lens voltage 8.1 kV
Linear detector voltage 2.986 kV
Deflection on
Deflection mass
Reflector voltage 1 21 kV
Reflector voltage 2 9.65 kV
Reflector detector voltage 1.969 kV
Instrument
Instrument type autoflex
Serial instrument number 264320.00433
Name of computer FLEX-PC
flexControl version flexControl 3.4.135.0
flexAnalysis version
a place of mind
UBC
CHEMISTRY

Synthesis and characterization of compound **21**

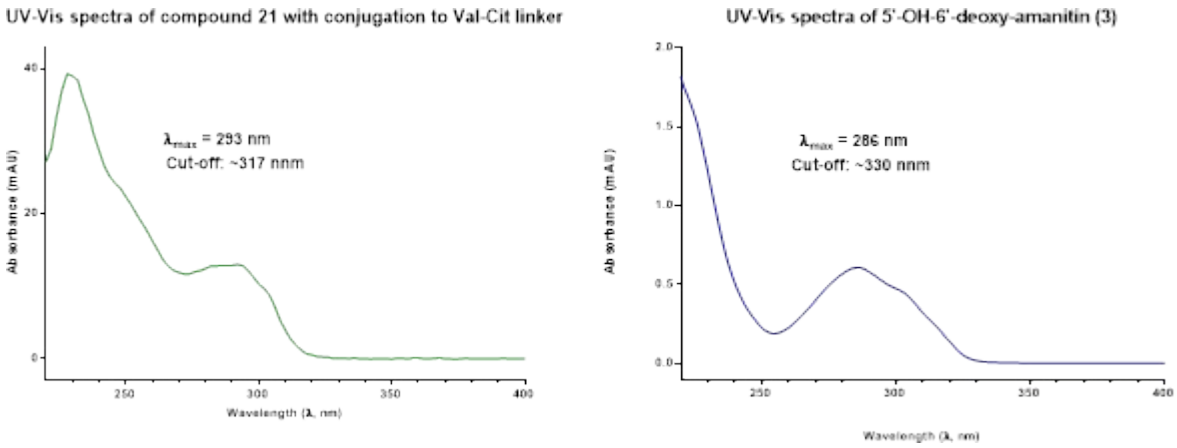


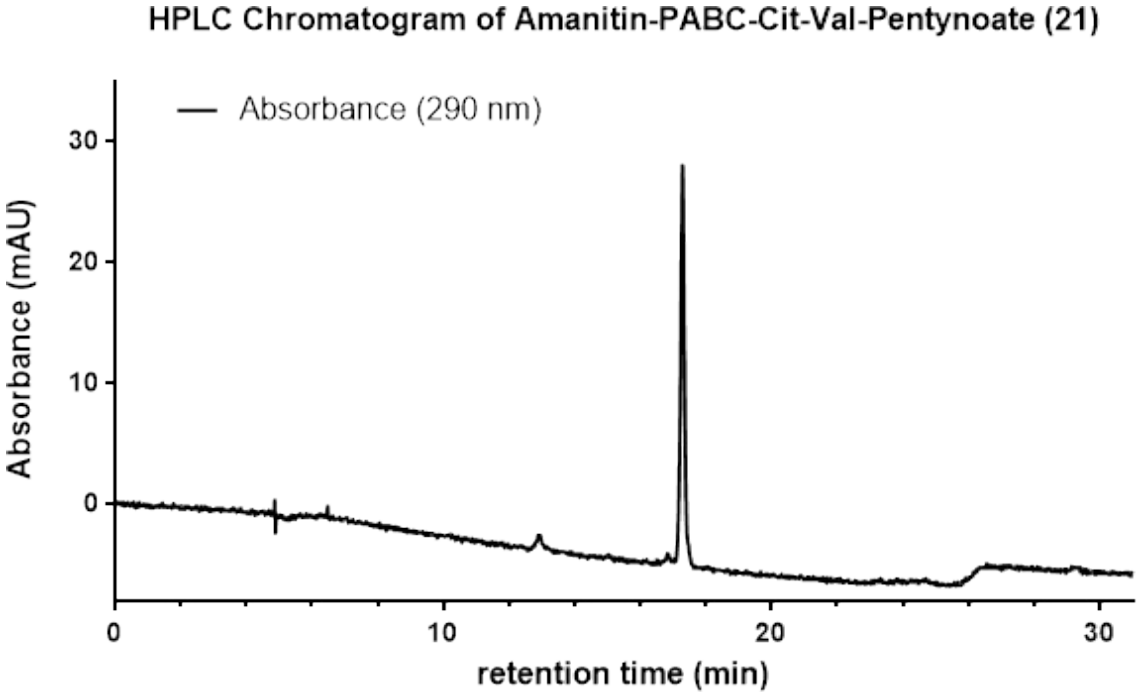
Amanitin-PABC-Cit-Val-Pentynoate (**21**). A 1.5-mL Eppendorf tube containing Cs_2CO_3 (2.5 eq., 174 µg, 535 nmol) was charged with 5'-OH-6'-deoxy-amanitin **3** (1 eq., 194 µg, 215 nmol) dissolved in 40 µL of pyridine. The reaction was heated at 60 °C for 40 minutes and then pyridine was evaporated under reduced pressure. Next, linker **18** (3.5 eq., 453 µg, 752 nmol) dissolved in 11.3 µL of pyridine was added to the reaction vessel containing Cs_2CO_3/amanitin solid, followed by DMAP (1 eq., 28 µg, 215 nmol) dissolved in 3.5 µL of pyridine. The reaction contents were stirred for 1 hour at 65 °C. The reaction contents were concentrated under reduced pressure, re-dissolved in DMF/ACN/H_2O, and purified using HPLC gradient A. The product was lyophilized to obtain 113 µg (82 nmol) of **21** as a white powder in 38% isolated yield. Quantification was done using extinction coefficient ε = 10,000 $mol^{-1}Lcm^{-1}$ in methanol at 290 nm.

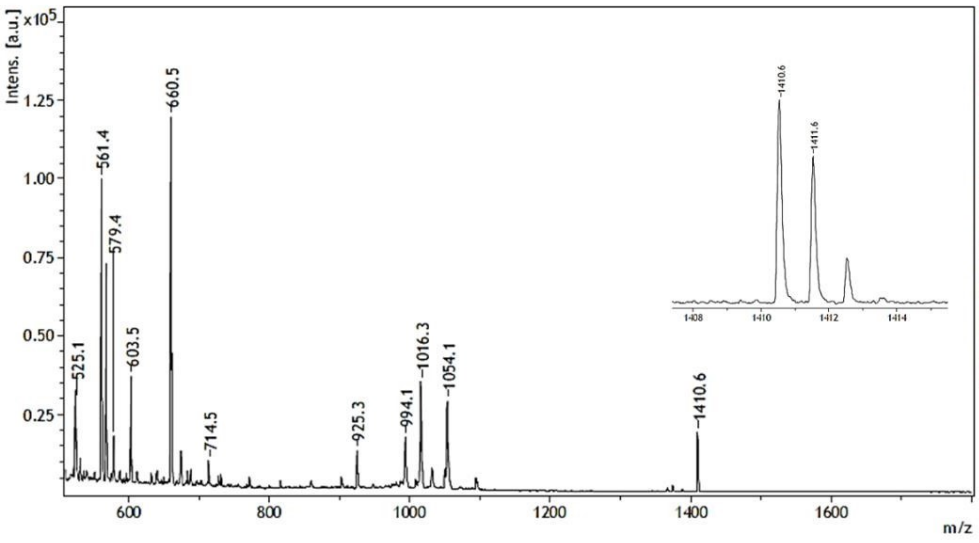
Note: pyridine used for this reaction was stirred with 4-toluenesulfonyl chloride (~2 g for 50 mL of pyridine) for 20 hours to quench any amine impurities that could result in linker degradation. This treatment was followed by vacuum distilled.

MALDI-TOF (m/z): $[M+Na]^+$ found 1410.6; calc. 1410.6 for $C_{63}H_{85}N_{15}O_{19}SNa$.

HPLC (gradient **A**) $\mathbf{t_R}$ = 17.3 min; λ_{max} = 290 nm.


UV-Vis spectra of compound 21 with conjugation to Val-Cit linker
λmax = 293 nm
Cut-off: ~317 nnm
Absorbance (mAU)
Wavelength (λ, nm)
UV-Vis spectra of 5'-OH-6'-deoxy-amanitin (3)
λmax = 286 nm
Cut-off: ~330 nnm
Absorbance (mAU)
Wavelength (λ, nm)


HPLC Chromatogram of Amanitin-PABC-Cit-Val-Pentynoate (21)
Absorbance (290 nm)
Absorbance (mAU)
retention time (min)



Target

Target type	0026755
Target serial number	0002294
Position	H21

Laser

Laser beam attenuation	36
Laser beam focus	9
Laser repetition rate	1000 Hz
Number of shots	8000

Spectrometer

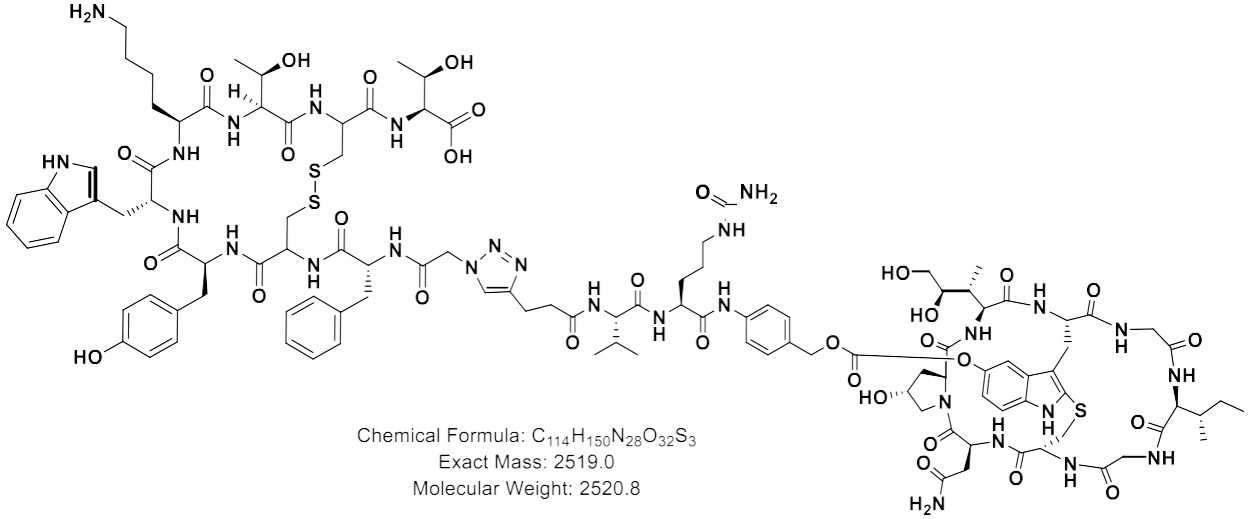
positive voltage polarity	POS
PIE delay	120 ns
Ion source voltage 1	19 kV
Ion source voltage 2	16.65 kV
Lens voltage	8.1 kV
Linear detector voltage	2.71 kV
Deflection on	
Deflection mass	
Reflector voltage 1	21 kV
Reflector voltage 2	9.65 kV
Reflector detector voltage	2.074 kV

Instrument

Instrument type	autoflex
Serial instrument number	264320.00433
Name of computer	FLEX-PC
flexControl version	flexControl 3.4.135.0
flexAnalysis version	

a place of mind
UBC
CHEMISTRY

Synthesis and characterization of compound **7**

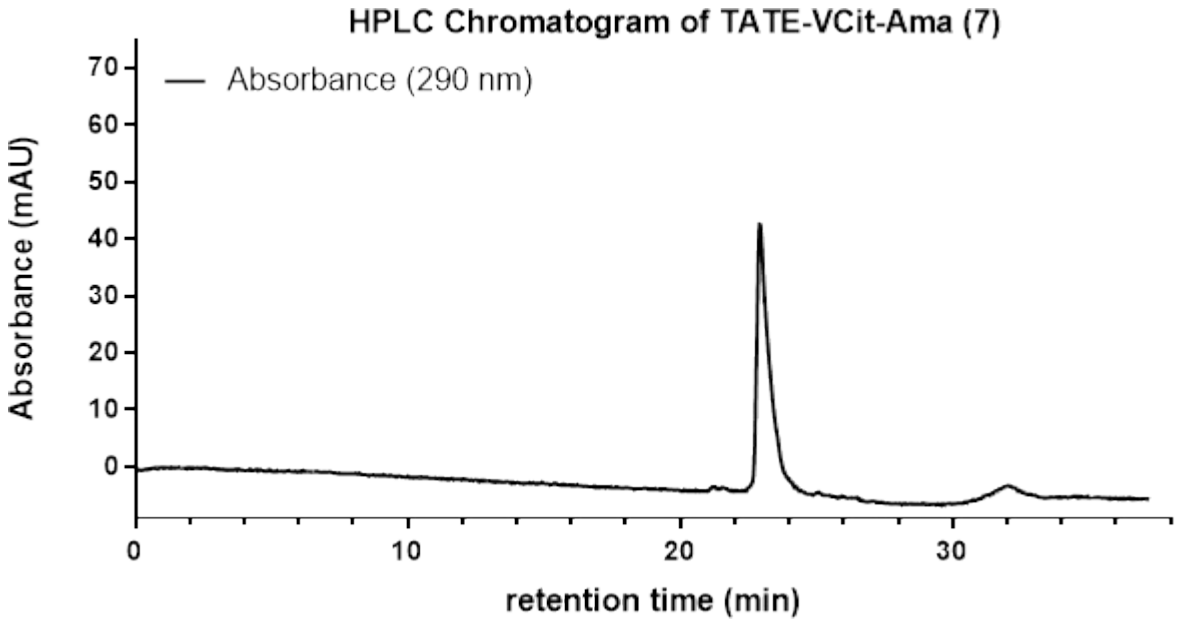


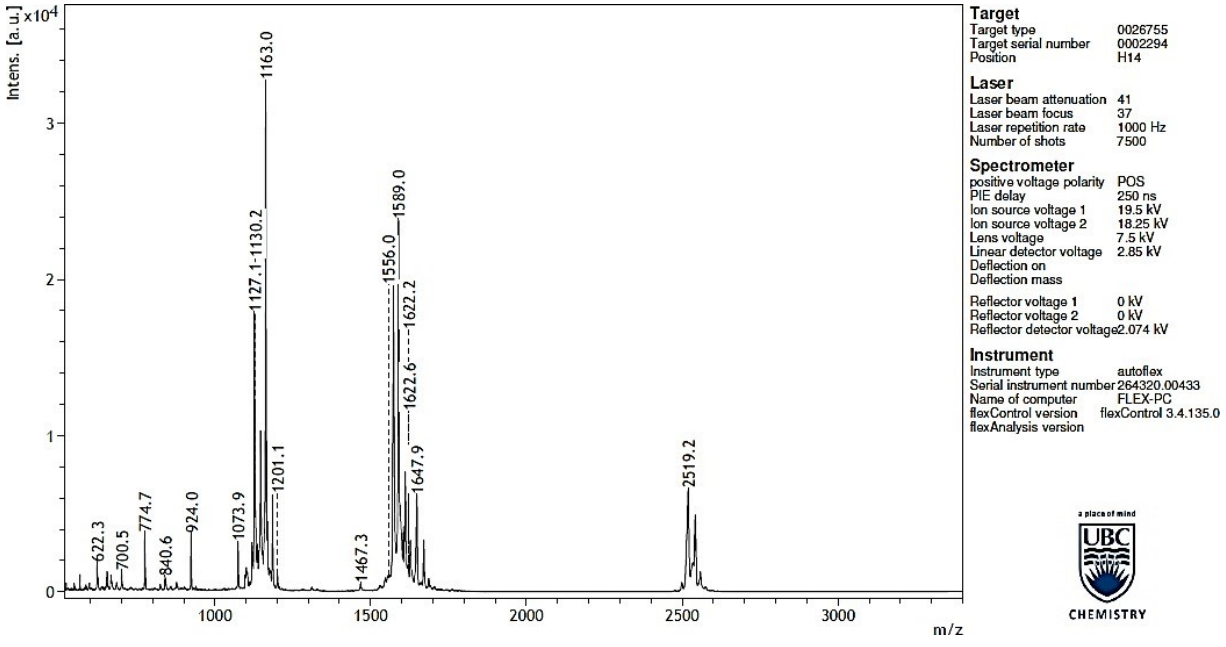
TATE-VCit-Amanitin (**7**). CuAAC conjugation of compound **21** (1 eq., 113 µg, 82 nmol) to TATE-N_3 **2** (1.5 eq., 194 µg, 170 nmol) was carried out following the protocol described for the synthesis of compound **19**. Upon completion, reaction contents were concentrated under reduced pressure, dissolved in 0.1% formic acid ACN/H_2O, and purified by HPLC using gradient J. Pure material was lyophilized to obtain 133 µg (53 nmol) of **7** as a white powder in 65% isolated yield. Quantification was done using extinction coefficient ε = 13,308 $mol^{-1}Lcm^{-1}$ in methanol at 290 nm.

Note: 0.1% TFA has to be used as an additive during the HPLC purification for effective separation; using formic acid as an additive results in poor separation.

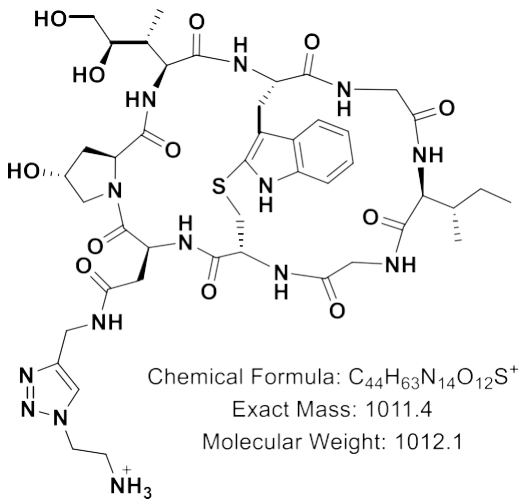
MALDI-TOF (m/z): $[M+H]^+$ found 2519.2; calc. 2520.0 for $C_{114}H_{151}N_{28}O_{32}S_3$.

HPLC (gradient **J**) $\mathbf{t_R}$ = 23.0 min; λ_{max} = 290 nm.


HPLC Chromatogram of TATE-VCit-Ama (7)
Absorbance (290 nm)
Absorbance (mAU)
retention time (min)


Intens. [a.u.]
x10^4
m/z
622.3
700.5
774.7
840.6
924.0
1073.9
1127.1-1130.2
1163.0
1201.1
1467.3
1556.0
1589.0
1622.2
1622.6
1647.9
2519.2
Target
Target type 0026755
Target serial number 0002294
Position H14
Laser
Laser beam attenuation 41
Laser beam focus 37
Laser repetition rate 1000 Hz
Number of shots 7500
Spectrometer
positive voltage polarity POS
PIE delay 250 ns
Ion source voltage 1 19.5 kV
Ion source voltage 2 18.25 kV
Lens voltage 7.5 kV
Linear detector voltage 2.85 kV
Deflection on
Deflection mass
Reflector voltage 1 0 kV
Reflector voltage 2 0 kV
Reflector detector voltage 2.074 kV
Instrument
Instrument type autoflex
Serial instrument number 264320.00433
Name of computer FLEX-PC
flexControl version flexControl 3.4.135.0
flexAnalysis version
a place of mind
UBC
CHEMISTRY

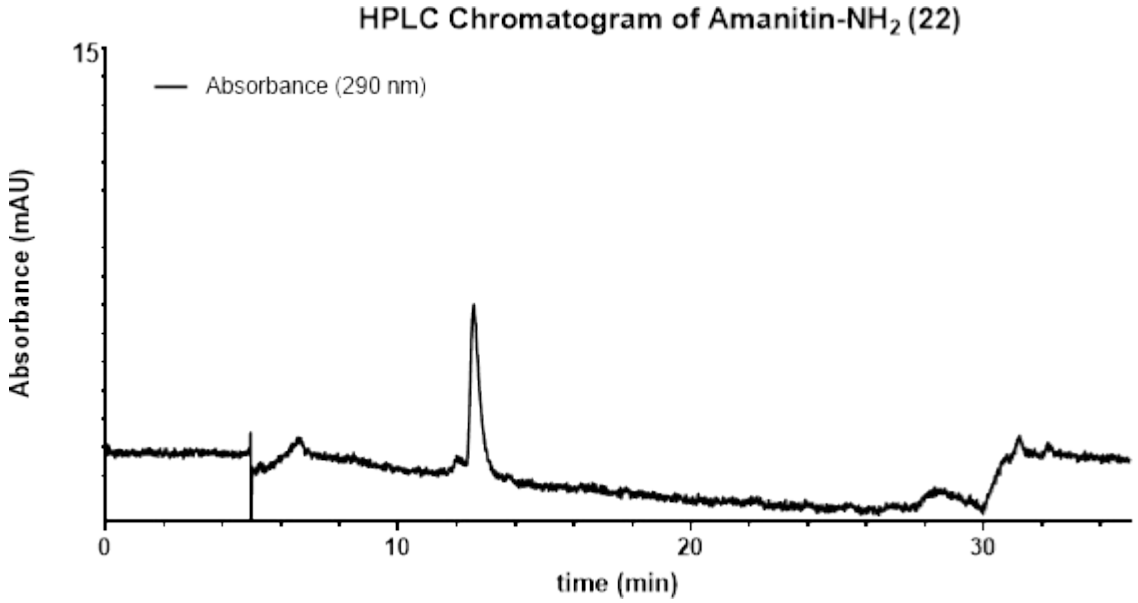
Synthesis and characterization of compound **22**

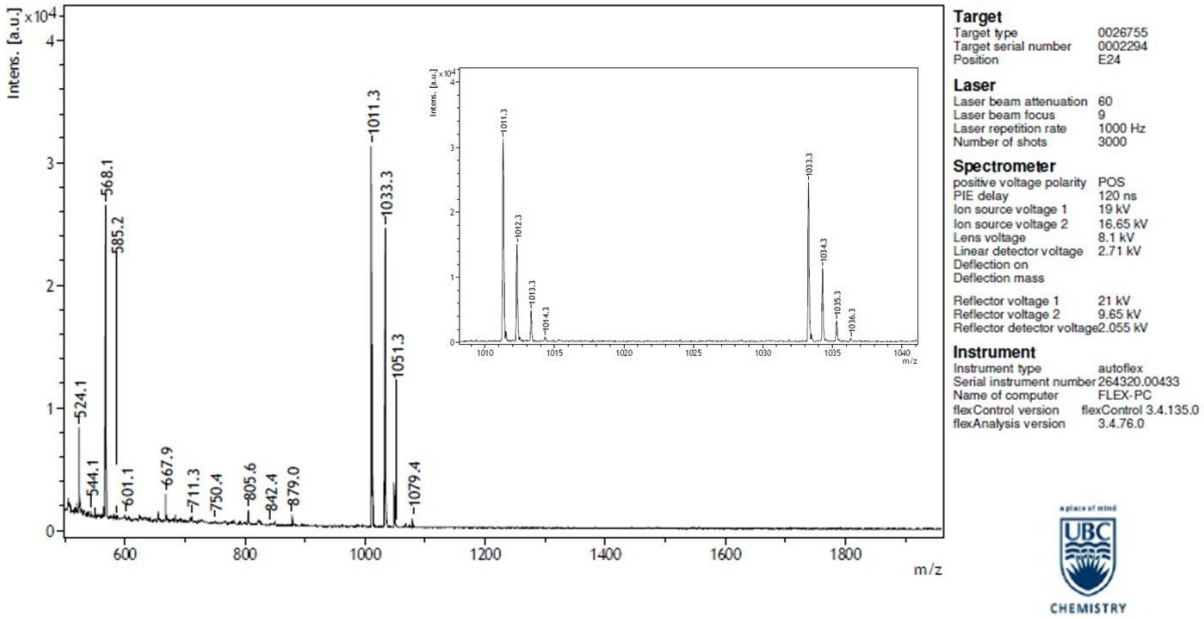


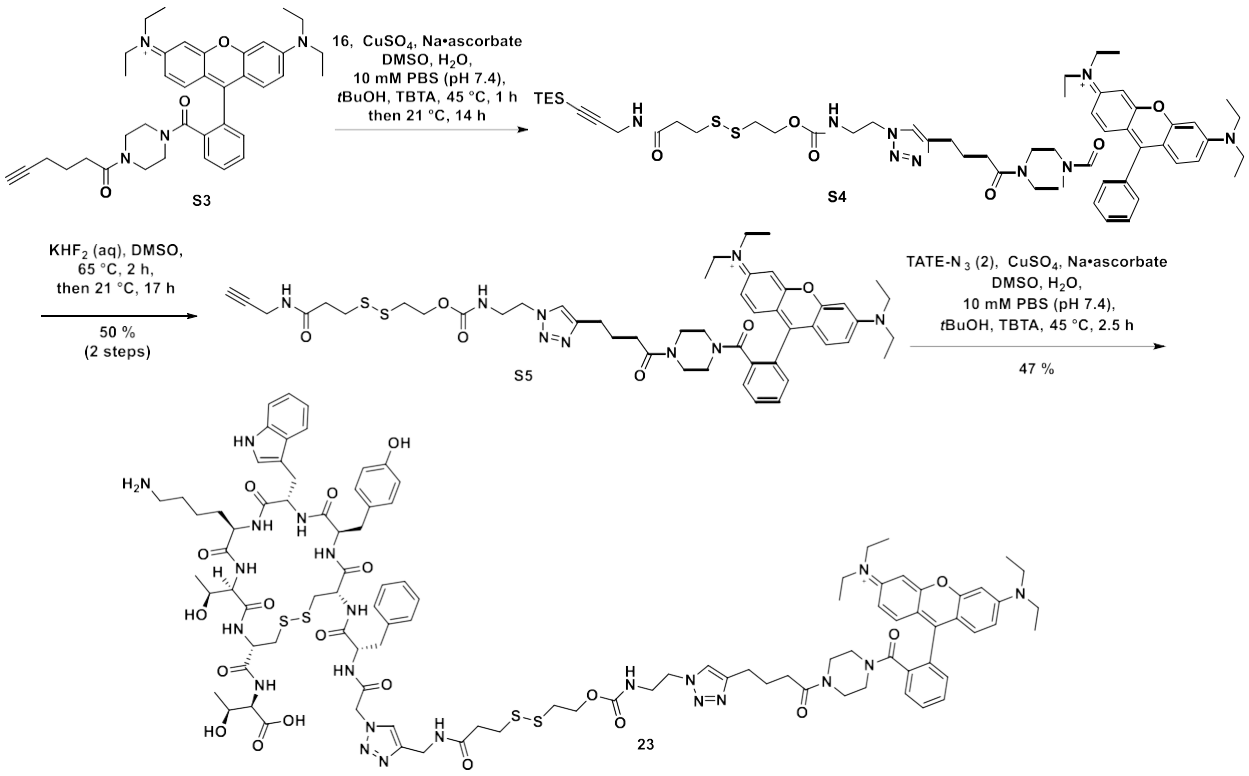
Amanitin-NH_2 (22). A 15-mL Falcon tube was charged with peptide-SS-Amanitin (1 eq., 80 nmol) dissolved in 8.0 mL of 10 mM PBS pH 7.4 buffer followed by dithiothreitol (30 eq., 2.4 µmol) in 13.2 µL of 10 mM PBS pH 7.4 buffer. The reaction mixture was incubated at 37 °C for 17 hours. The reaction contents were lyophilized, redissolved in 1:1 MeOH:H_2O, and purified by HPLC using gradient K. The product was lyophilized to obtain 39 µg (38 nmol) of **22** in 48% isolated yield. Quantification was done using extinction coefficient ε = 10,000 $mol^{-1}Lcm^{-1}$ in methanol at 290 nm.

MALDI-TOF (m/z): $[M+H]^+$ found 1011.3; calc. 1011.4 for $C_{44}H_{63}N_{14}O_{12}S$.

HPLC (gradient **K**) $\mathbf{t_R}$ = 12.8 min; λ_{max} = 290 nm.

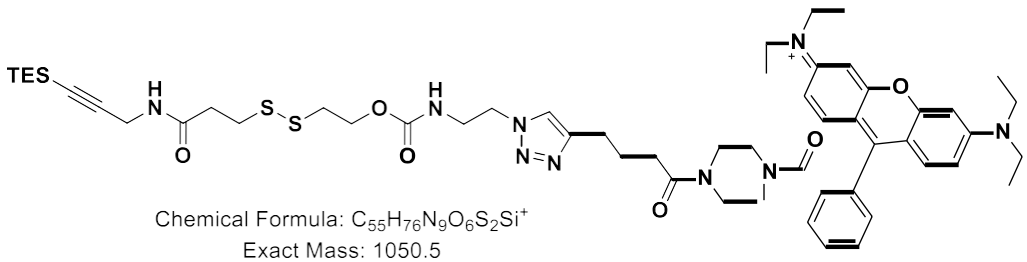

HPLC Chromatogram of Amanitin-NH2 (22)
Absorbance (290 nm)
Absorbance (mAU)
15
0
10
20
30
time (min)


Intens. [a.u.]
x10^4
524.1
544.1
568.1
585.2
601.1
667.9
711.3
750.4
805.6
842.4
879.0
1011.3
1033.3
1051.3
1079.4
m/z
Target
Target type 0026755
Target serial number 0002294
Position E24
Laser
Laser beam attenuation 60
Laser beam focus 9
Laser repetition rate 1000 Hz
Number of shots 3000
Spectrometer
positive voltage polarity POS
PIE delay 120 ns
Ion source voltage 1 19 kV
Ion source voltage 2 16.65 kV
Lens voltage 8.1 kV
Linear detector voltage 2.71 kV
Deflection on
Deflection mass
Reflector voltage 1 21 kV
Reflector voltage 2 9.65 kV
Reflector detector voltage 2.055 kV
Instrument
Instrument type autoflex
Serial instrument number 264320.00433
Name of computer FLEX-PC
flexControl version flexControl 3.4.135.0
flexAnalysis version 3.4.76.0
UBC
CHEMISTRY



Scheme S1. Synthesis of conjugate TATE-SS-Rhod **23** for use in fluorescence microscopy studies.

Synthesis and characterization of compound **S4**

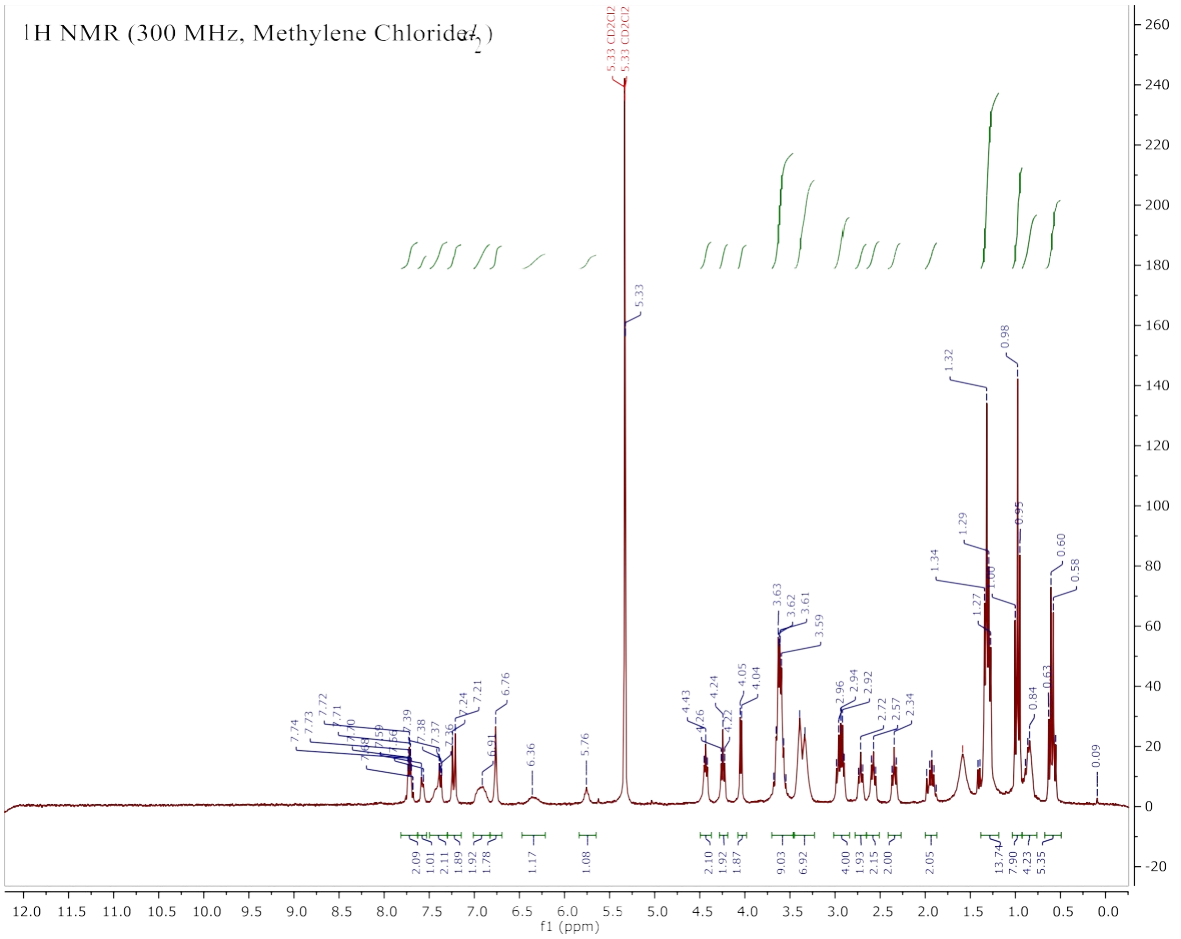


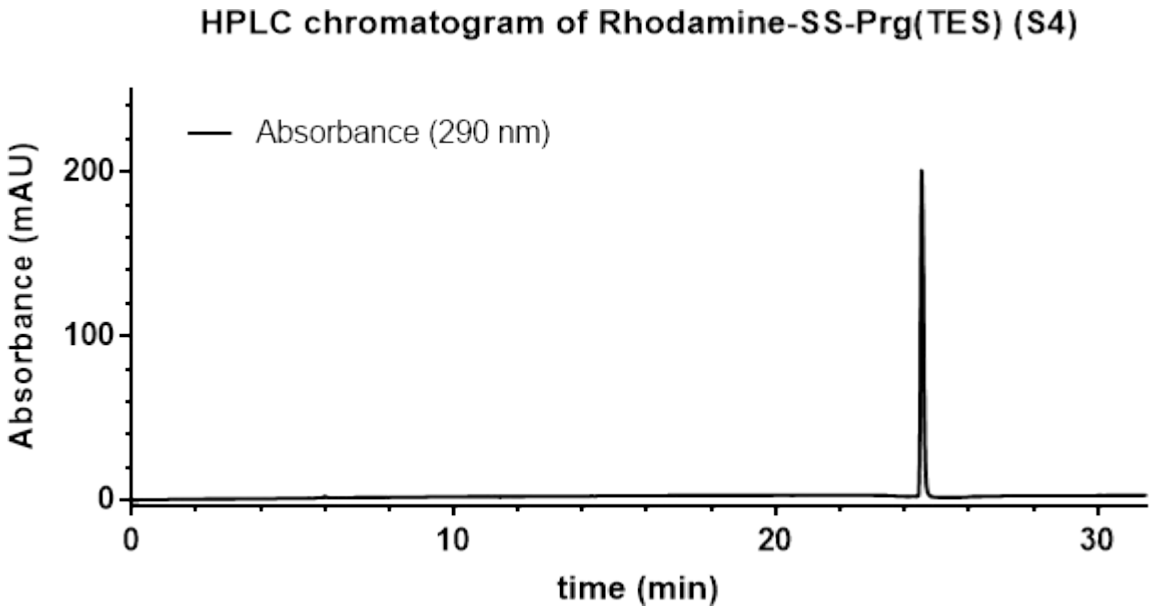
Rhodamine-SS-Prg(TES) (**S4**). Click Conjugation. $CuSO_4$/TBTA/Na-ascorbate solution was prepared first in the following manner: 20 µL of 400 mM aqueous $CuSO_4$ (0.5 eq., 1.3 mg, 8.26 µmol) were combined with 41 µL of 200 mM TBTA solution in DMSO (0.5 eq, 4.35 mg, 8.26 µmol) and allowed to rest for 5 mins. The blue clear $CuSO_4$/TBTA solution was then mixed with 13.5 µL of DMSO and 33 µL of 800 mM aqueous sodium ascorbate (1.6 eq., 5.2 mg, 26.4 µmol) to give a light beige clear solution. A 5-mL round bottom flask was charged with rhodamine-hexynoate **S3** (1 eq., 10 mg, 16.52 µmol) followed by bio-reducible disulfide linker **16** (1.4 eq., 10.3 mg, 23.14 µmol) dissolved in 500 µL of *t*BuOH, 130 µL of H_2O, and 140 µL of 10 mM pH 7.4 PBS buffer. The mixture was sonicated at 45 until all of the materials were dissolved. $CuSO_4$/TBTA/Na-ascorbate mixture was then added to the reaction flask and the reaction contents were sonicated at 45 C for 1 hour and stirred at 21 for 14 hours. The reaction contents were concentrated under reduced pressure, re-dissolved in 0.1% formic acid ACN/H_2O, and purified using gradient A. The product was lyophilized to obtain 9.2 mg (8.76 µmol) of **S4** as a pink solid in 53% isolated yield. Quantification was done using extinction coefficient ε = 78,700 $mol^{-1}Lcm^{-1}$ in ethanol at 560 nm.

^{1}H NMR ^{1}H NMR (300 MHz, Methanol-d_4) δ 7.58 (d, J = 8.5 Hz, 2H), 7.32 (d, J = 8.5 Hz, 2H), 4.63 – 4.46 (m, 3H), 4.25 (d, J = 7.3 Hz, 1H), 3.28 – 3.06 (m, 2H), 2.53-2.50 (m, 4H), 2.30 (t, J = 1.6 Hz, 1H), 2.10 (p, J = 6.9 Hz, 1H), 2.02 – 1.84 (m, 1H), 1.84 – 1.70 (m, 1H), 1.60 (dt, J = 8.9, 6.7 Hz, 2H), 1.00 (dd, J = 6.7, 3.3 Hz, 6H).

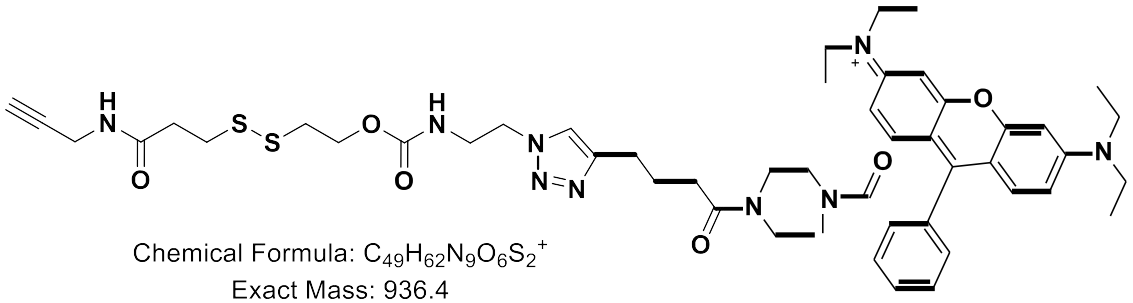
HRMS (ESI-TOF, m/z): $[M]^+$ found 1050.5128; calc. 1050.5129 for $C_{55}H_{76}N_9O_6S_2Si$.

HPLC (gradient **A**) $\mathbf{t_R}$ = 24.6 min; λ_{max} = 562 nm.


1H NMR (300 MHz, Methylene Chloride-d2)
f1 (ppm)


HPLC chromatogram of Rhodamine-SS-Prg(TES) (S4)
Absorbance (290 nm)
Absorbance (mAU)
time (min)

Synthesis and characterization of compound **S5**

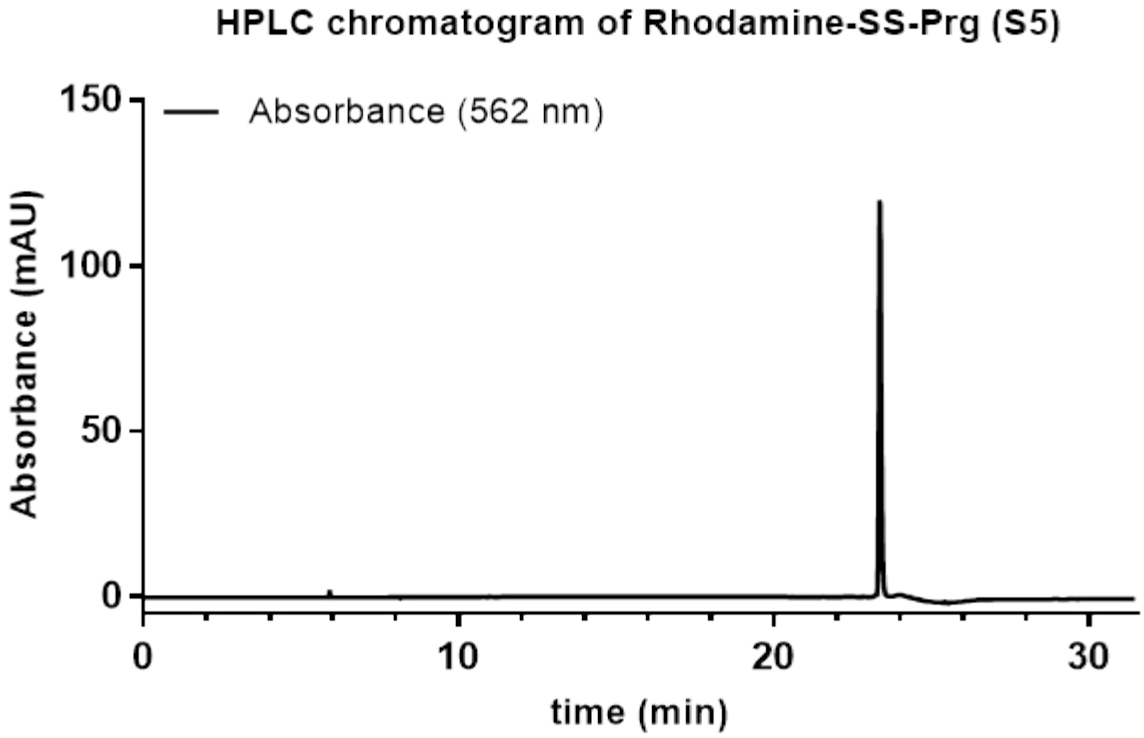


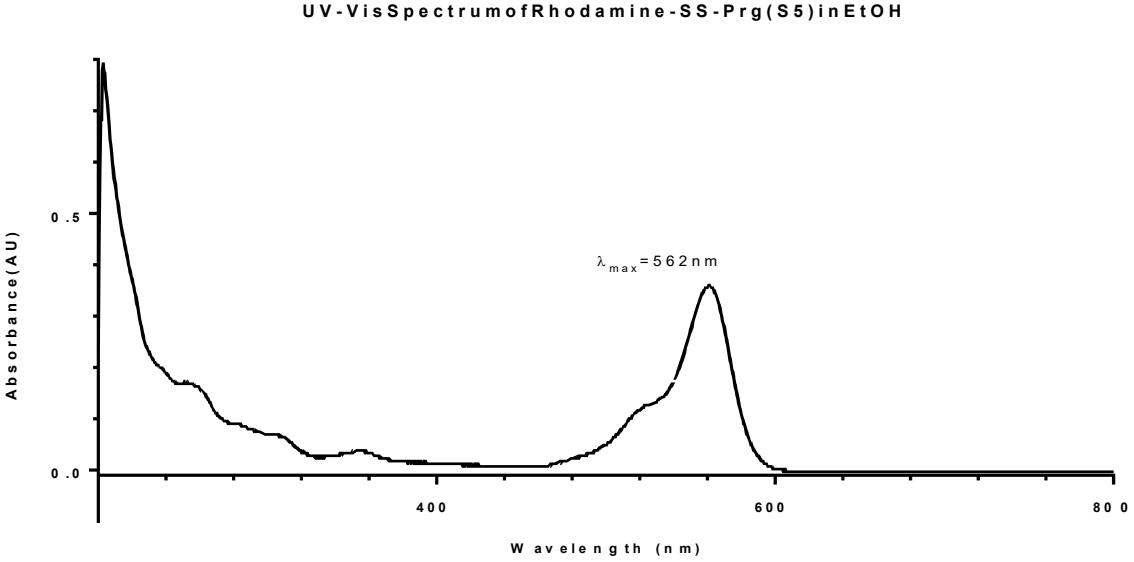
Chemical Formula: $C_{49}H_{62}N_9O_6S_2^+$
Exact Mass: 936.4
Molecular Weight: 937.2

Rhodamine-SS-Prg (**S5**). A 1.5-mL Eppendorf tube was charged with Rhodamine-SS-Prg(TES) (**S4**) (1 eq., 4.2 mg, 4 µmol) in 100 µL of DMSO followed by 10 µL of aqueous 3M KHF_2 (excess). The reaction mixture was warmed to 65 °C to give a clear pink solution. The reaction was run for 2 hours at 65 °C followed by 17 hours at 21 °C. Upon completion, the reaction contents were diluted with DMC (8 mL) and washed with milliQ water (3x4 mL) and brine (1x4 mL). The organic layer was concentrated under reduced pressure and HPLC analysis of the product was performed using gradient **A**. The product was found to be ~95% pure based on the HPLC analysis. The product was carried to the next step without further purification. Quantification was done using extinction coefficient ε = 78,700 $mol^{-1}Lcm^{-1}$ in ethanol at 560 nm.

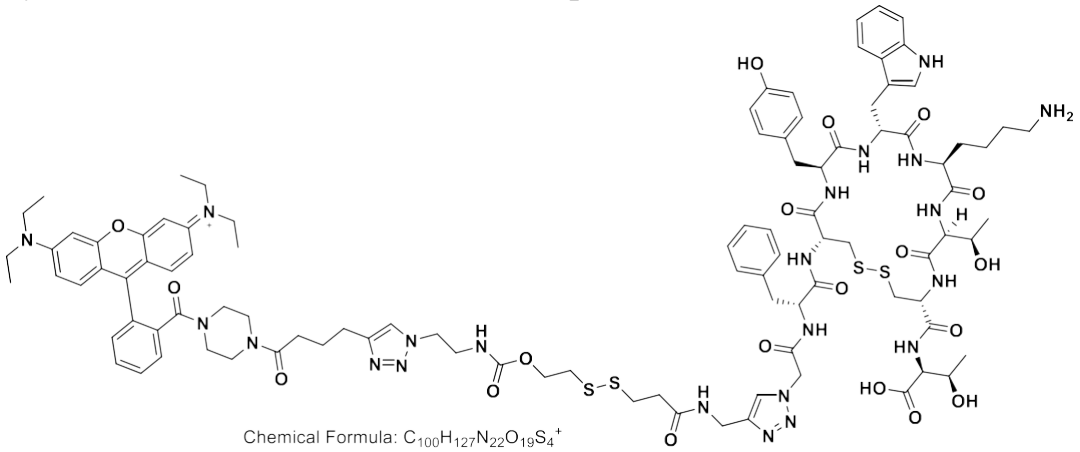
LRMS (ESI, m/z): $[M]^+$ found 936.6; calc.936.4 for $C_{49}H_{62}N_9O_6S_2$.

HPLC (gradient **A**) $\mathbf{t_R}$ = 23.2 min; λ_{max} = 562 nm.


HPLC chromatogram of Rhodamine-SS-Prg (S5)
Absorbance (562 nm)
Absorbance (mAU)
150
100
50
0
0
10
20
30
time (min)


UV-VisSpectrumofRhodamine-SS-Prg(S5)inEtOH
Absorbance(AU)
0.5
0.0
λmax=562nm
400
600
800
Wavelength (nm)

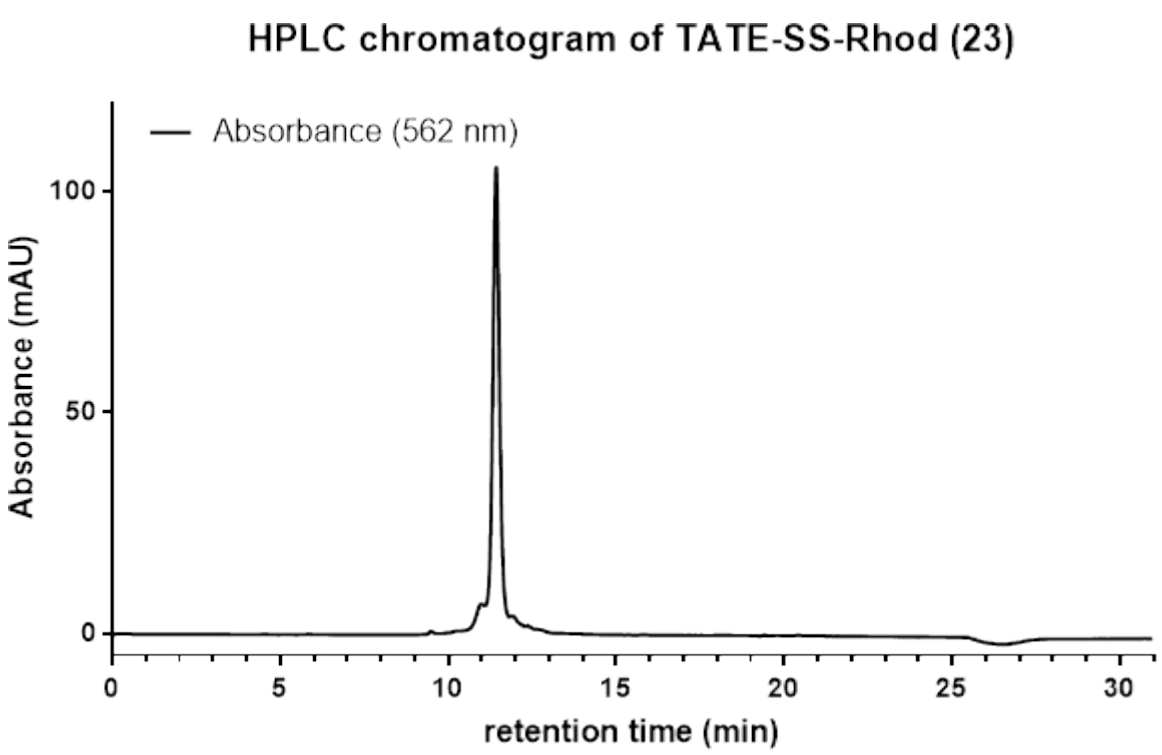
Synthesis and characterization of compound **23**

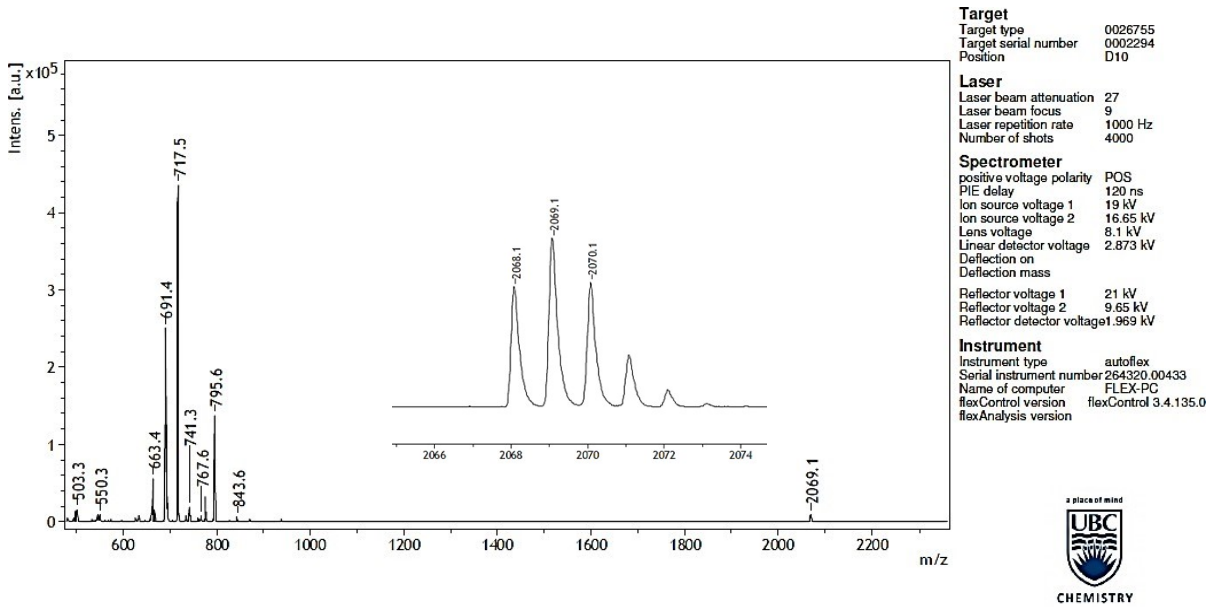


TATE-SS-Rhodamine (**23**). $CuSO_4$/TBTA/Na-ascorbate solution was prepared first in the following manner: aqueous solution of $CuSO_4$ (0.74 eq., 0.2 mg, 10 µL, 1.23 µmol) was combined with and TBTA (0.74 eq, 0.65 mg, 15 µL, 1.23 µmol) dissolved in DMSO and allowed to rest for 5 mins. The clear blue $CuSO_4$/TBTA solution was then mixed with 5 µL of DMSO and aqueous sodium ascorbate (2.37 eq., 0.78 mg, 15 µL, 3.95 µmol) to give a light beige clear solution. A 1.5-mL Eppendorf tube was charged with Rhodamine-SS-Prg **S5** (1.2 eq., 1.9 mg, 2.0 µmol) followed by TATE-N_3 **2** (1 eq., 2.0 mg, 1.67 µmol) dissolved in 350 µL of *t*BuOH, 50 µL of H_2O, and 100 µL of 10 mM pH 7.4 PBS buffer. The mixture was sonicated at 45 C until all of the materials were dissolved. $CuSO_4$/TBTA/Na-ascorbate mixture was then added to the reaction flask and the reaction contents were agitated in a sonicator at 45 °C for 2.5 hours. The reaction contents were concentrated under reduced pressure, re-dissolved in 0.1% TFA ACN/H_2O, and purified using gradient **B**. The material was lyophilized to obtain 1.6 mg (785 nmol mmol) of **23** as a pink solid in 47% yield. Quantification was done using extinction coefficient ε = 78,700 $mol^{-1}Lcm^{-1}$ in ethanol at 560 nm.

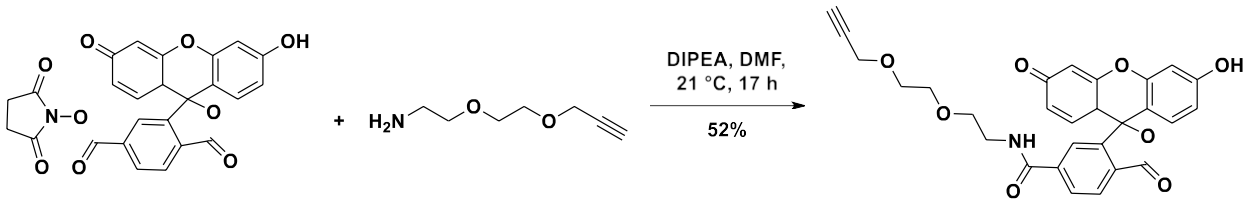
MALDI-TOF (m/z): $[M]^+$ found 2069.1; calc. 2068.9 for $C_{100}H_{127}N_{22}O_{19}S_4$.

HPLC (gradient **B**) $\mathbf{t_R}$ = 11.4 min; λ_{max} = 562 nm.


HPLC chromatogram of TATE-SS-Rhod (23)
Absorbance (562 nm)
Absorbance (mAU)
100
50
0
0
5
10
15
20
25
30
retention time (min)


Intens. [a.u.]
x10^5
5
4
3
2
1
0
503.3
550.3
663.4
691.4
717.5
741.3
767.6
795.6
843.6
2069.1
600
800
1000
1200
1400
1600
1800
2000
2200
m/z
2068.1
2069.1
2070.1
2066
2068
2070
2072
2074
Target
Target type 0026755
Target serial number 0002294
Position D10
Laser
Laser beam attenuation 27
Laser beam focus 9
Laser repetition rate 1000 Hz
Number of shots 4000
Spectrometer
positive voltage polarity POS
PIE delay 120 ns
Ion source voltage 1 19 kV
Ion source voltage 2 16.65 kV
Lens voltage 8.1 kV
Linear detector voltage 2.873 kV
Deflection on
Deflection mass
Reflector voltage 1 21 kV
Reflector voltage 2 9.65 kV
Reflector detector voltage 1.969 kV
Instrument
Instrument type autoflex
Serial instrument number 264320.00433
Name of computer FLEX-PC
flexControl version flexControl 3.4.135.0
flexAnalysis version
a place of mind
UBC
CHEMISTRY

Synthesis and characterization of compound **S6**

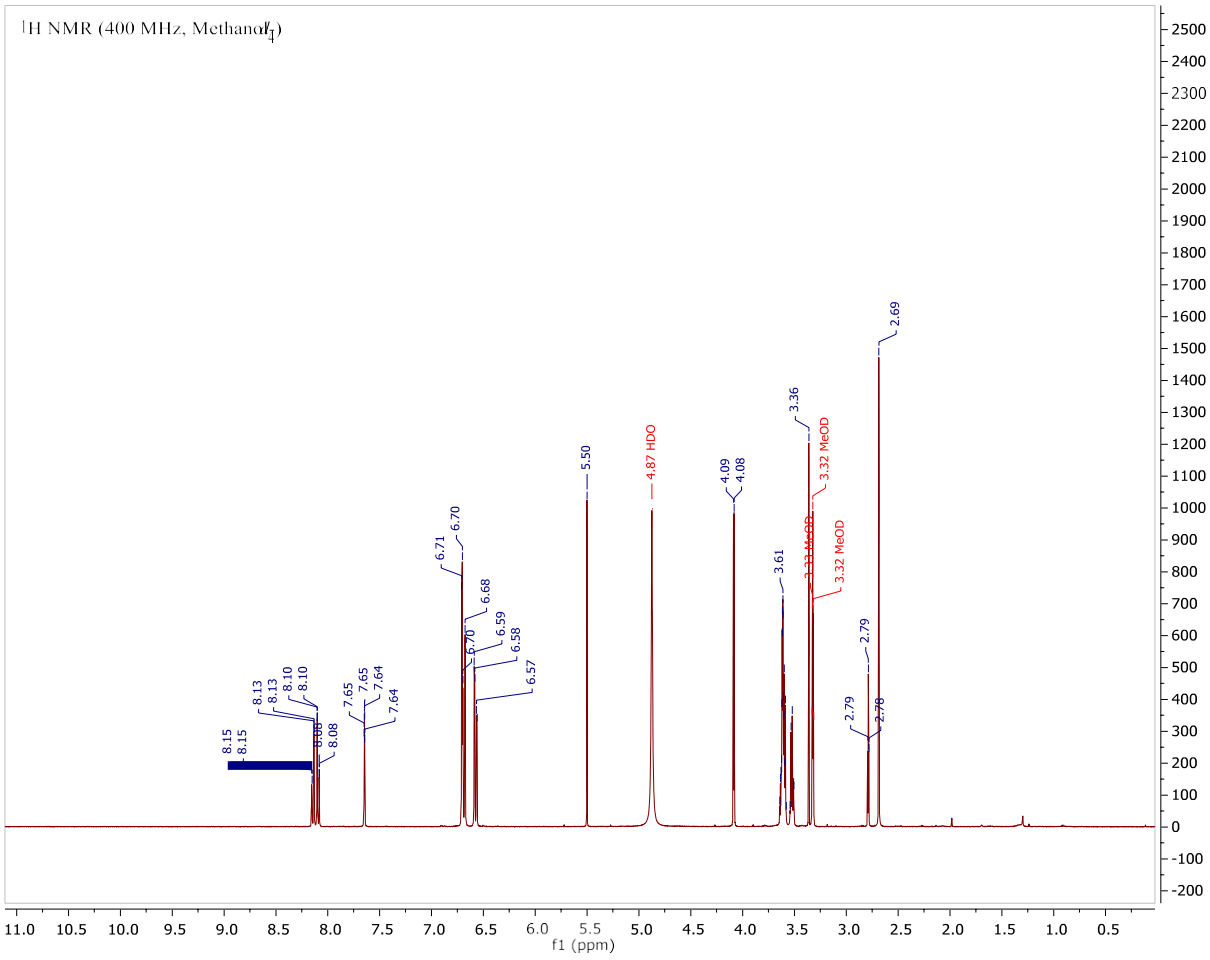


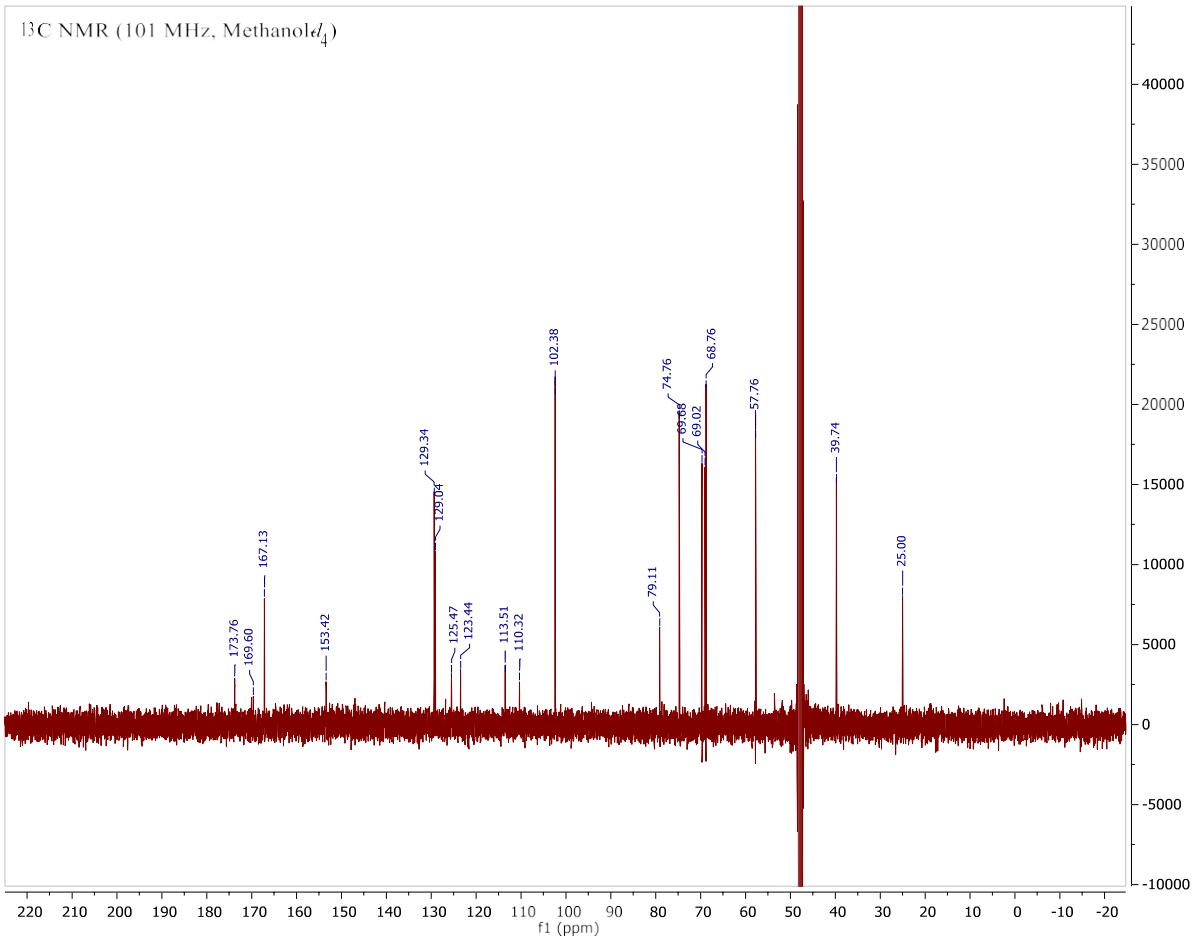
FITC-PEG$_2$ (S6). 6-FAM-NHS (14 mg, 29.6 µmol, 1 eq.) was dissolved in 170 µL of DMF and to this, 5.5 mg (38.45 µmol, 1.3 eq.) of PEG-linker were added in 20 µL of DMF. As soon as linker was added to 6-FAM-NHS in DMF, the solution changed colour from yellow to deep orange. Next, DIPEA was added to the reaction solution and the reaction was run in the dark at room temperature for 15 hours. TLC (7% MeOH/DCM, 2 drops acetic acid in 10 mL of solvent) of the reaction mixture showed full conversion of starting material; notably, the product moved with the same R_f as 6-FAM-NHS (R_f ~0.3). The reaction was stopped and DMF was first removed by running a silica plug with MeOH/DCM (3% MeOH in DCM, 120 ml). The product was then eluted out of the silica gel plug with 15% MeOH/DCM and re-purified using high quality Merck-grade silica gel.

^{1}H NMR (400 MHz, Methanol-d_4) δ 8.18 – 8.06 (m, 2H), 7.65 (dd, J = 1.5, 0.7 Hz, 1H), 6.73 – 6.65 (m, 4H), 6.57 (dd, J = 8.8, 2.4 Hz, 2H), 5.50 (s, 1H), 4.08 (d, J = 2.4 Hz, 2H), 3.67 – 3.55 (m, 6H), 3.52 (t, J = 5.1 Hz, 2H), 3.36 (s, 1H), 2.79 (t, J = 2.4 Hz, 1H), 2.69 (s, 2H).

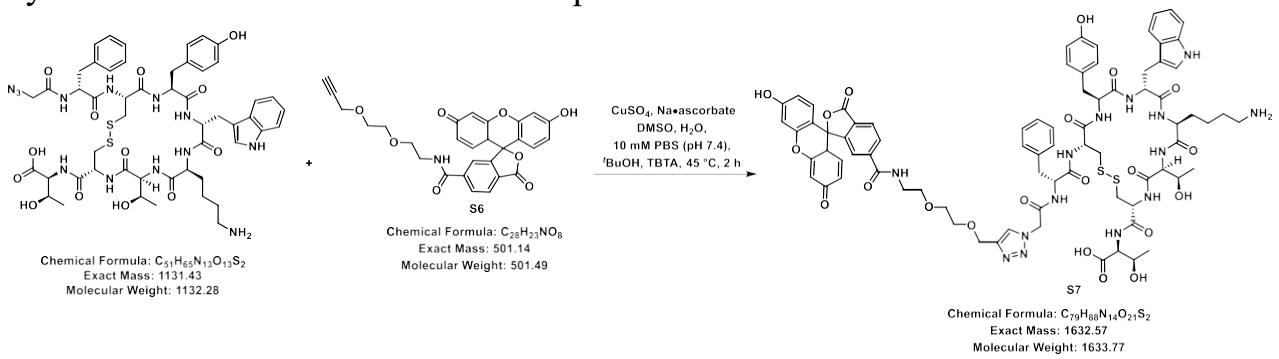
^{13}C NMR (101 MHz, Methanol-d_4) δ 25.00, 39.74, 57.76, 68.76, 69.02, 69.68, 74.76, 79.11, 102.38, 110.32, 113.51, 123.44, 125.47, 129.04, 129.34, 153.42, 167.13, 169.60, 173.76.

LRMS (ESI, m/z): $[M]^+$ found 524.1; calc.524.1 for $C_{28}H_{23}NO_8Na$.


1H NMR (400 MHz, Methanol-d4)
8.15
8.15
8.13
8.13
8.10
8.10
8.08
7.65
7.65
7.64
7.64
6.71
6.70
6.70
6.68
6.59
6.58
6.57
5.50
4.87 HDO
4.09
4.08
3.61
3.36
3.32 MeOD
3.32 MeOD
2.79
2.79
2.78
2.69
f1 (ppm)


13C NMR (101 MHz, Methanol-d4)
173.76
169.60
167.13
153.42
129.34
129.04
125.47
123.44
113.51
110.32
102.38
79.11
74.76
69.68
69.02
68.76
57.76
39.74
25.00
40000
35000
30000
25000
20000
15000
10000
5000
0
-5000
-10000
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10 -20
f1 (ppm)

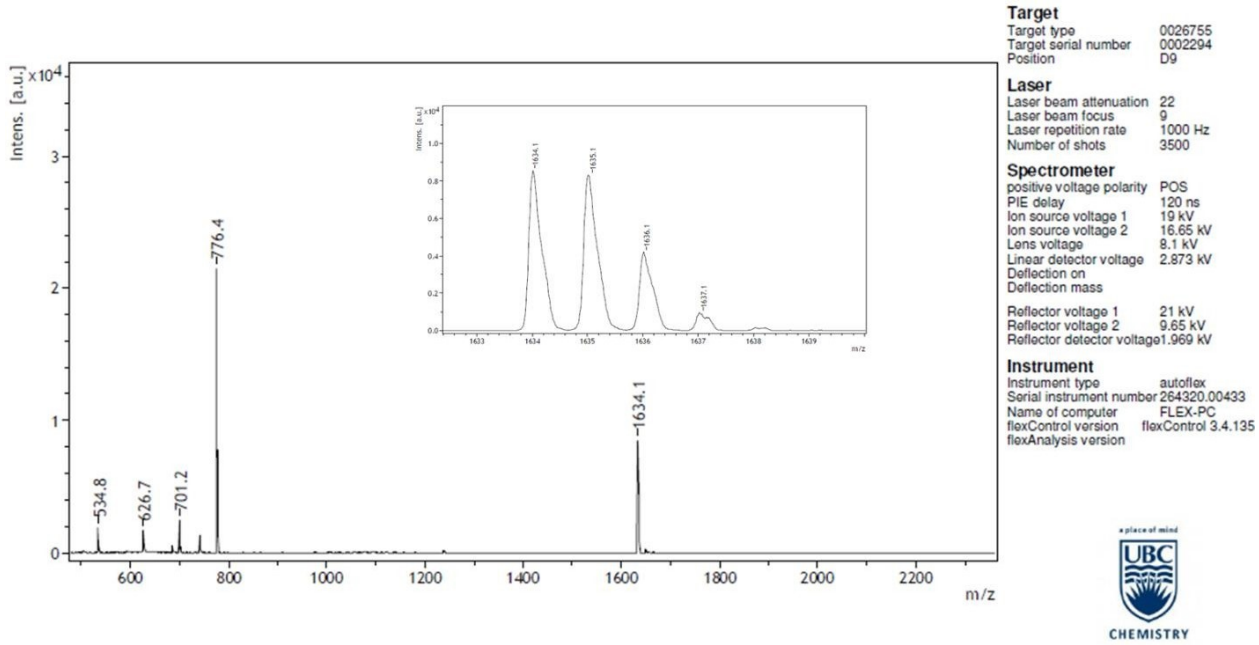
Synthesis and characterization of compound **S7**



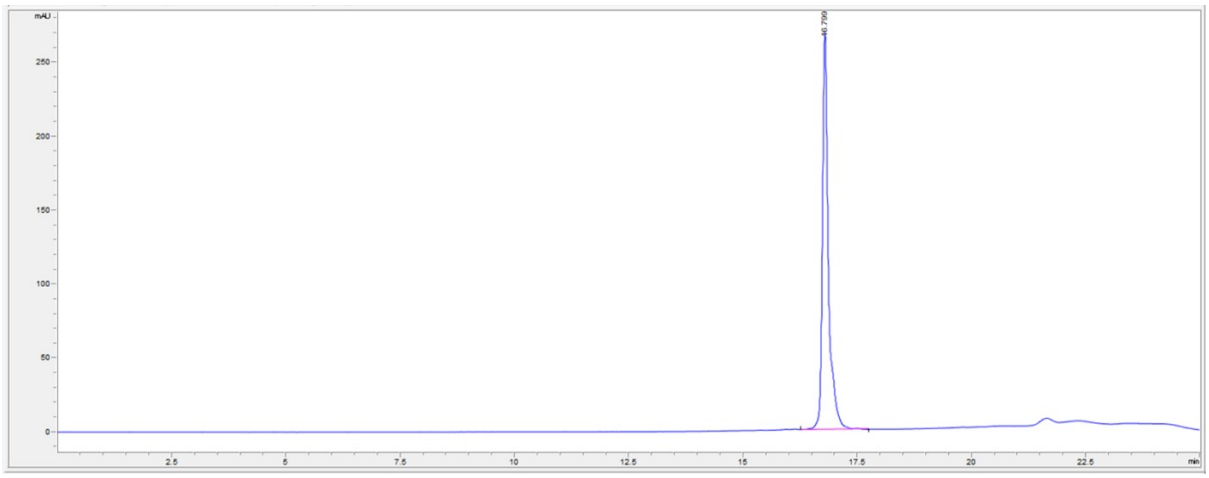
TATE-PEG$_2$-FITC (**S7**). CuAAC conjugation of compound FITC-PEG$_2$ **S6** (1 eq., 0.6 mg, 1.2 µmol) to TATE-N$_3$ **2** (1 eq., 1.4 mg, 1.2 µmol) was carried out following the protocol described for the synthesis of compound **19**. Upon completion, reaction contents were concentrated under reduced pressure, dissolved in ACN/5 mM phospahtre buffer pH 7.4, and purified by HPLC using gradient L. To exchange phosphate buffer for water, the product containing HPLC eluent was passed though a C-18 SEP-Pak column and the retained product was washed with 4 column volumes of water and then eluted with 2:3 ACN/ water. Pure material was lyophilized to obtain 0.9 mg (550 nmol) of **S7** as a yellow powder in 46% isolated yield. Quantification was done using extinction coefficient ε = 92 300 mol^{-1}Lcm^{-1} in basic ethanol at 499 nm.

]**MALDI**-TOF (m/z): $[M+H]^+$ found 1634.1; calc. 1634.7 for $C_{78}H_{89}N_{14}O_{21}S_2$.

HPLC (gradient **L**) $\mathbf{t_R}$ = 16.8 min; λ_{max} = 499 nm.



HPLC Chromatogram of TATE-PEG2-FITC (absorbance at 499 nm)



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