Supporting Information

Solid-phase synthesis of a novel phalloidin analog with on-bead and off-bead biological activity

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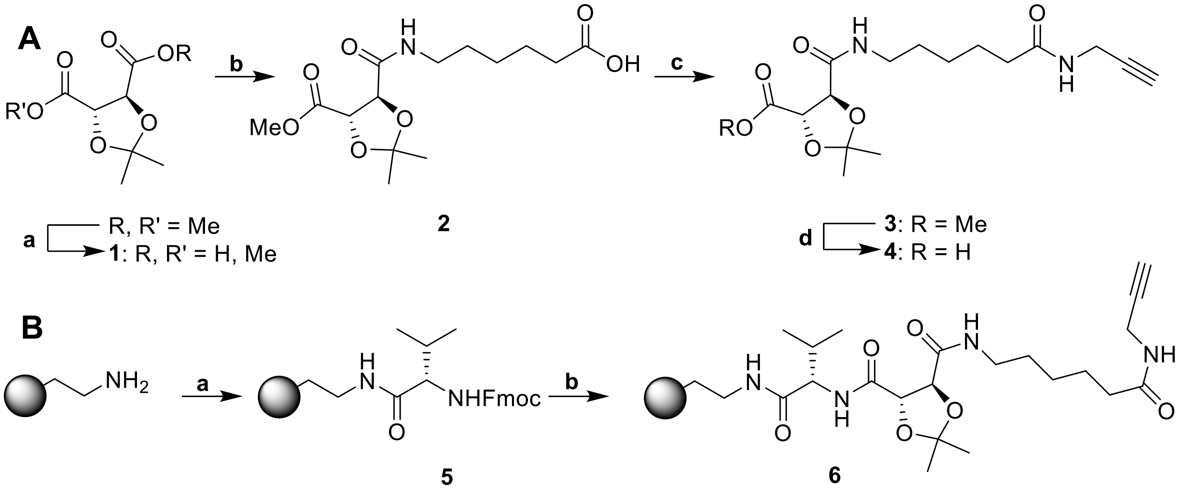
1. Materials and detailed methods

1.1. General

All reactions requiring the exclusion of air and/or moisture were conducted in flame-dried glassware under an argon atmosphere. Unless stated otherwise, solvents used for reactions such as DCM, DMF, EtOAc, DMPU, MTBE, CPME and MeCN were HPLC grade solvents dried over flamed-dried 3 Å or 4 Å molecular sieves and stored under argon.[1] Reactions requiring a set temperature were performed using a mineral oil bath and a temperature controlled hot plate (IKA Ceramag Midi equipped with an IKA ETS-D4 Fuzzy thermometer). Reactions conducted below room temperature were performed in an ice/water bath (≈ 4°C) or dry ice/acetone bath (-78°C). Solvents were evaporated under reduced pressure using a Büchi rotary evaporator. Lyophilization of water or water/MeCN mixtures, with or without formic acid, proceeded by sublimation below 200 mTorr after freezing the solution in a dry ice/acetone bath. A brine solution refers to a saturated NaCl (aq) solution. TentaGel MB-NH_2 refers to microbead (MB) grafted copolymers consisting of a low crosslinked polystyrene matrix onto which polyethylene glycol (PEG), in the range of 3000 Da, is grafted and functionalized with terminal amine (particle size is of 280 – 320 µm; capacity is of 0.2 - 0.3 mmol/g giving 3.5 nmol/bead and ca. 65550 beads/g). All reagents and solvents were purchased at the highest quality grade from commercial suppliers and used without prior purification unless otherwise stated. All chemicals and products were stored at 4^{o}C or –20^{o}C under argon. Reaction progress was followed by thin layer chromatography (TLC) on alumina backed 0.25 mm silica gel 60 aluminium sheets containing F-254 indicator. TLC analyte retention factor was reported as R_f that refers to a ratio-to-solvent front measured on plates. Visualization on TLC was monitored by different methods; using UV lamp at 254 and 365 nm; iodine crystals embedded in silica; potassium permanganate; cerium molybdate (Hanessian's stain); ninhydrin; dinitrophenylhydrazine (DNP); bromocresol green and cinnamaldehyde according to literature procedures.[2] For acid-labile compounds such as tritylated Hpi molecules, TLC plates were pre-run in 1%(*v*/*v*) TEA together with the noted solvent system. Analytical RP-HPLC was performed using an Agilent 1100 HPLC, equipped with a photodiode array detector using reverse phase semi-prep columns C18 (4.6 x 250 mm, 5 µm, 80 Å) at a flow rate of 2 ml/min with absorbance detection at 229 nm (amide), 253 nm (EDANS), 265 nm (Fmoc) and/or 280 nm (Hpi), 290 nm (tryptathionine), 301 nm (Fmoc), 340 or 342 nm (EDANS). Linear gradient was achieved by combining eluent A and B as the mobile phase. Eluent A was 0.1% (*v*/*v*) formic acid (FA) in Milli-Q water and Eluent B was 0.1% (*v*/*v*) formic acid (FA) in MeCN. RP-HPLC analyte chromatogram retention time was reported as t_r (min). Flash chromatography was performed on silica gel F60 (230-400 mesh, 43-60 µm). For acid-labile compounds such as tritylated Hpi molecules, the silica gel was resuspended in 1:3 (*v*/*v*) TEA/ 10% (*v*/*v*) EtOH in DCM and stirred overnight, then filtered and washed with various organic solvents and air dried overnight. Manual reverse phase column chromatography was performed on Sep-Pak (C18) gel (Waters, Delaware). Pressure was applied manually using a syringe. Elution was achieved with a dropwise flow rate with various proportions of Eluent A, 0.1% (*v*/*v*) formic acid (FA) in Milli-Q water, and Eluent B, 0.1% (*v*/*v*) formic acid (FA) in MeCN as the mobile phase. The product elution was monitored by TLC, ESI-MS, and/or RP-HPLC (18). Purification by RP-HPLC was performed on same system as the analytical RP-HPLC (C18) described above. Low-resolution mass spectra (LR-MS) and high-resolution mass spectra (HR-MS) in electrospray ionization (ESI) mode were acquired using positive or negative ionization mode in MeOH or MeCN on a Waters ZQ equipped with ESCI ion source and on a Micromass LCT time-of-flight spectrometer respectively. Nuclear magnetic resonance spectra were recorded in deuterated solvents. Proton (^{1}H-NMR), and carbon (^{13}C-NMR) nuclear magnetic resonance spectra were recorded using a 400 MHz or 300MHz and 100 MHz and 75MHz NMR spectrometer respectively using standard pulse sequences. Chemical shifts (δ) for all spectra were reported in parts per million (ppm) relative to tetramethylsilane referenced to the residual solvent peak signal. UV spectra were recorded on a spectrophotometer in 1 mL quartz cuvettes. Colorimetric resin analysis was performed by a Kaiser or Green Malachite test according to literature procedures.[3] Resin loading was determined by Fmoc cleavage for Fmoc bearing resins.[4] Unless stated otherwise, the Fmoc/CAM solid-phase peptide synthesis (SPPS) procedure was performed by manual solid-phase peptide synthesis carried out in a Zeba^{TM} spin desalting column (Pierce, 5 mL) with vortex mixing on TentaGel MB-NH_2 resin using standard Fmoc chemistry. If a mini Zeba^{TM} spin desalting column (Pierce, 0.5

mL) was used, the volumes were scaled down to one ninth to one tenth and reagent equivalents were scaled according.

1.2. Tartrate-based linker preparation for solid phase CuAAC



Scheme *S1* Reagents and conditions: **Panel A**: **a**) KOH, 1:10 (*v*/*v*) MeCN/H_2O, 4°C to RT, 68%; **b**) in two steps: *i*) HOSu, EDC•HCl, DMF, 4°C to RT, 25 h, used without further purification; *ii*) 6-aminohexanoic acid, Na_2CO_3, 1:1 (*v*/*v*) 2-MeTHF/H_2O, 4°C to RT, 21 h, 24% over two steps; **c**) propargylamine, EDC•HCl, Oxyma, DMF, DIEA, 10 h 30 min, 37%; **d**) NaOH, 2:1 (*v*/*v*) water/MeCN, 4°C to RT, 18 h, 76%. **Panel B**: **a**) TentaGel resin, N^{α}-Fmoc-Val-OH, COMU, Oxyma, DMF, DIEA, 2 x 30 min, 0.23 mmol/g; **b**) 20% (*v*/*v*) piperidine in DMF, 1 x 5 min, 1 x 10 min; **c**) Tartaric acid-propargylamide unit (**4**), COMU, Oxyma, DIEA, DMF, 2 x 30 min.

Methyl-(4*S*,5*S*)-2,2-dimethyl-1,3-dioxolane-4-carboxylic acid-5-carboxylate (1). To a clear pale yellow solution of dimethyl (4*S*,5*S*)-2,2-dimethyl-1,3-dioxolane-4,5-dicarboxylate (5.02 g, 22.9 mmol) was added dropwise into 1:10 (*v*/*v*) MeCN/H_2O (560 mL), 5M KOH (aq) (126 mL, 25.2 mmol). The solution was cooled in an ice/water bath and stirred for 1 hour and then acidified to pH 2 with aqueous 85%(*w/w*) *ortho*-phosphoric acid and extracted with EtOAc (12 x 40 mL). The organic layers were pooled, dried over anhydrous $MgSO_4$, filtered and evaporated to dryness under reduced pressure to afford the mono tartrate ester (**1**) (3.2 g, 68%) as a thick clear gum. R_f (**1**) = 0.25 (0.3:1:3.7:5 AcOH/Acetone/Heptane/EtOAc). **LR-MS** (ESI-single quadrupole) *m/z*: calcd for $C_8H_{11}O_6$ $[M\text{-H}]^-$: 203.1, found 203.3. **^{1}H-NMR** (300 MHz, CD_2Cl_2): δ 7.00 (br s, 1H), 4.83 (d, *J* = 5.2 Hz, 1H), 4.79 (d, *J* = 5.2 Hz, 1H), 3.81 (s, 3H), 1.48 (s, 3H), 1.46 (s, 3H).

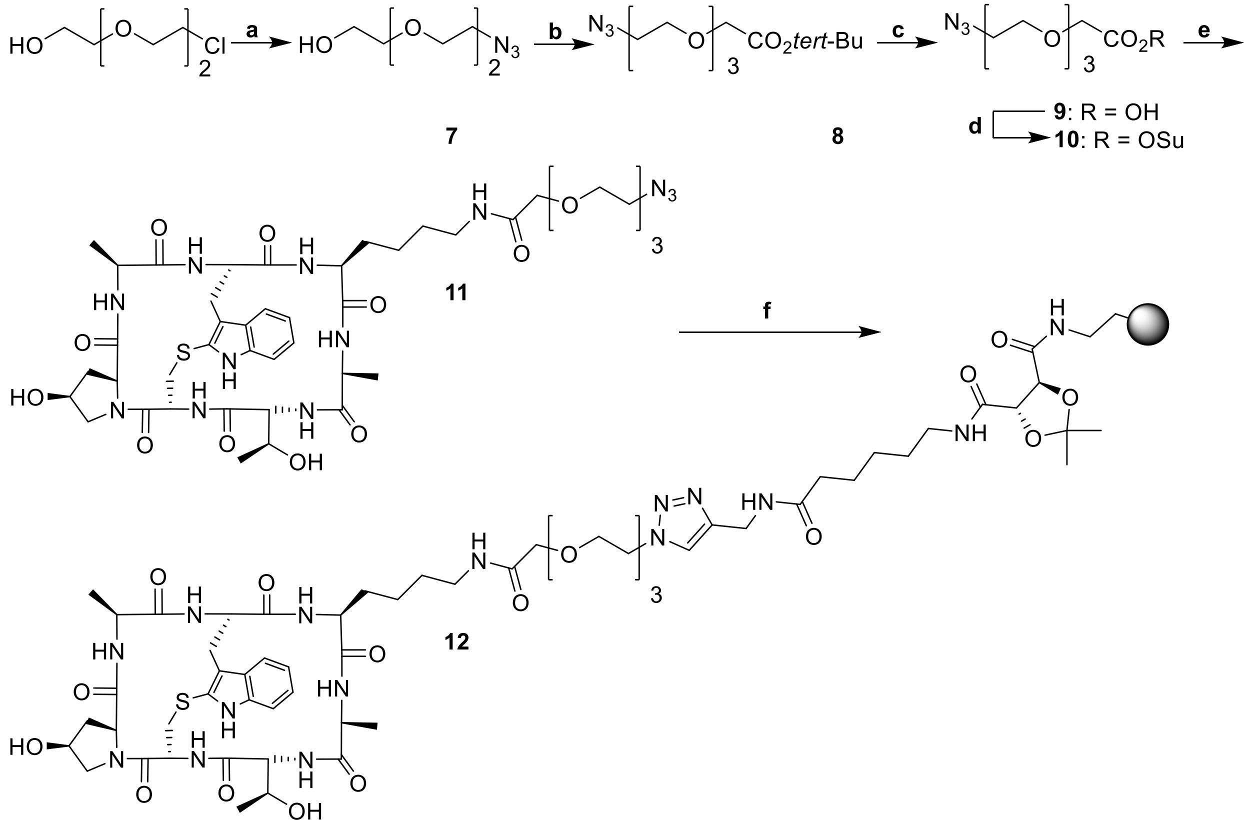
Methyl-(4*S*,5*S*)-2,2-dimethyl-1,3-dioxolane-4-carboxylate-5-*N*-(hexanoic acid)-carboxamide (2). EDC•HCl (2.91 g, 15.2 mmol) was added to a clear yellow solution of methyl-(4*S*,5*S*)-2,2-dimethyl-1,3-dioxolane-4-carboxylic acid-5-carboxylate (**1**) (2.58 g, 12.6 mmol) and *N*-hydroxysuccinimide (1.74 g, 15.2 mmol) in dry DMF (30 mL) in an ice/water bath. The resulting clear mixture was stirred at RT over 25 h in the dark and diluted in EtOAc (130 mL). The organic layer was washed with 0.1N HCl (aq) (3 x 50 mL), cold saturated $NaHCO_3$ (aq) (3 x 50 mL) and brine (3 x 50 mL). The organic layer was dried over anhydrous $MgSO_4$ and the volatiles were evaporated under reduced pressure to afford methyl-(4*S*,5*S*)-2,2-dimethyl-1,3-dioxolane-4-(2',5'-dioxopyrrolidin-1'-yl)-carboxylate-5-*N*-(hexanoic acid)-carboxamide (2.603g, 68%) as a clear yellowish oil. The crude product, dissolved in dry 2-MeTHF (40 mL) in an ice/water bath, was treated with a clear solution of 6-aminohexanoic acid (1.13 g, 8.64 mmol) and Na_2CO_3 (1.10 g, 10.4 mmol) in water (40 mL) at pH 9. The mixture was stirred for 21 h at RT in the dark, after which time, the aqueous layer was decanted, diluted with water (50 mL) and acidified to pH 2.5 with 1N HCl (aq) (145 mL) on an ice/water bath. The aqueous layer was extracted with EtOAc (7 x 50 mL) and the organic layers were pooled. The organic layer was washed with 0.1N HCl (aq) (3 x 150 mL), brine (1 x 150 mL), dried over anhydrous $MgSO_4$ and the volatiles were evaporated under reduced pressure to yield a thick clear yellow oil. The crude product was resolved on silica by flash chromatography (0.05:9.95 AcOH/EtOAc) to afford the tartrate hexanoic acid (**2**) (1.0 g, 26% over two steps) as a yellow oil. R_f (**2**) = 0.29 (0.05:9.95 AcOH/EtOAc). **HR-MS** (ESI-TOF) *m*/*z*: calcd for $C_{14}H_{24}NO_7$ $[M+H]^+$: 318.1547, found: 318.1553. **^{1}H-NMR** (300 MHz, CD_2Cl_2): δ 6.59 (br s, 1H), 4.71 (br s, 2H), 3.80 (s, 3H), 3.28 (q, *J* = 6.8 Hz, 2H), 2.36 (t, *J* = 7.3 Hz, 2H), 1.71-1.61 (m, 2H), 1.58-1.51 (m, 2H), 1.48 (s, 3H), 1.46 (s, 3H), 1.42-1.35 (m, 2H). **^{13}C-NMR** (75 MHz, APT, CD_2Cl_2): δ 178.30 (**C**), 170.75 (**C**), 169.53 (**C**), 113.39 (**C**), 78.05 (**C**H), 77.60 (**C**H), 52.71 (**C**H_3), 38.99 (**C**H_2), 33.68 (**C**H_2), 29.25 (**C**H_2), 26.56 (**C**H_3), 26.29 (**C**H_2), 26.13 (**C**H_3), 24.34 (**C**H_2).

Methyl-(4*S*,5*S*)-2,2-dimethyl-1,3-dioxolane-4-carboxylate-5-*N*-(1'-*N'*-[2''-propyn-1''-yl]-hexanamide-6'-yl)-carboxamide (3). Methyl-(4*S*,5*S*)-2,2-dimethyl-1,3-dioxolane-4-carboxylate-5-*N*-(hexanoic acid)-carboxamide (**2**) (1.04 g, 3.28 mmol) was treated with a solution of Oxyma (0.470 g, 3.28 mmol), dry DMF (30 mL) and EDC•HCl (0.630 g, 3.28

mmol) in an ice/water bath. To the clear yellow mixture was added propargyl amine (0.191 mL, 2.98 mmol) and DIEA (0.520 mL, 2.98 mmol). The clear orange mixture was stirred at RT for 10 h 30 min and diluted with EtOAc (160 mL). The organic layer was washed with 0.1 N HCl (aq) (3 x 50 mL), saturated $NaHCO_3$ (aq) (3 x 50 mL), brine (3 x 50 mL), dried over anhydrous $MgSO_4$ and the volatiles were evaporated under reduced pressure to yield a dark orange oil. The crude product was resolved on silica by flash chromatography (0.5:9.5 Heptane/EtOAc) to afford the title compound (**3**) (0.39 g; 37%) as a clear oil yellow. R_f (**3**) = 2.9 (EtOAc). **HR-MS** (ESI-TOF) *m*/*z*: calcd for $C_{17}H_{26}N_2O_6Na$ $[M+Na]^+$: 377.1685, found: 377.1689. **^{1}H-NMR** (300 MHz, CD_2Cl_2): δ 6.63 (br s, 1H), 6.13 (br s, 1H), 4.70 (br s, 2H), 4.00 (dd, *J* = 2.5; 5.6 Hz, 2H), 3.79 (s, 3H), 3.27 (q, *J* = 6.8 Hz, 2H), 2.28 (t, *J* = 2.5 Hz, 1H), 2.19 (t, *J* = 7.4 Hz, 2H), 1.69-1.59 (m, 2H), 1.56-1.49 (m, 2H), 1.47 (s, 3H), 1.45 (s, 3H), 1.39-1.31 (m, 2H). **^{13}C-NMR** (75 MHz, APT, CD_2Cl_2): δ 172.41 (**C**), 170.78 (**C**), 169.47 (**C**), 113.38 (**C**), 78.09 (**C**H), 77.59 (**C**H), 70.87 (**C**H_2), 52.72 (**C**H_3), 38.90 (**C**H_2), 36.03 (**C**H_2), 29.32 (**C**H_2), 28.91 (**C**H_2), 26.57 (**C**H_3), 26.38 (**C**H), 26.14 (**C**H_3), 25.04 (**C**H_2).

(4*S*,5*S*)-2,2-Dimethyl-1,3-dioxolane-4-carboxylic acid-5-*N*-(1'-*N'*-[2''-propyn-1''-yl]-hexanamide-6'-yl)-carboxamide (4). Methyl-(4*S*,5*S*)-2,2-dimethyl-1,3-dioxolane-4-carboxylate-5-*N*-(1'-*N'*-[2''-propyn-1''-yl]-hexanamide-6'-yl)-carboxamide (**3**) (0.347 g, 0.980 mmol), dissolved in 2:1 (*v*/*v*) water/MeCN (15 mL), was treated with an aqueous solution of 4.9 M NaOH (0.250 mL, 1.23 mmol) in an ice/water bath. The mixture was stirred at RT for 18 h, diluted with water (60 mL) and extracted with EtOAc (1 x 40 mL), Et_2O (1 x 40 mL), and EtOAc (2 x 20 mL). The aqueous layer was acidified to pH 2.5 with 1M HCl (aq) (160 mL) in an ice/water bath and extracted with EtOAc (13 x 50 mL). The organic layers were pooled, dried over anhydrous $MgSO_4$ and the volatiles were evaporated under reduced pressure to afford the crude title product (**4**) (0.25 g, 76%) as a yellowish clear oil. R_f (**4**) = 0.2 (0.5:1.5:8 AcOH/EtOH/EtOAc). **HR-MS** (ESI-TOF) *m*/*z*: calcd for $C_{16}H_{24}N_2O_6Na$ $[M+Na]^+$: 363.1536, found: 363.1532. **^{1}H-NMR** (300 MHz, $CDCl_3$): δ 6.90 (br s, 1H), 5.67 (br s, 1H), 4.55 (s, 2H), 4.08 (dd, *J* = 2.6; 5.2 Hz, 2H), 3.51-3.32 (m, 2H), 2.27-2.22 (m, 3H), 1.77-1.70 (m, 2H), 1.67-1.59 (m, 2H), 1.57 (s, 3H), 1.52 (s, 3H), 1.47-1.39 (m, 2H). **^{13}C-NMR** (75 MHz, APT, $CDCl_3$): δ 172.33 (**C**), 171.57 (**C**), 167.80 (**C**), 113.20 (**C**), 76.38 (**C**H), 75.14 (**C**H), 71.84 (**C**H$_2$), 39.44 (**C**H$_2$), 36.00 (**C**H$_2$), 29.34 (**C**H$_2$), 29.00 (**C**H$_2$), 26.39 (**C**H$_3$), 26.25 (**C**H), 26.16 (**C**H$_3$), 24.76 (**C**H$_2$).

1.3. Lysine modification of [Lys^7]-phalloidin



Scheme *S2* Reagents and conditions: **a**) NaN$_3$, DMF, 100^{o}C, 48 h, 76%; **b**) BrCH$_2$CO$_2$C(CH$_3$)$_3$, 1:1 (*v*/*v*) toluene/ 9.5 M NaOH (aq), 4^{o}C to RT, NBu$_4$HSO$_4$, 22 h, 73%; **c**) 1:1 (*v*/*v*) TFA/DCM, 4^{o}C to RT, 2 h; **d**) DSC, DCM, TMP, 12 h, 50%; **e**) NHS ester (**10**), Na$_2$CO$_3$, water/MeCN/MeTHF, 0^{o}C to RT, ON, 80%; **f**) Propargyl-tartrate on TentaGel Macrobead resin (**6**), CuSO$_4$, aqueous ascorbic acid/sodium ascorbate, 1:1:1 (*v*/*v*/*v*) H$_2$O/*tert*-BuOH/DMF, pH ≈ 5 to 7, ON.

8-Azido-3,6-dioxa-1-octanol (7). To a solution of NaN$_3$ (1.15 g, 17.7 mmol) in dry DMF (38 mL) was added 8-chloro-3,6-dioxa-1-octanol (1.66 mL, 11.4 mmol) under argon. The resulting mixture was stirred at 100^{o}C for 48 hours. The light-yellow mixture was diluted with 5% (*w*/*v*) LiCl (aq) (100 mL) and extracted with EtOAc (6 x 75 mL). The organic layers were combined into 200 mL portions and each portion was washed with brine (50 mL). All organic layers were then pooled, dried over anhydrous MgSO$_4$, and solvent was removed under reduced pressure. The crude oil was resolved on silica by flash chromatography gel with a loading solvent system 1:1 EtOAc/Heptane and eluted with 8:2 EtOAc/Heptane to afford product 2-(2-(2-azidoethoxy)ethoxy)ethanol (**7**) (1.5 g, 76%) as clear lightly yellow oil. R*f* (**7**) = 0.14 (7:3 EtOAc/Heptane). R*f* (**7**) = 0.11 (3.5:6.5 Heptane/EtOAc). **LR-MS** (ESI-single quadrupole) *m/z*: calcd for $C_6H_{13}N_3O_3Na$ [*M*+Na]$^+$: 198.1, found 198.3. **^{1}H-NMR** (300 MHz, $CDCl_3$): δ 3.76 (dd, *J* = 5.8; 11.1 Hz, 2H), 3.72-3.66 (m, 6H), 3.64 (t, *J* = 4.7 Hz, 2H), 3.42 (t, *J* = 4.7 Hz, 2H), 2.29 (t, *J* = 6.2 Hz, 1H). **^{13}C-NMR** (75 MHz, APT, $CDCl_3$): δ 72.62 (**C**H_2), 70.83 (**C**H_2), 70.56 (**C**H_2), 70.22 (**C**H_2), 61.94 (**C**H_2), 50.81 (**C**H_2). The spectroscopic data agree with literature reports.[5, 6]

11-azido-3,6,9-trioxa-1-*tert*-butyl-undecanoate (8). To a solution of 8-azido-3,6-dioxa-1-octanol (**7**) (1.03 mL, 5.85 mmol) in toluene (18 mL) was added tetrabutylammonium hydrogen sulfate (0.993 g, 2.92 mmol) and *tert*-butyl bromoacetate (2.59 mL, 17.5 mmol). The yellow clear mixture was cooled in an ice/water bath, 9.5 M NaOH (aq) (17 mmol, 18 mL) was added and the resulting solution was vigorously stirred for 22 h at room temperature. The mixture was diluted with water (100 mL) and extracted with Et_2O (6 x 30 mL). The organic layers were pooled, washed with brine (3 x 75 mL), dried over anhydrous $MgSO_4$ and all volatiles were evaporated under reduced pressure. The resulting crude product was applied to on silica by flash chromatography with loading solvent system 9:1 Heptane/EtOAc and the desired product was resolved by flash chromatography and eluted with 7:3 Heptane/EtOAc to afford the title compound (**8**) (1.2 g, 73%) as a clear colorless oil. R*f* (**8**) = 0.15 (3:7 EtOAc/Heptane). **HR-MS** (ESI-TOF) *m*/*z*: calcd for $C_{12}H_{23}N_3O_5Na$ [*M*+Na]$^+$: 312.1535, found: 312.1539. **^{1}H-NMR**($CDCl_3$, 300 MHz): δ = 4.02 (s, 2H), 3.78- 3.61 (m, 10H), 3.39 (t, *J* = 5.0 Hz, 2H), 1.47 (s, 9H). **^{13}C-NMR** (75 MHz, APT, $CDCl_3$): δ = 169.79 (**C**), 81.66 (**C**), 71.48 (**C**H_2), 70.81 (**C**H_2), 70.16 (**C**H_2), 69.17 (**C**H_2), 63.13 (**C**H_2), 50.81 (**C**H_2), 42.84 (**C**H_2), 28.24 (**C**H_3). The spectroscopic data agree with literature reports.[5, 6]

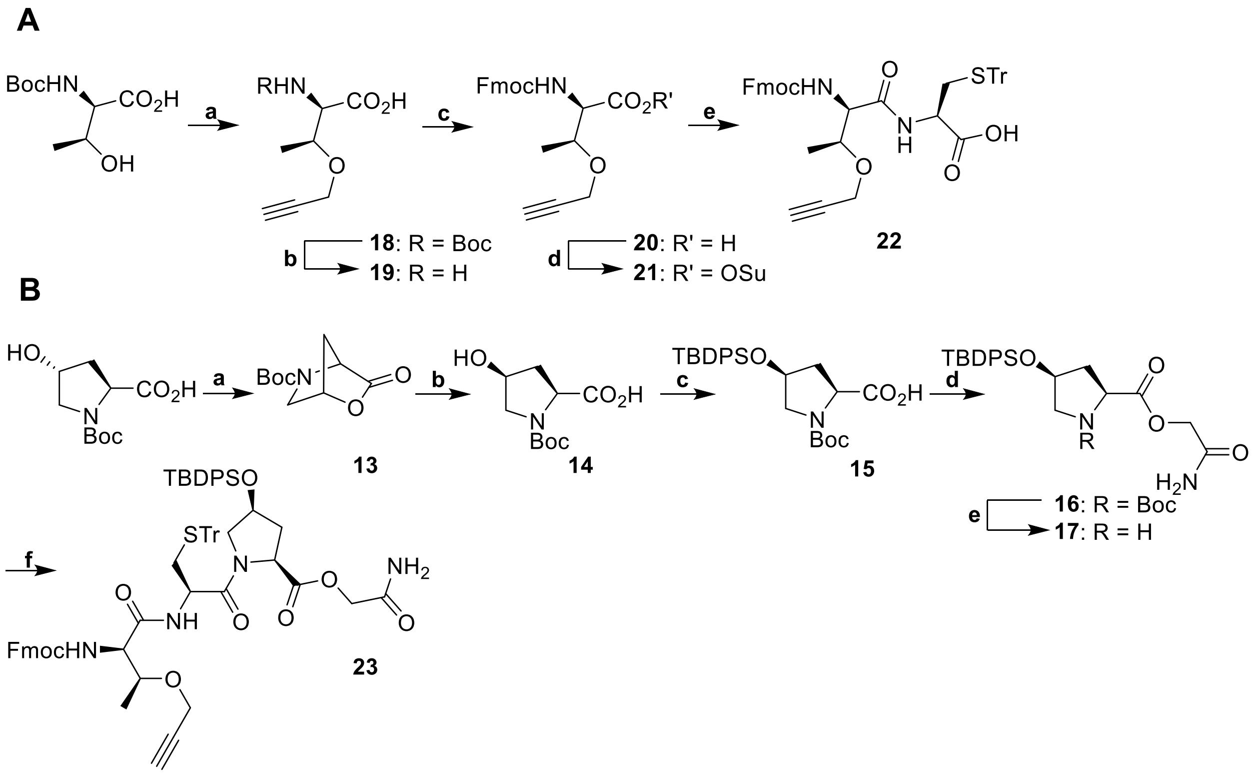
11-azido-3,6,9-trioxa-1-undecanoic acid (9). To a solution of 11-azido-3,6,9-trioxa-1-*tert*-butyl-undecanoate (**8**) (0.1 g, 0.348 mmol) in dichloromethane (10 mL) was slowly added TFA (10 mL, 135 mmol). The resulting clear yellow solution was stirred for 2 h and then, diluted with toluene (5 mL) followed by evaporation under reduced pressure. The resulting crude brown oil was re-dissolved in toluene (5 mL), evaporated under reduced pressure and the procedure was repeated twice. The crude brown oil was applied on silica by flash chromatography with 0.05:1:8.95 AcOH/Heptane/EtOAc and purified by flash chromatography to afford the title compound (**9**) (0.24 g, 90%) as a yellow clear oil. R_f (**9**) = 0.12 (0.1:1.5:8.4 AcOH/EtOH/AcOEt). **HR-MS** (ESI-TOF) *m*/*z*: calcd for $C_8H_{15}N_3O_5Na$ $[M+Na]^+$: 256.0909, found: 256.0909. **^{1}H-NMR** (CD_2Cl_2, 300 MHz): $\delta = 4.15$ (s, 2H), 3.75- 3.73 (m, 2H), 3.69-3.65 (m, 8H), 3.40 (t, *J* = 5.0 Hz, 2H). **^{13}C-NMR** (75 MHz, APT, CD_2Cl_2): $\delta = 172.80$ (**C**), 71.31 (**C**H_2), 70.66 (**C**H_2), 70.48 (**C**H_2), 70.29 (**C**H_2), 70.00 (**C**H_2), 68.74 (**C**H_2), 50.87 (**C**H_2). The spectroscopic data agree with literature reports.[6]

11-azido-3,6,9-trioxa-1-(2',5'-dioxopyrrolidin-1'-yl)-undecanoate (10). A mixture of 11-azido-3,6,9-trioxa-1-undecanoic acid (**9**) (0.059 g, 0.252 mmol), *N*,*N'*-disuccinimidyl carbonate (0.097 g, 0.377 mmol) and 2,4,6-trimethylpyridine (0.100 ml; 0.755 mmol) dissolved in MeCN (5 mL) was stirred for 22 h. The clear yellow mixture was diluted in EtOAc (60 mL), washed with 0.1 N HCl (aq) (3 x 40 mL), brine (3 x 40 mL), dried over anhydrous $MgSO_4$ and all volatiles were evaporated under reduced pressure. The crude turbid oil was resolved on silica by flash chromatography with 3:7 EtOAc/Heptane to afford the title compound (**10**) (0.04 g, 50%) as a clear oil contaminated with *N*-hydroxysuccinamide. R_f (**10**) = 0.26 (3:7 EtOAc/Heptane). **HR-MS** (ESI-TOF) *m*/*z*: calcd for $C_{12}H_{18}N_4O_7Na$ $[M+Na]^+$: 353.1073, found: 353.1071. **^{1}H-NMR** ($CDCl_3$, 300 MHz): $\delta =$ 4.54 (s, 2H), 3.83- 3.80 (m, 2H), 3.74-3.69 (m, 8H), 3.41 (t, *J* = 5.0 Hz, 2H), 2.87 (s, 4H). **^{13}C-NMR** (75 MHz, APT, $CDCl_3$): $\delta = 168.93$ (**C**), 166.17 (**C**), 71.49 (**C**H_2), 70.83 (**C**H_2,), 70.80 (**C**H_2), 70.19 (**C**H_2), 66.69 (**C**H_2), 50.83 (**C**H_2), 25.73 (**C**H_2).

Bicyclic(Ala^1-DThr^2-[Cys^3-*cis*-4-Hyp^4-Ala^5-2-mercapto-Trp^6]-Lys^7{*N*$^\varepsilon$-11'-azido-3',6',9'-trioxa-1'-undecanamide})[*S*-3→6] (11). A clear solution of 11-azido-3,6,9-trioxa-1-*N*-hydroxysuccinamide-undecanoate (**10**) (1 mM, 1 mL, 1.5 µmol) in 5:95 (*v*/*v*) 2-MeTHF/MeCN, cooled in an ice/water bath, was treated with a clear aqueous solution of bicyclic(Ala^1-DThr^2-[Cys^3-*cis*-4-Hyp^4-Ala^5-2-mercapto-Trp^6]-Lys^7)[*S*-3→6] (1 mM, 1 mL, 1.1 µmol) and Na_2CO_3 (0.3 mg, 2.8 µmol) pH 8.5. The mixture was vigorously stirred for 12 h in the dark at room temperature. The pH was lowered to 4 by addition of acetic acid (0.5 mL) and saturated $NaHCO_3$ (aq) (0.25 mL) following by evaporation to dryness under reduced pressure. The crude solid was dissolved in 1:9 (*v*/*v*) MeCN/water (0.5 mL) and the desired product was purified by RP-HPLC (C18) with a gradient of 15% to 95% of eluent 0.05% (*v*/*v*) TFA in MeCN against eluent 0.1% (*v*/*v*) TFA in water over 17 min at 1 ml/min to afford the bicyclic(Ala^1-DThr^2-[Cys^3-*cis*-4-Hyp^4-Ala^5-2-mercapto-Trp^6]-Lys^7{*N*^{ε}-11'-azido-3',6',9'-trioxa-1'-undecanamide})[*S*-3→6] (**11**) (0.90 µmol, 80%) as a white powder following lyophilisation. Quantification was done using the phalloidin coefficient of extinction $E^{1\%}$ = 0.597 (295 nm in water). **HR-MS** (ESI-TOF) *m*/*z*: calcd for $C_{43}H_{62}N_{12}O_{13}NaS$ [*M*+Na]$^+$: 1009.4154, found: 1009.4178. **RP-HPLC** (C18), solvent A: 0.1% TFA in Milli-Q water; solvent B: 0.05% TFA in MeCN: t_r = 8.929 min (A_{2292} 99.5%) with gradient of 15% to 95% solvent B over 17 min at a flow rate of 1 mL/min; UV-Vis spectra with $λ_{max}$ = 292 nm.

Propargyl-tartrate based linker CuAAC with the Lys(*N*^{ε}-11'-azido-3',6',9'-trioxa-1'-undecanamide)^7-phalloidin derivative. Prior to use in CuAAC, solvents were degassed by argon bubbling and the reaction vessel was a caped glass vial flushed with argon. The synthesis was initiated from TentaGel MB-NH_2 (3.6 mg, ≈ 0.8 µmol). In a mini-spin desalting column (0.5 mL, Pierce), the resin was filtered with 1:1:2 (*v*/*v*/*v*) H_2O/*tert*-BuOH/DMF (5 mL), then the resin was shaken 3 x 10 minutes in 1:1:2 (*v*/*v*/*v*) H_2O/*tert*-BuOH/DMF (0.5 mL) and drained by filtration each time. The swollen resin was resuspended in a fresh yellow mixture made of bicyclic(Ala^1-DThr^2-[Cys^3-*cis*-4-Hyp^4-Ala^5-2-mercapto-Trp^6]-Lys^7{*N*^{ε}-11'-azido-3',6',9'-trioxa-1'-undecanamide})[S-3→6] (**11**) (0.90 µmol), aqueous ascorbic acid/sodium ascorbate pH 5 (0.15 M, 0.2 mL, 0.0293 mmol), and aqueous $CuSO_4$ (6 mM, 0.08 mL, 0.5 µmol) in 1:2 (*v*/*v*) *tert*-BuOH/DMF (0.6 mL). The resulting mixture was shaken for 37 h 30 min in the dark and turned a turbid brown. Upon completion of reaction, estimated by Kaiser test, the resin was drained by filtration and washed according to the following procedure: The resin was filtered with 1:1:2 (*v*/*v*/*v*) H_2O/*tert*-BuOH/DMF (5 ml). The resin was resuspended in 1:1:2 (*v*/*v*/*v*) H_2O/*tert*-BuOH/DMF (0.6 mL), shaken, drained by filtration and procedure was repeated nine more times with fresh solvent. The filtration and shaking procedure was repeated using 1:2 (*v*/*v*) water/MeCN, 0.05% (*w*/*v*) EDTA in 1.5:1 (*v*/*v*) water/MeCN pH 7, 1:2 (*v*/*v*) water/MeCN, 1:1 (*v*/*v*) MeCN/EtOH. EtOH, DCM to afford the colorless resin (**12**) which was dried *in vacuo* and stored at 4°C.

1.4. Tripeptide preparation as precursor of phalloidin derivative



Scheme *S3* Reagents and conditions: **Panel A**: **a**) *i*) dry DMF, NaH, 4^{o}C, 30 min, Ar; 80% (w/v) propargylbromide in toluene, 4^{o}C, 4 h 30 min, Ar; 50%; **b**) TFA/DCM, 1 h; **c**) Fmoc-Oxyma, sat $NaCO_{3(aq)}$, acetone/MeCN, 4^{o}C to RT, ON, 84%; **d**) DSC, TMP, 1:1 dry MeCN/dry CPME, 4^{o}C to RT, ON, 90%; **e**) H-Cys(Tr)-OH, Na_2CO_3, MeCN/CPME/water, 4^{o}C to RT, ON, 88%. **Panel B**: **a**) DIAD, PPh_3, THF, 4^{o}C to RT, 20 h, 72%; **b**) LiOH, 2:2:3 (*v*/*v*/*v*) THF/EtOH/H_2O, 25 h, acidic work-up, quantitative; **c**) TBDPSCl, imidazole, DMF, ice/water bath to RT, 5 days, 41%; **d**) *i*) Cs_2CO_3, 2:1 (*v*/*v*) EtOH/water, 1h 30 min; *ii*) P_2O_5 dry vaccum, 1 h; *iii*) bromoacetamide, DMF, 14 h 30 min, ≈ 60^{o}C, 97%; **e**) *i*) 1:2 (*v*/*v*) TFA/DCM, 1 h; *ii*) DIPEA, 1:9 (*v*/*v*) MeCN/EtOAc, 4^{o}C, 5 min, used immediately as crude. **f**) H-*cis*-Hyp(TBDPS)-*O*Cam (**17**), COMU, Oxyma; DIPEA, 2:4 (*v*/*v*) MeCN/EtOAc, 4^{o}C to RT, ON; 61%.

2'-Methyl-2'-propanyl (1*S*,4*S*)-3-oxo-2-oxa-5-azabicyclo [2.2.1] heptane-5-carboxylate (13). A solution of N^{α}-Boc-4-*trans*-Hyp-OH (1.00 g, 4.30 mmol) and triphenylphosphine (1.36 g, 5.20 mmol) in dry THF (22 mL) was treated with DIAD (1.0 mL, 5.2 mmol) under argon in an ice/water bath in dark. After 10 min, the yellow clear mixture was stirred at RT for 20 h and the volatiles were evaporated under reduced pressure to yield a turbid white/yellow oil. The oil was resuspended in 4:6 (*v*/*v*)

EtOAc/Hexane, filtered and the volatiles were evaporated under reduced pressure to yield a yellow oil. The crude oil was applied to silica and the desired compound was purified twice on silica by flash chromatography (loaded with 3:7 EtOAc/hexane, eluted with 4:6 EtOAc/hexane) to give the desired lactone (**13**) contaminated with diisopropylhydrazinodicarboxylate (DIHD) and triphenylphosphine oxide. The mixture was further resolved on silica by flash chromatography (loaded with 4:6 Et_2O/hexane, eluted with 1:1 Et_2O/hexane followed by 7:3 Et_2O/hexane) to afford the title lactone (**13**) (0.59 g, 72 %) as a white solid. R_f (**13**) = 0.3 (1:1 EtOAc/Hexane). **HR-MS** (ESI-TOF) *m*/*z*: calcd for $C_{10}H_{15}NO_4Na$ $[M+Na]^+$: 236.0901, found: 236.0899. **^{1}H-NMR** ($CDCl_3$, 300 MHz): δ =.5.10 (s, 1H), 4.58 (br s, 1H), 3.56 (d, *J* = 11 Hz, 1H), 3.48 (d, *J* = 11 Hz, 1H), 2.23 (d, *J* = 10.6 Hz, 1H), 2.04 (d, *J* = 10.6 Hz, 1H), 1.50 (s, 9H). The spectroscopic data agree with literature reports.[7]

(2*S*,4*S*)-4-Hydroxy-1-[(2'-methylpropan-2'-yl)oxycarbonyl]-pyrrolidine-2-carboxylic acid (14). A solution of 2'-methyl-2'-propanyl (1*S*,4*S*)-3-oxo-2-oxa-5-azabicyclo[2.2.1] heptane-5-carboxylate (**13**) (0.564 g, 2.65 mmol) in 1:1 (*v*/*v*) THF/EtOH (12 mL) was treated with an aqueous solution of 0.38 M LiOH (9.0 mL, 7.9 mmol) and stirred for 23 h at RT. The mixture was diluted with water (30 mL), acidified to pH 2 with 1N HCl (aq) (3.5 mL) in an ice/water bath and extracted with EtOAc (6 x 15 mL). The organic layers were pooled, dried over anhydrous $MgSO_4$ and the volatiles were evaporated under reduced pressure to yield a white solid. The crude solid was triturated with heptane to afford the desired *cis*-Hyp isomer (**14**) (0.61 g, quantitative) as a white solid. R_f (**14**) = 2.1 (1:0.5:8.5 AcOH/EtOH/DCM). **HR-MS** (ESI-TOF) *m*/*z*: calcd for $C_{10}H_{17}NO_5Na$ $[M+Na]^+$: 254.1009, found: 254.1004. **^{1}H-NMR** ($CDCl_3$, 300 MHz): δ =.4.46 (br s, 2H), 3.77-3.52 (m, 2H), 2.48-2.30 (m, 2H), 1.51-1.47 (s, 9H). The spectroscopic data agree with literature reports.[8]

(2*S*,4*S*)-4-[*Tert*-butyldiphenylsilyl]oxy-1-[(2'-methylpropan-2'-yl)oxycarbonyl]-pyrrolidine-2-carboxylic acid (15). A solution of (2*S*,4*S*)-4-hydroxy-1-[(2'-methylpropan-2'-yl)oxycarbonyl]-pyrrolidine-2-carboxylic acid (**14**) (0.500 g, 2.16 mmol) and imidazole (0.650 g, 9.55 mmol) in dry DMF (7 mL) was treated with *tert*-butyldimethylsilyl chloride (0.720 ml, 2.81 mmol) under argon in an ice/water bath and stirred at room temperature for 5 days. The mixture was diluted with water (60 mL) in an ice/water bath, acidified with 1N HCl (aq) to pH 2, and extracted with ethyl acetate (4 x

20 mL). The organic layers were pooled, washed with saturated NH_4Cl (aq) (3 x 40 mL), brine (3 x 40 mL), dried over anhydrous $MgSO_4$ and the volatiles were evaporated under reduced pressure to yield a crude solid. The residue was resolved on silica by flash chromatography (0.1:1:8.9 AcOH/EtOAc/Heptane) to afford the desired silyl Hyp (**15**) (0.42 g, 41 %) as a white solid. R_f (**15**) = 0.15 (0.1:7:2.9 AcOH/Heptane/EtOAc). **HR-MS** (ESI-TOF) *m*/*z*: calcd for $C_{26}H_{35}NO_5NaSi$ [*M*+Na]$^+$: 492.2181, found: 492.2182. **^{1}H-NMR** (CD_2Cl_2, 300 MHz): δ =.10.32 (br s, 1H), 7.68 (d, *J* = 6.1 Hz, 4H), 7.48-7.41 (m, 6H), 4.37-4.33 (m, 2H), 3.39 (br s, 2H), 2.23 (br s, 2H), 1.44 (s, 9H), 1.07 (s, 9H). **^{13}C-NMR** (75 MHz, APT, CD_2Cl_2): δ = rotamers: 177.66 (**C**), 175.23 (**C**), 155.89 (**C**), 153.79 (**C**), 135.89 (**C**H), 135.83 (**C**H)), 133.26 (**C**), 130.05 (**C**H), 129.99 (**C**H), 127.92 (**C**H), 127.80 (**C**H), 81.04 (**C**), 80.36 (**C**), 71.54 (**C**H), 71.00 (**C**H), 58.40 (**C**H), 57.95 (**C**H), 54.88 (**C**H_2), 54.27 (**C**H_2), 39.11 (**C**H_2), 37.61 (**C**H_2), 28.30 (**C**H_3), 28.16 (**C**H_3), 26.63 (**C**H_3), 18.93 (**C**).

(2*S*,4*S*)-4-[*Tert*-butyldiphenylsilyl]oxy-1-[(2’-methylpropan-2’-yl)oxycarbonyl]-pyrrolidine-2-(ethanamide-2’’–yl-)-carboxylate (16). A solution of (2*S*,4*S*)-4-[*tert*-butyldiphenylsilyl]oxy-1-[(2’-methylpropan-2’-yl)oxycarbonyl]-pyrrolidine-2-carboxylic acid (**15**) (0.044 g, 0.094 mmol) in EtOH (3.5 mL) and water (1.5 mL) was treated with Cs_2CO_3 (0.015 g, 0.047 mmol), stirred for 1 h 30 min and the volatiles were evaporated under reduced pressure. The crude salt was dried *in vacuo* under P_2O_5 for an hour. The dried salt was dissolved in dry DMF (5 mL) under argon and treated with 2-bromoacetamide (0.026 g, 0.19 mmol). The resulting mixture was stirred for 14 h 30 min at 63°C. The mixture was cooled to room temperature, additional 2-bromoacetamide (0.003 g, 0.024 mmol) was added and the resulting mixture was then stirred for an additional 6 h 30 min at 63°C. The addition procedure was repeated but stirred for 19 h 30 min and the volatiles were evaporated under reduced pressure. The crude product was resolvedresolved on silica by flash chromatography (loaded in 2:8 EtOAc/Heptane, eluted in 4:6 EtOAc/Heptane) to afford the carbamoyl methyl ester (**16**) (0.05 g, 97%) as a white gum. R_f (**16**) = 0.18 (1:1 Heptane/EtOAc). **HR-MS** (ESI-TOF) *m*/*z*: calcd for $C_{28}H_{38}N_2O_6NaSi$ [*M*+Na]$^+$: 549.2401, found: 549.2397. **^{1}H-NMR** (CD_2Cl_2, 300 MHz): δ = 7.64 (d, *J* = 6.4 Hz, 4H), 7.47-7.39 (m, 6H), 5.41 (br s, 1H), 5.34 (br s, 1H), 5.02 (d, *J* = 15.6 Hz, 1H), 4.43 (dd, *J* = 3.7; 8.8 Hz, 1H), 4.36 (br s, 1H), 4.25 (d, *J* = 15.6 Hz, 1H), 3.43 (dd, *J* = 1.7; 11.5 Hz, 1H), 3.35 (dd, *J* = 4.2; 11.5 Hz, 1H), 2.29 (ddd, *J* = 13.2; 3.3;

4.3 Hz, 1H), 2.19 (ddd, *J* = 13.2; 4.2; 4.3 Hz, 1H), 1.46 (s, 9H), 1.03 (s, 9H). ^{13}**C-NMR** (75 MHz, APT, CD_2Cl_2): δ = 171.46 (**C**), 170.01 (**C**), 155.68 (**C**), 135.87 (**C**H), 135.72 (**C**H), 133.61 (**C**), 133.02 (**C**), 130.09 (**C**H), 129.98 (**C**H), 127.93 (**C**H), 127.82 (**C**H), 80.77 (**C**), 71.76 (**C**H), 62.62 (**C**H_2), 58.02 (**C**H), 54.96 (**C**H_2), 38.56 (**C**H_2), 28.25 (**C**H_3), 26.55 (**C**H_3), 18.92 (**C**).

(2*R*,3*S*)- 2-(([(2’-Methyl-2’-propanyl)oxy]carbonyl)amino)-3-(prop-2’’-yn-1’’-yloxy)-butanoic acid (18). Boc-D-Thr-OH (0.5 g, 2.28 mmol) was dried in vacuo under P_2O_5 overnight, then dissolved in dry DMF (20 mL) under argon and cooled in an ice/water bath. To the clear mixture was added 60% (*w/w*) NaH in mineral oil (3 x 0.091 g, 6.84 mmol, 5 min between addition) and the turbid bubbly mixture was stirred for 1 h under argon and in an ice/water bath. A solution of 80% (*w/v*) propargyl bromide in toluene (0.3 mL, 2.69 mmol) was added to the turbid white mixture, dropwise, over 10 min and the resulting solution was stirred for 4 h 30 min under argon in an ice/water bath. The brown clear mixture was diluted slowly with ice-cold water (60 mL) in an ice/water bath. The resulting solution (pH 13) was extracted with 8:2 Heptane/EtOAc (6 x 20 mL). The aqueous layer was acidified to pH 2.5 with aqueous 85% (*w/w*) *o*-phosphoric acid (4.5 mL) in an ice/water bath and extracted with EtOAc (3 x 20 mL, 4 x 10 mL). The brown clear organic layers were pooled, washed with saturated KH_2PO_4 (aq), dried over anhydrous $MgSO_4$ and evaporated under reduced pressure to yield a crude brown oil. The crude residue was resolved on silica by flash chromatography (loaded 0.2:4:5.8 AcOH/Heptane/$CHCl_3$, eluted with 0.2:3:6.8 AcOH/Heptane/$CHCl_3$) to afford the threonine propargyl ether (**18**) (0.36 g, 45%) as a brown clear oil. R_f (**18**) = 0.05 (0.2:1:8.8 AcOH/Heptane/$CHCl_3$). **HR-MS** (ESI-TOF) *m/z*: calcd for $C_{12}H_{19}NO_5Na$ $[M+Na]^+$: 280.1157, found: 280.1161. ^{1}**H-NMR** (CD_2Cl_2, 300 MHz): δ = 9.46 (br s, 1H), 5.31 (br s, 1H), 4.35-4.29 (m, 2H), 4.24-4.10 (m, 2H), 2.51 (t, *J* = 2.1 Hz, 1H), 1.45 (s, 9H), 1.25 (d, *J* = 6.1 Hz, 3H). ^{13}**C-NMR** (75 MHz, APT, CD_2Cl_2): δ = 175.93 (**C**), 156.44 (**C**), 80.42 (**C**), 79.28 (**C**), 74.98 (**C**H), 74.40 (**C**H), 58.16 (**C**H), 56.46 (**C**H_2), 28.41 (**C**H_3), 16.23 (**C**H_3).

(2*R*,3*S*)- 2-(*N*-(9*H*-fluoren-9’-ylmethoxycarbonyl)amino)-3-(prop-2’’-yn-1’’-yloxy)-butanoic acid (20). A solution of (2*R*,3*S*)- 2-(([(2’-Methyl-2’-propanyl)oxy]carbonyl)amino)-3-(prop-2’’-yn-1’’-yloxy)-butanoic acid (**18**) (0.246 g, 1.03 mmol) in CPME (12 mL), toluene (1 mL) and TFA (8 mL) was stirred for 2 h and the volatiles were evaporated under reduced pressure. The crude brown oil was dissolved in toluene (5 mL), the volatiles were evaporated under reduced pressure and the procedure was repeated twice. A solution of the crude brown oil in DCM (12 mL), toluene (1 mL) and TFA (2 mL) was stirred for 2 h 30 min and the volatiles were evaporated under reduced pressure. The crude brown oil was dissolved in toluene (5 mL), the volatiles were evaporated under reduced pressure and the procedure was repeated twice to afford the ammonium salt (2*R*,3*S*)- 2-amino-3-(prop-2''-yn-1''-yloxy)-butanoic acid (**19**) (quantitative) as a brown oil. R*f* (**19**) = 0.07 (1:1:3 AcOH/*i*-PrOH/$CHCl_3$). The crude ammonium salt (2*R*,3*S*)- 2-amino-3-(prop-2''-yn-1''-yloxy)-butanoic acid (**19**) (1.03 mmol) was dissolved in 2:1 (*v/v*) water/MeCN (15 mL), basified to pH 8 with saturated $NaHCO_3$ (aq) (12 mL) and treated with a white turbid solution of Fmoc-Oxyma (0.206 g, 0.555 mmol) in 1:3 acetone/MeCN (20 mL) in an ice/water bath. The resulting clear yellow mixture was stirred for 8 h at RT, followed by addition of Fmoc-Oxyma (0.412 g, 1.11 mmol) and further stirred for 16 h at RT. The white-yellow turbid solution was diluted in water (50 mL), basified to pH 9 with saturated $CaCO_3$ (aq) (150 mL) and extracted with 8:2 Heptane/CPME (4 x 25 mL). In an ice/water bath, the clear aqueous layer was acidified to pH 1 with aqueous 85% (*w/w*) *o*-phosphoric acid (0.1 mL), became turbid and turned white and was extracted with EtOAc (5 x 40 mL). The organic layers were pooled, washed with saturated KH_2PO_4 (aq) (1 x 50 mL), brine (1 x 50 mL) and dried over anhydrous $MgSO_4$. All volatiles were evaporated under reduced pressure and the crude solid was resolved on silica by flash chromatography (loaded with 8:2 Heptane/EtOAc, eluted with 0.1:7.9:2 AcOH/Heptane/EtOAc) to afford (2*R*,3*S*)- 2-(*N*-(9*H*-fluoren-9'-ylmethoxycarbonyl)amino)-3-(prop-2''-yn-1''-yloxy)-butanoic acid (**20**) (0.33 g, 84%) as a white foamy solid. R*f* (**20**) = 0.18 (0.1:3.9:6 AcOH/Heptane/EtOAc). **RP-HPLC** (C18): t_r = 30.41 min (A_{301} 99%) with gradient of 20% to 95% solvent B over 25 min, 95% to 100% solvent B over 5 min at a flow rate of 1 mL/min; UV-Vis spectra with λ_{max} = 266 nm, 290 nm, 301 nm. **HR-MS** (ESI-TOF) *m*/*z*: calcd for $C_{22}H_{21}NO_5Na$ $[M+Na]^+$: 402.1313, found: 402.1317. **^{1}H-NMR** (CD_2Cl_2, 300 MHz): δ = 10.52 (br s, 1H), 7.81 (d, *J* = 7.4 Hz, 2H), 7.66-7.64 (m, 2H), 7.43 (dd, *J* = 7.3; 8.1 Hz, 2H), 7.34 (dd, *J* = 7.3; 8.1 Hz, 2H), 5.63 (d, *J* = 9.3 Hz, 1H), 4.46-4.11 (m, 7H), 2.51 (t, *J* = 2.1 Hz, 1H), 1.27 (d, *J* = 6.1 Hz, 3H). **^{13}C-NMR** (75 MHz, APT, CD_2Cl_2): δ = 175.37 (**C**), 158.87 (**C**), 143.93 (**C**), 141.41 (**C**), 127.82

(**C**H), 127.29 (**C**H), 125.24 (**C**H), 120.06 (**C**H), 79.27 (**C**), 74.80 (**C**H), 74.15 (**C**H), 67.32 (**C**H_2), 58.65 (**C**H), 56.38 (**C**H_2), 47.29 (**C**H), 15.85 (**C**H_3).

1-(2''',5'''-dioxopyrrolidin-1'''-yl)-(2*R*,3*S*)-2-(*N*-(9*H*-fluoren-9'-ylmethoxycarbonyl)amino)-3-(prop-2''-yn-1''-yloxy)-butanoate (21). A solution of (2*R*,3*S*)-2-(*N*-(9*H*-fluoren-9'-ylmethoxycarbonyl)amino)-3-(prop-2''-yn-1''-yloxy)-butanoic acid (**20**) (0.390 g, 1.03 mmol) in 1:1(*v/v*) MeCN/CPME (20 mL) was treated with *N,N'*-disuccinimidyl carbonate (0.390 g, 1.52 mmol) and 2,4,6-trimethylpyridine (0.300 mL, 2.27 mmol) in an ice/water bath. The clear yellow mixture was stirred for 12 h at RT and diluted with 1:1 (*v/v*) Heptane/EtOAc (80 mL). The organic layer was washed with saturated KH_2PO_4 (aq) (3 x 30 mL), saturated $NaHCO_3$ (aq) (1 x 30 mL), brine (3 x 30 mL) and dried over anhydrous $MgSO_4$. The volatiles were evaporated under reduced pressure and the crude solid was resolved on silica by flash chromatography (7:3 Heptane/EtOAc) to afford the *N*-hydroxysuccinimide ester (**21**) (0.43 g, 87%) as a white solid. R_f (**21**) = 0.06 (7:3 Heptane/EtOAc). *m*/*z*: calcd for $C_{26}H_{24}N_2O_7Na$ $[M+Na]^+$: 499.1483, found: 499.1481.

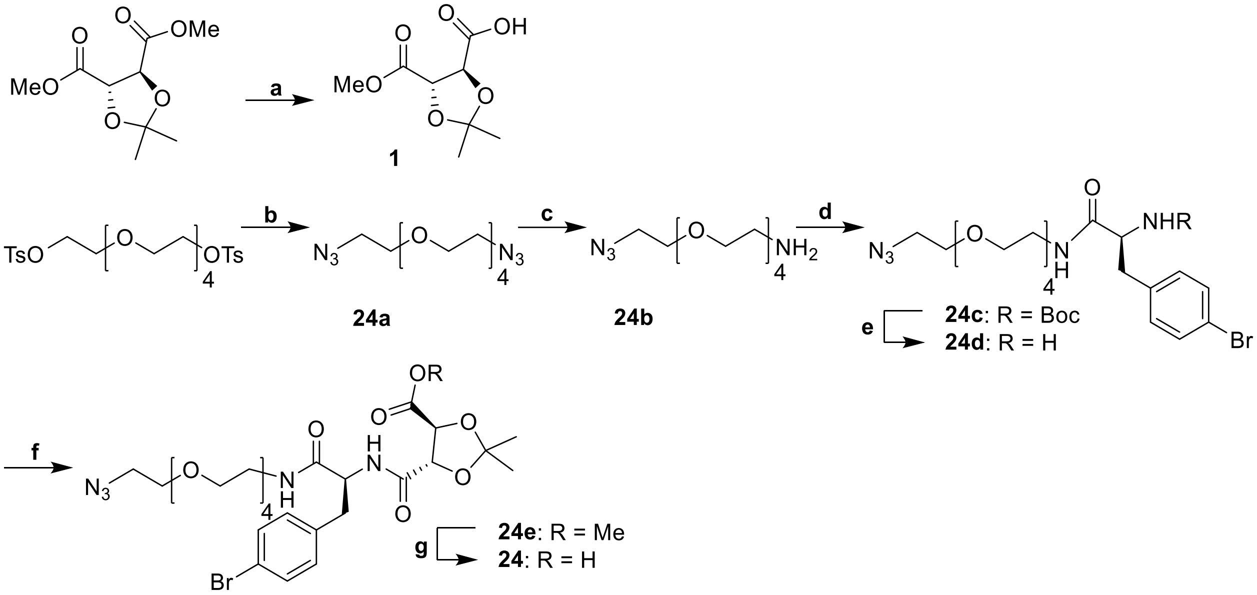
***N*$^{\alpha}$-Fmoc-*O*$^{\beta}$-propargyl-D-threonyl-*S*$^{\beta}$-trityl-cysteine (22).** A solution of 1-(2''',5'''-dioxopyrrolidin-1'''-yl)-(2*R*,3*S*)-2-(*N*-(9*H*-fluoren-9'-ylmethoxycarbonyl)amino)-3-(prop-2''-yn-1''-yloxy)-butanoate (**21**) (0.488 g, 1.02 mmol) in MeCN (30 mL) and CPME (0.3 mL) was treated with a solution of *S*$^{\beta}$-trityl-cysteine (0.410 g, 1.23 mmol) and Na_2CO_3 (0.119 g, 1.23 mmol) in 1:1 (*v/v*) MeCN/water (20 mL), in an ice/water bath. The clear yellow mixture was stirred for 4 h at RT and pH 8.5, then additional *S*$^{\beta}$-trityl-cysteine (0.082 g, 0.246 mmol) was added and further stirred for 1 h. The solution was acidified to pH 3 with aqueous 3% (*v/v*) *o*-phosphoric acid (6.3 mL), diluted with water (15 mL) and extracted with EtOAc (5 x 20 mL). The organic layers were pooled, washed with saturated KH_2PO_4 (aq) (3 x 20 mL), brine (1 x 20 mL) and dried over anhydrous $MgSO_4$. The volatiles were evaporated under reduced pressure and the crude product was resolved on silica by flash chromatography (loaded 0.1:4:5.9 AcOH/EtOAc/Heptane, eluted with 0.1:6.9:3 AcOH/EtOAc/Heptane) to afford the dipeptide (**22**) (0.68 g, 91%) as a white solid. R_f (**22**) = 0.15 (0.1:6.9:3 AcOH/EtOAc/Heptane). **HR-MS** (ESI-TOF) *m*/*z*: calcd for $C_{44}H_{39}N_2O_6S$ $[M\text{-}H]^-$: 723.2534, found: 723.2529. Contaminated with EtOAc: **^{1}H-NMR** (CD_2Cl_2, 400 MHz): δ = 7.80 (d, *J* = 7.5 Hz, 2H), 7.66-7.59 (m, 2H), 7.43-7.20 (m, 19H), 7.00 (d, *J* = 6.7 Hz, 1H), 5.83 (d, *J* = 6.8 Hz, 1H), 4.41 (dd, *J* = 7.1; 11.5 Hz, 2H), 4.36-4.15 (m, 6H), 2.73-2.63 (m, 2H), 2.44 (br s, 1H), 1.17 (d, *J* = 6.1 Hz, 3H). Contaminated with heptane: **^{13}C-NMR** (75 MHz, APT, CD_2Cl_2): δ = 172.95 (**C**), 170.02 (**C**), 156.48 (**C**), 144.33 (**C**), 143.84 (**C**), 141.40 (**C**), 129.55 (**C**H), 128.15 (**C**H), 127.83 (**C**H), 127.21 (**C**H), 127.00 (**C**H), 125.24 (**C**H), 120.06 (**C**H), 79.63 (**C**), 74.83 (**C**H), 74.56 (**C**H), 67.35 (**C**H_2), 67.04 (**C**H), 58.34 (**C**H), 56.99 (**C**H_2), 47.26 (**C**H), 33.25 (**C**H_2), 15.55 (**C**H_3).

***N*$^{\alpha}$-Fmoc-*O*$^{\beta}$-propargyl-D-threonyl-*S*$^{\beta}$-trityl-cysteinyl-(1-carbamoylmethyl)-*O*$^{\gamma}$-(*tert*-butyldiphenylsilyl)-*cis*-hydroxyprolinate ester (23).** A solution of (2*S*,4*S*)-4-[*tert*-butyldiphenylsilyl]oxy-1-[(2’-methylpropan-2’-yl)oxycarbonyl]-pyrrolidine-2-(ethanamide-2’’–yl-)-carboxylate (**16**) (0.054 g, 0.103 mmol) in DCM (4 mL) was treated with TFA (2 mL) and stirred for 1 hour. To the mixture was added toluene (1 mL) and the volatiles were evaporated under reduced pressure. The crude oil was re-dissolved in toluene (2 mL), the volatiles evaporated under reduced pressure and the procedure was repeated once to afford the crude ammonium salt (2*S*,4*S*)-4-[*tert*-butyldiphenylsilyl]oxy-pyrrolidine-2-(ethanamide-2’–yl-)-carboxylate (**17**) (quantitative) as a light brown solid. **LR-MS** (ESI-single quadrupole) *m*/*z*: calcd for $C_{23}H_{30}N_2O4NaSi$ [*M*+Na]$^+$: 449.2, found: 449.4.

A solution of crude ammonium salt (2*S*,4*S*)-4-[*tert*-butyldiphenylsilyl]oxy-pyrrolidine-2-(ethanamide-2’–yl-)-carboxylate (**17**) in 4:1 (*v/v*) EtOAc/MeCN (10 mL) was basified to pH 6 with DIPEA (0.036 mL, 0.206 mmol) in an ice/water bath. To the solution was added Oxyma (0.016 g, 0.113 mmol), dipeptide (**22**) (0.082 g, 0.113 mmol), COMU (0.048 g, 0.113 mmol), DIPEA (0.018 mL, 0.113 mmol) and the resulting clear yellow mixture was stirred for 16 h. Additional COMU (0.02 g, 0.056 mmol) and DIPEA (0.036 mL, 0.226 mmol) were added and the yellow clear solution of pH 9 was further stirred for 5 h 30 min. The mixture was diluted with EtOAc (85 mL), washed with saturated KH_2PO_4 (aq) (3 x 40 mL), saturated $CaCO_3$ (aq) (3 x 40 mL), brine (3 x 40 mL) and dried over anhydrous $MgSO_4$. The volatiles were evaporated under reduced pressure and the crude product was resolved on silica by flash chromatography (loaded 0.05:0.05:1:8.8 AcOH/EtOH/Heptane/DCM, eluted with 0.05:0.1:1:8.85 AcOH/EtOH/Heptane/DCM) to afford the tripeptide (**23**) (0.078 g, 61%) as a white solid. R_f (**23**) = 0.3 (1:1 EtOAc/DCM). **HR-MS** (ESI-TOF) *m*/*z*: calcd for $C_{67}H_{68}N_4O_9NaSSi$ [*M*+Na]$^+$: 1155.4377, found: 1155.4374. Contaminated with heptane: **^{1}H-NMR** (CD_2Cl_2, 400 MHz): δ = 7.79 (d, *J* = 7.6

Hz, 2H), 7.68-7.61 (m, 6H), 7.50-7.17 (m, 26H), 6.60 (br s, 1H), 5.82 (d, *J* = 8.4 Hz, 1H), 5.35 (br s, 1H), 4.86 (d, *J* = 15.5 Hz, 1H), 4.53 (dd, *J* = 3.8; 9.2 Hz, 1H), 4.46 (br s, 1H), 4.36-4.28 (m, 3H), 4.19-4.04 (m, 6H), 3.72 (dd, *J* = 4.9; 10.7 Hz, 1H), 3.38 (dd, *J* = 2.6; 10.7 Hz, 1H), 2.73 (dd, *J* = 7.2; 12.7 Hz, 1H), 2.57 (dd, *J* = 7.6; 12.7 Hz, 1H), 2.32 (br s, 1H), 2.29-2.23 (m, 1H), 2.17-2.10 (m, 1H), 1.13 (d, *J* = 6.2 Hz, 3H), 1.03 (s, 9H). **^{13}C-NMR** (75 MHz, APT, CD_2Cl_2): δ = 171.99 (**C**), 170.98 (**C**), 170.15 (**C**), 156.39 (**C**), 144.35 (**C**), 144.04 (**C**), 141.38 (**C**), 141.35 (**C**), 135.80 (**C**H), 135.77 (**C**H), 133.04 (**C**), 132.99 (**C**), 130.25 (**C**H), 130.18 (**C**H), 129.58 (**C**H), 128.22 (**C**H), 127.97 (**C**H), 127.73 (**C**H), 127.13 (**C**H), 127.03 (**C**H), 125.46 (**C**H), 125.40 (**C**H), 119.95 (**C**H), 79.89 (**C**), 74.55 (**C**H), 74.32 (**C**H), 71.68 (**C**H), 67.24 (**C**H_2), 67.01 (**C**H), 62.51 (**C**H_2), 59.02 (**C**H), 58.39 (**C**H), 56.53 (**C**H_2), 55.78 (**C**H_2), 47.31 (**C**H), 37.42 (**C**H_2), 32.99 (**C**H_2), 26.61 (**C**H_3), 18.90 (**C**), 16.02 (**C**H_3).

1.5. Tartrate-based linker with isotopic probe for solution phase CuAAC



Scheme *S4* <u>Reagents and conditions</u>: **a**) KOH, MeOH, 4°C to RT, 2 h, 60 %; **b**) NaN_3, TBAI, Na_2CO_3 anhydrous DMF, Ar, temperature ~ 60°C, reaction time ~ 2 h, yield ~ 96%; **c**) PPh_3, 85% (*w*/*w*) H_3PO_4(aq.), water, CPME, 4°C, 22 h, 72%; **d**) N^{α}-Boc-Phe(4-Br)-OH, Oxyma, COMU, dry EtOAc, dry MeCN, DIEA, Ar, 4°C to RT, 22 h, 63%; **e**) 1:4 (v/v) TFA/DCM, 4°C to RT, 3 h 30 min; **f**) Tartrate methyl ester (**1**), COMU, Oxyma, DIEA, dry EtOAc, dry MeCN, 4°C to RT, Ar, 68 % (over two steps); **g**) 3 M NaOH in MeOH, 9:1 (v/v) DCM/MeOH, 2 h, 4°C to RT, 90 %.

(4*S*,5*S*)-2, 2-Dimethyl-1, 3-dioxolane-4, 5-dicarboxylate-4-methyl ester (1). To a clear pale yellow solution of dimethyl (4*S*,5*S*)-2,2-dimethyl-1,3-dioxolane-4,5-dicarboxylate-dimethyl ester (5.02 g, 22.9 mmol) in 1:10 (*v*/*v*) MeCN/H_2O (560 mL), 5M KOH (aq) (126 mL, 25.2 mmol) was added dropwise. The solution was cooled in an ice/water bath and stirred for 1 hour. The solution cooled in ice/water bath was acidified to pH ~ 2 with saturated aqueous orthophosphoric acid and extracted with EtOAc (12 x 40 mL). The organic layers were pooled, dried over anhydrous $MgSO_4$, filtered and evaporated to dryness under reduced pressure to afford (4*S*,5*S*)-2,2-dimethyl-1,3-dioxolane-4,5-dicarboxylate-4-methyl ester (**1**) (3.18 g, 15.6 mmol) as a thick clear gum in 68% yield, which was resolved on silicy by flash chromatography, R_f ~ 0.25 (0.3:1:3.7:5 AcOH/Acetone/Heptan/EtOAc). LR-MS (ESI-single quadrupole) *m/z*: calcd for $C_8H_{11}O_6$ $[M - H]^-$: 203.1, found 203.3. **^{1}H-NMR** (300 MHz, CD_2Cl_2): δ 7.00 (br s, 1H), 4.83 (d, *J* = 5.2 Hz, 1H), 4.79 (d, *J* = 5.2 Hz, 1H), 3.81 (s, 3H), 1.48 (s, 3H), 1.46 (s, 3H).

1,14-Diazido-3,6,9,12-tetraoxatetradecane (24a). A solution of 3,6,9,12-tetraoxatetradecane-1,14-diyl bis(4-methylbenzenesulfonate) (5.02 g, 9.18 mmol), TBAI (0.180 g, 0.460 mmol), and Na_2CO_3 (0.990 g, 9.20 mmol) in dry DMF (200 mL) was treated with NaN_3 (1.79 g, 27.5 mmol) under argon. The reaction mixture was warmed up to 70^{o}C and stirred overnight for 20 h. The reaction mixture was cooled in ice/water bath, diluted with saturated Na_2CO_3 (aq) (400 mL) and extracted with EtOAc (5 x 100 mL). The organic layers were pooled, dried over anhydrous $MgSO_4$, filtered and evaporated under reduced pressure to afford 1,14-diazido-3,6,9,12-tetraoxatetradecane (**24a**) (2.60 g; 9.03 mmol) as a clear yellow oil in 98% yield and the crude product was resolved on silica by flash chromatography R_f ~ 0.25 (1:9 Heptane/Et_2O). HR-MS (ESI-TOF) *m/z*: calcd for $C_{10}H_{20}N_6O_4Na$ $[M + Na]^+$: 311.1444, found 311.1442. **^{1}H-NMR** (300 MHz, CD_2Cl_2): δ 3.67-3.61 (m, 16H), 3.38 (t, *J* = 5.2 Hz, 4H). **^{13}C-NMR** (75 MHz, APT, CD_2Cl_2): δ 70.71 (**C**H_2), 70.68 (**C**H_2), 70.61 (**C**H_2), 69.98 (**C**H_2), 50.89 (**C**H_2).

14-Azido-3,6,9,12-tetraoxatetradecane-1-amine (24b). To a solution of 1,14-diazido-3,6,9,12-tetraoxatetradecane (**24a**) (2.50 g, 8.70 mmol) in H_3PO_4 (aq)/KH_2PO_4 (aq) (180 mL) pH ~1, cooled in ice/water bath, a solution of PPh_3 (2.23 g, 8.7 mmol) in CPME (90 mL) was added dropwise, and the mixture was stirred vigorously. The resulting turbid biphasic solution was stirred overnight at room temperature, the aqueous layer decanted, and the latter was extracted with CPME (3 x 60 mL). The subsequent aqueous layer was basified to pH ~ 12 with 1 N NaOH, and extracted with DCM (5 x 60 mL). The organic layers were pooled, dried over anhydrous $MgSO_4$ and evaporated under reduced pressure to afford 14-azido-3,6,9,12-tetraoxatetradecane-1-amine (**24b**) (1.50 g, 5.72 mmol) as a thick yellow oil in 66% yield and the crude product was resolved on silica by flash chromatography R_f ~ 0.2 (0.1:2:7.9 TEA/EtOH/DCM). HR-MS (ESI-TOF) *m/z*: calcd for $C_{10}H_{23}N_4O_4$ $[M + H]^+$: 263.1719, found 263.1722. Contaminated with $OPPh_3$ and S.M.: **^{1}H-NMR** (300 MHz, CD_2Cl_2): δ 3.67-3.57 (m, 16H), 3.47-3.44 (t, *J* = 5.2 Hz, 2H), 3.40-3.36 (t, *J* = 5.1 Hz, 2H). **^{13}C-NMR** (75 MHz, APT, CD_2Cl_2): δ 73.76 (**C**H_2), 72.76 (**C**H_2), 70.91 (**C**H_2), 70.71 (**C**H_2), 70.63 (**C**H_2), 70.49 (**C**H_2), 70.44 (**C**H_2), 70.39 (**C**H_2), 70.25 (**C**H_2), 69.99 (**C**H_2), 61.52 (**C**H_2), 50.90 (**C**H_2), 42.01 (**C**H_2), 40.19 (**C**H_2).

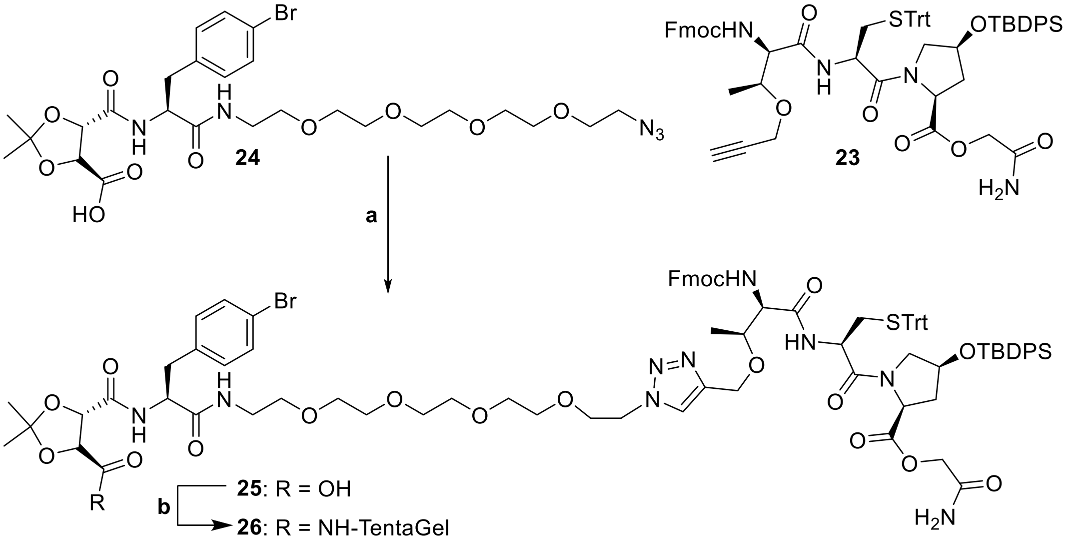
(2*S*)-3-(4’-Bromophenyl)-2-(([(2’’-methyl-2’’-propanyl)oxy]carbonyl)amino)-1-*N*-(14’’’-azido-3’’’,6’’’,9’’’,12’’’-tetraoxatetradecane-1’’’-)-propanamide (24c). 1-Amino-14-azido-3,6,9,12-tetraoxatetradecane (**24b**) (1.50 g, 5.72 mmol), COMU (3.43 g, 8.00 mmol), Oxyma Pure (0.896 g, 6.30 mmol) and (2*S*)-3-(4’-bromophenyl)-2-(([(2’’-methyl-2’’-propanyl)oxy]carbonyl)amino)-propanoic acid (2.16 g, 6.30 mmol) were dissolved in dry 4:1 (*v*/*v*) EtOAc/MeCN (55 mL) and cooled in an ice/water bath. To the yellow clear mixture, DIPEA (3.50 mL, 20.0 mmol) was added and the mixture was stirred at room temperature for 18 hours. The clear dark orange reaction mixture was diluted with saturated Na_2CO_3 (aq) (150 mL) and extracted with EtOAc (5 x 50 mL). The organic layers were pooled, dried over anhydrous $MgSO_4$, filtered and evaporated under reduced pressure to yield a thick yellow gum that was resolved on silica by flash chromatography (4:2:4 to 1:1:0 EtOAc/Heptane/DCM) to afford (2*S*)-3-(4’-bromophenyl)-2-(([(2’’-methyl-2’’-propanyl)oxy]carbonyl)amino)-1-*N*-(14’’’-azido-3’’’,6’’’,9’’’,12’’’-tetraoxatetradecane-1’’’-)-propanamide (**24c**) (1.68 g, 2.86 mmol) as a gummy yellow solid in 50% yield, R_f ~ 0.1 (4:6 EtOAc/Heptane). HR-MS (ESI-TOF) *m/z*: calcd for $C_{24}H_{38}N_5O_7BrNa$ $[M + Na]^+$: 610.1852, found 610.1860. Contaminated with *N*,*N*-dimethylmorpholine-4-carboxamide: **^{1}H-NMR** (300 MHz, CD_2Cl_2): δ 7.43 (d, *J* = 8.3 Hz, 2H), 7.11 (d, *J* = 8.3, 2H), 6.48 (br s, 1H), 5.19 (br s, 1H), 4.33-4.26 (m, 1H), 3.67-3.37 (m, 20H), 3.05 (dd, *J* =6.4; 13.1 Hz, 1H), 2.94 (dd, *J* =6.4; 13.1 Hz, 1H), 1.39 (s, 9H). **^{13}C-NMR** (75 MHz, APT, CD_2Cl_2): δ

170.81 (**C**), 155.19 (**C**), 136.42 (**C**), 131.55 (**C**H), 131.38 (**C**H), 120.64 (**C**), 79.81 (**C**), 70.68 (**C**H$_2$), 70.65 (**C**H$_2$), 70.55 (**C**H$_2$), 70.36 (**C**H$_2$), 69.96 (**C**H$_2$), 69.68 (**C**H$_2$), 66.60 (**C**H$_2$), 55.59 (**C**H), 50.89 (**C**H$_2$), 47.49 (**C**H$_2$), 39.37 (**C**H$_2$), 38.28 (**C**H$_2$), 28.10 (**C**H$_3$).

(4*S*,5*S*)-2,2-Dimethyl-1,3-dioxolane-4-carboxylate methyl ester-5-*N*-{(2’*S*)-3’-(4’’-bromophenyl)-1’-*N’*-(14’’’’-azido-3’’’’,6’’’’,9’’’’,12’’’’-tetraoxatetradecane-1’’’’-)-propanamide)}-carboxamide (24e). A clear solution of (2*S*)-3-(4’-bromophenyl)-2-(([(2’’-methyl-2’’-propanyl)oxy]carbonyl)amino)-1-*N*-(14’’’-azido-3’’’,6’’’,9’’’,12’’’-tetraoxatetradecane-1’’’-)-propanamide (**24c**) (1.35 g, 2.3 mmol) in DCM (100 mL) was treated with TFA (50 mL). The resulting clear pale-yellow solution was stirred for 2 hours and evaporated under reduced pressure to yield thick yellow oil/gum. The crude product (**24d**) was used without further purification. The crude product (**24d**) was dissolved in 1:1:1 (v/v/v) dry EOAc/dry MeCN/ dry DMF (22 mL), cooled to 4^{o}C and DIPEA (0.8 ml, 4.6 mmol) was added. A solution of 1-methyl-2,3-*O*-isopropylidene-D-tartrate monoester (**1**) (0.63 g, 2.51 mmol) in dry EtOAc (15 mL) was added to the mixture, followed by COMU (1.17 g, 2.7 mmol), Oxyma (0.325 g, 2.28 mmol) and DIPEA (1.4 mL, 8 mmol) resulting in a solution of pH ~ 9. The solution was stirred at RT overnight, and diluted with EtOAc (150 mL). The organic layer was washed with sat KH_2PO_4 (aq) (3 x 50 mL), sat $NaHCO_3$ (aq) (8 x 50 mL) and brine (3 x 50 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered and the volatiles were evaporated to dryness under reduced pressure to yield a thick yellow oil, which was resolved on silica by flash chromatography (1:1 EtOAc/Heptane to EtOAc) to afford (4*S*,5*S*)-2,2-dimethyl-1,3-dioxolane-4-carboxylate methyl ester-5-*N*-{(2’*S*)-3’-(4’’-bromophenyl)-1’-*N’*-(14’’’’-azido-3’’’’,6’’’’,9’’’’,12’’’’-tetraoxatetradecane-1’’’’-)-propanamide)}-carboxamide (**24e**) (1.38 g, 2.04 mmol) as a thick yellow gum in 89% yield, R$_f$ ~ 0.1 (1:9 Heptane/EtOAc. HR-MS (ESI-TOF) *m/z*: calcd for $C_{27}H_{40}N_5O_{10}BrNa$ [M + Na]$^+$: 696.1856, found 696.1858. ^{1}**H-NMR** (300 MHz, CD_2Cl_2): δ 7.43 (d, *J* = 8.3 Hz, 2H), 7.11 (d, *J* = 8.3, 3H), 6.55 (br s, 1H), 4.68 (d, *J* = 5.3 Hz, 1H), 4.63-4.58 (m, 1H), 4.53 (d, *J* = 5.2 Hz, 1H), 3.79 (s, 3H), 3.66-3.38 (m, 20H), 3.10 (dd, *J* = 6.9; 14.7 Hz, 1H), 2.99 (dd, *J* = 6.9; 14.7 Hz, 1H), 1.41 (s, 3H), 1.36 (s, 3H). ^{13}**C-NMR** (75 MHz, APT, CD_2Cl_2): δ 170.55 (**C**), 169.33 (**C**), 169.23 (**C**), 135.82 (**C**), 131.65 (**C**H), 131.34 (**C**H), 120.89 (**C**), 113.54 (**C**), 77.89 (**C**H), 77.58 (**C**H), 70.71 (**C**H$_2$), 70.65 (**C**H$_2$), 70.56 (**C**H$_2$), 70.36 (**C**H$_2$), 69.97 (**C**H$_2$), 69.52 (**C**H$_2$), 66.60 (**C**H$_2$), 53.71 (**C**H), 52.76 (**C**H$_3$), 50.88 (**C**H$_2$), 39.47 (**C**H$_2$), 38.05 (**C**H$_2$), 29.79 (**C**H$_2$), 26.39 (**C**H$_3$), 26.01 (**C**H$_3$).

(4*S*,5*S*)-2,2-Dimethyl-1,3-dioxolane-4-carboxylic acid-5-*N*-{(2'*S*)-3'-(4''-bromophenyl)-1'-*N'*-(14'''-azido-3''',6''',9''',12'''-tetraoxatetradecane-1'''-yl)-propanamide-2'-yl}-carboxamide (24). A clear pale yellow solution of (4*S*,5*S*)-2,2-dimethyl-1,3-dioxolane-4-carboxylate methyl ester-5-*N*-{(2'*S*)-3'-(4''-bromophenyl)-1'-*N'*-(14''''-azido-3'''',6'''',9'''',12''''-tetraoxatetradecane-1''''-)-propanamide)}-carboxamide (**24e**) (1.37 g, 2.04 mmol) in 9:1 (*v*/*v*) DCM/MeOH, at 4°C, was treated with dropwise addition of a clear solution of 1N NaOH in MeOH (12.25 mL, 12.24 mmol). The resulting solution was stirred for 1 h, cooled in an ice/water bath, acidified to pH ~ 4 with acetic acid and evaporated under reduced pressure to yield a crude white solid. The crude product was dissolved in saturated KH_2PO_4 (aq) (200 mL), acidified to pH ~ 3 with aqueous saturated *ortho*-phosphoric acid and extracted with EtOAc (5 x 30 mL). The organic layers were pooled, dried over anhydrous $MgSO_4$, filtered and evaporated under reduced pressure to afford ((4*S*,5*S*)-2,2-dimethyl-1,3-dioxolane-4-carboxylic acid-5-*N*-{(2'*S*)-3'-(4''-bromophenyl)-1'-*N'*-(14''''-azido-3'''',6'''',9'''',12''''-tetraoxatetradecane-1''''-)-propanamide)}-carboxamide (**24**) (1.35 g, 2.04 mmol) as a thick yellow gum in quantitative yield, R_f ~ 0.3 (0.2:0.8:9 AcOH/EtOH/DCM). HR-MS (ESI-TOF) *m/z*: calcd for $C_{26}H_{37}N_5O_{10}Br$ $[M - H]^-$: 658.1724, found 658.1720. **^{1}H-NMR** (300 MHz, CD_2Cl_2): δ 7.45 (d, *J* = 8.4 Hz, 2H), 7.40 (d, *J* = 8.5, 1H), 7.12 (d, *J* = 8.4 Hz, 2H), 6.62 (br s, 1H), 4.71 (q, *J* = 7.0 Hz, 1H), 4.52 (d, *J* = 8.5 Hz, 1H), 4.36 (d, *J* = 8.5 Hz, 1H), 3.66-3.37 (m, 21H), 3.16 (dd, *J* = 7.0; 13.8 Hz, 1H), 3.04 (dd, *J* = 7.0; 13.8 Hz, 1H), 1.49 (s, 3H), 1.38 (s, 3H). **^{13}C-NMR** (75 MHz, APT, CD_2Cl_2): δ 170.91 (**C**), 169.27 (**C**), 168.15 (**C**), 135.28 (**C**), 131.77 (**C**H), 131.33 (**C**H), 121.18 (**C**), 113.28 (**C**), 76.45 (**C**H), 75.86 (**C**H), 70.69 (**C**H$_2$), 70.65 (**C**H$_2$), 70.54 (**C**H$_2$), 70.49 (**C**H$_2$), 70.31 (**C**H$_2$), 69.95 (**C**H$_2$), 69.40 (**C**H$_2$), 53.85 (**C**H), 50.87 (**C**H$_2$), 39.65 (**C**H$_2$), 38.05 (**C**H$_2$), 29.80 (**C**H$_2$), 26.05 (**C**H$_3$), 25.83 (**C**H$_3$).

1.6. Solution phase CuAAC with linker and tripeptide and loading on resin



Scheme *S5* Reagents and conditions: **a**) $CuSO_4$, cesium ascorbate/ascorbate, CPME, water, RT, Ar, 17 h 30 min, 73%; **b**) 0.5:1:3.5 (*v*/*v*/*v*) dry DCM/dry MeCN/dry EtOAc, DIPEA, PyBOP, HOB*t*•H_2O, pH ≈ 9.5, ON, 90%.

***N*$^{\alpha}$-Fmoc-*O*$^{\beta}$-[5-(1'-(18'-(4''-bromophenyl)-16'-oxo-3',6',9',12'-tetraoxa-15'-azaoctadecan-17'-yl)carbamoyl)-2,2-dimethyl-1,3-dioxolane-4-carboxylic acid)-1*H*-[1',2',3']-triazol-4'-yl)methyl]-D-threonyl-*S*$^{\beta}$-trityl-cysteinyl-1'''-(carbamoylmethyl)-4'''-(*tert*-butyldiphenylsilyl)oxy-*cis*-hydroxyprolinate (25).** Under argon, to a clear yellow solution of propargylated tripeptide (**23**) (0.120 g, 0.106 mmol) in degassed (argon bubbling) CPME (2 mL) was added a clear yellow solution of 0.52 M azido tartrate-based linker (**24**) (0.23 mL, 0.12 mmol) in degassed (argon bubbling) CPME and a clear blue solution of 0.125 M $CuSO_4$ (aq) (0.090 mL, 0.011 mmol) in degassed (argon bubbled) milli-Q water. Upon addition of a clear colorless solution of 0.125 M cesium ascorbate/ascorbic acid (1.50 mL, 0.187 mmol) in degassed (argon bubbled) milli-Q water, the mixture turned a turbid brown with the appearance of a white precipitate in minutes. The resulting turbid mixture was vigorously stirred for 3 h 45 min under argon, then additional solution of 0.125 M cesium ascorbate/ascorbic acid (1.50 mL, 0.187 mmol) in degassed (argon bubbled) milli-Q water was added followed by CPME (3.1 mL), (argon bubbled) milli-Q water (1 mL) and 0.125 M $CuSO_4$ (aq) (0.090 mL, 0.011 mmol) in degassed (argon bubbled) milli-Q water. The resulting turbid brown/white mixture was further stirred for 19 h, then 0.25 M cesium ascorbate/ascorbic acid (1.00 mL, 0.250 mmol) in degassed (argon bubbled) milli-Q water was added. The resulting turbid brown/white mixture was further stirred for 3 h, diluted with 1:10 (*v*/*v*) DCM/EtOAc (110 mL). The organic layer was washed with KH_2PO_4 (aq) pH 2 (1 x 25 mL) as well as brine (2 x 25 mL). The organic layer was dried over anhydrous $MgSO_4$ and all volatiles were evaporated under reduced pressure. The resulting off-white crude solid was resolved on silica by flash chromatography with a loading solvent system of 0.2:0.5:9.3 AcOH/EtOH/DCM and eluted with 0.2:1:8.8 AcOH/EtOH/DCM to afford the triazolyl tripeptide tartrate-based linker (**25**) (0.14 g, 73%) as a foamy beige solid. To wash out residual acetic acid, the triazolyl tripeptide tartrate-based linker (**25**) was dissolved in 1:5 (*v/v*) DCM/EtOAc (120 mL), washed with brine (3 x 30 mL), dried over anhydrous $MgSO_4$, and all volatiles were evaporated under reduced pressure to afford the triazolyl tripeptide tartrate-based linker (**25**) (0.08 g, 40%) as a beige foamy solid. R_f (**25**) = 0.12 (0.2:1:8.8 AcOH/EtOH/DCM). **HR-MS** (ESI-TOF) *m*/*z*: calcd for $C_{93}H_{105}BrN_9O_{19}SSi$ [*M*-H]$^-$: 1790.6206; 1792.6185 found: 1790.6216; 1792.6169. **LR-MS** (MALDI-TOF) *m/z*: calcd for $C_{93}H_{106}BrN_9O_{19}NaSSi$ [*M*+Na]$^+$: 1814.6; 1816.6, found 1814.5, 1816.5. **^{1}H-NMR** (CD_2Cl_2, 400 MHz): δ 7.80 (d, *J* = 7.7 Hz, 2H), 7.70 (d, 1H, *J* = 7.2 Hz), 7.75 (s, 1H), 7.63-7.60 (m, 6H), 7.48-7.12 (m, 31H), 6.69 (d, *J* = 9.3 Hz, 1H), 5.96 (d, *J* = 7.1 Hz, 1H), 5.35 (br s, 1H), 4.91 (d, *J* = 15.6 Hz, 1H), 4.77-4.68 (m, 2H), 4.57-4.54 (m, 2H), 4.47-4.05 (m, 10H), 3.85 (t, *J* = 5.1 Hz, 2H), 3.70-3.33 (m, 20H), 3.19-3.14 (m, 1H), 3.08-2.99 (m, 1H), 2.70-2.66 (m, 1H), 2.59-2.49 (m, 2H), 2.22 (br s, 2H), 1.48 (d, 3H), 1.38 (d, 3H), 1.14 (d, *J* = 5.7 Hz, 3H), 1.03 (s, 9H). **^{13}C-NMR** (100 MHz, APT, CD_2Cl_2): δ 171.00 (**C**), 170.78 (**C**), 170.31 (**C**), 170.15 (**C**), 169.75 (**C**), 169.47 (**C**), 169.34 (**C**), 168.65 (**C**), 156.21 (**C**), 144.65 (**C**), 144.34 (**C**), 144.24 (**C**), 144.19 (**C**), 143.94 (**C**), 141.39 (**C**), 141.38 (**C**), 135.80 (**C**H), 135.74 (**C**H), 135.70 (**C**), 135.39 (**C**), 133.02 (**C**), 133.01 (**C**), 131.76 (**C**H), 131.66 (**C**H), 131.39 (**C**H), 131.36 (**C**H), 130.24 (**C**H), 130.21 (**C**H), 129.54 (**C**H), 128.24 (**C**H), 128.19 (**C**H), 128.08 (**C**H), 127.98 (**C**H), 127.80 (**C**H), 127.17 (**C**H), 127.02 (**C**H), 125.29 (**C**H), 123.87 (**C**H), 121.15 (**C**), 120.97 (**C**), 120.03 (**C**H), 113.28 (**C**), 113.27 (**C**), 76.89 (**C**H), 76.72 (**C**H), 76.59 (**C**H), 76.07 (**C**H), 75.07 (**C**H), 71.69 (**C**H), 70.72 (**C**H_2), 70.66 (**C**H_2), 70.56 (**C**H_2), 70.55 (**C**H_2), 70.52 (**C**H_2), 70.50 (**C**H_2), 70.47 (**C**H_2), 70.45 (**C**H_2), 70.37 (**C**H_2), 70.33 (**C**H_2), 69.97 (**C**H_2), 69.55 (**C**H_2), 69.44 (**C**H_2), 67.13 (**C**H_2), 67.08 (**C**H), 62.94 (**C**H_2), 62.61 (**C**H_2), 58.35 (**C**H), 58.17 (**C**H), 55.46 (**C**H_2), 53.75 (**C**H), 50.89 (**C**H_2), 50.31 (**C**H_2), 47.29 (**C**H), 39.64 (**C**H_2), 39.57 (**C**H_2), 38.07 (**C**H_2), 37.95 (**C**H_2), 37.39 (**C**H_2), 33.26 (**C**H_2), 29.81 (**C**H_2), 26.63 (**C**H_3), 26.07 (**C**H_3), 26.02 (**C**H_3), 25.91 (**C**H_3), 18.91 (**C**), 15.72 (**C**H_3).

Resin loading. The TentaGel MB-NH_2 resin (0.158 g, ≈ 39.5 µmol) was filtered with 0.5:1:3.5 (*v*/*v*/*v*) dry DCM/dry MeCN/dry EtOAc (10 mL). The resin was resuspended in 0.5:1:3.5 (*v*/*v*/*v*) dry DCM/dry MeCN/dry EtOAc (6 mL), shaken for 30 minutes, drained by filtration and the procedure was repeated twice with fresh solvent. The resin was resuspended in a solution of triazolyl tripeptide tartrate-based linker (**25**) (2 equivalents, 0.015 M), PyBOP (6 eq.), HOBt hydrate (3 eq.) and DIPEA (29 eq.) in 0.5:1:3.5 (*v*/*v*/*v*) dry DCM/dry MeCN/dry EtOAc to give pH 9.5. After 16 hours of shaking in the dark, a resin sample was taken and a Kaiser test was applied, qualitatively showing no residual free amine on resin. All materials dissolved in solvent were drained by filtration and the resin was further filtered with 0.5:1:3.5 (*v*/*v*/*v*) dry DCM/dry MeCN/dry EtOAc (10 ml). The resin was resuspended in 0.5:1:3.5 (*v*/*v*/*v*) dry DCM/dry MeCN/dry EtOAc (6 mL), shaken, drained by filtration and the procedure was repeated six more times with fresh solvent. The resin was resuspended in 1:2:2 (*v*/*v*/*v*) Ac_2O/2,4,6-trimethylpyridine/dry EtOAc (5 mL) at pH 7 and shaken for 1 h, then drained by filtration. The resin was washed according to the following procedure: the resin was filtered with 0.5:1:3.5 (*v*/*v*/*v*) dry DCM/dry MeCN/dry EtOAc (15 ml). The resin was resuspended in 0.5:1:3.5 (*v*/*v*/*v*) dry DCM/dry MeCN/dry EtOAc (6 mL), shaken, drained by filtration and the procedure was repeated six more times with fresh solvent. The filtration and the shaking procedures were repeated using dry MeCN, dry EtOAc, dry DCM to yield the triazolyl tripeptide tartrate-based linker resin N^{α}-Fmoc-O^{β}-[5-(1'-(18'-(4''-bromophenyl)-16'-oxo-3',6',9',12'-tetraoxa-15'-azaoctadecan-17'-yl)carbamoyl)-2,2-dimethyl-1,3-dioxolane-4-*N*-(TentaGel MB)-carboxamide)-1*H*-[1',2',3']-triazol-4'-yl)methyl]-D-threonyl-S^{β}-trityl-cysteinyl-1'''-(carbamoylmethyl)-4'''-(*tert*-butyldiphenylsilyl)oxy-*cis*-hydroxyprolinate (**26**) (0.205 g, ≈ 35.5 µmol, 90%).

1.7. Solid Phase Peptide Synthesis of Leu^7, Thr^2 modified phalloidin (30)

Fmoc-deprotection procedure:

The synthesis was initiated from triazolyl tripeptide tartrate-based linker resin (**26**) (0.205 g, ≈ 35.5 μmol). In general, if the previous step was not done in DMF then the resin was shaken 3 x 10 minutes in dry DMF and drained by filtration each time. The resin was resuspended in a clear solution made of 20% (*v/v*) diethylamine in dry DMF (6 mL) at pH 11, shaken for 2 hours 30 minutes, drained by filtration and filtered with dry DMF (15 ml). The resin was resuspended in dry DMF (6 mL), shaken, drained by filtration and the procedure was repeated six more times with fresh solvent. The filtration and the shaking procedures were repeated using 1:1 (*v/v*) EtOH/EtOAc, dry MeCN, dry EtOAc, dry DCM. A Kaiser test was performed. If the following step was peptide coupling, then the resin was further washed with 0.5:1:3.5 (*v/v/v*) dry DCM/dry MeCN/dry EtOAc.

Fmoc-protected amino acid coupling procedure:

The synthesis was initiated from N^{α}-Fmoc-deprotected of triazolyl tripeptide tartrate-based linker resin (**26**) (≈ 35.5 μmol). In general, the freshly N^{α}-Fmoc-deprotected resin was resuspended in a clear yellow solution of N^{α}-Fmoc-protected amino acid (5 eq., 0.04 M), Oxyma Pure (5 eq.), COMU (5 eq.), DIPEA (12 eq.) in 0.5:1:3.5 (*v/v/v*) dry DCM/dry MeCN/dry EtOAc at pH 9.5. The resin was shaken for a maximum of one hour and a half. Upon completion of the reaction, estimated by Kaiser test, the resin was drained by filtration. The resin was washed according to the following procedure: The resin was filtered with 0.5:1:3.5 (*v/v/v*) dry DCM/dry MeCN/dry EtOAc (15 ml). The resin was resuspended in 0.5:1:3.5 (*v/v/v*) dry DCM/dry MeCN/dry EtOAc (6 mL), shaken, drained by filtration and the procedure was repeated six more times with fresh solvent. A Kaiser test was performed.

Capping procedure:

Following each coupling step, a capping step was performed as follows. The resin was resuspended in 1:2:2 (*v/v/v*) Ac_2O/2,4,6-trimethylpyridine/dry EtOAc (5 mL) at pH 7 and shaken for 1 h, then drained by filtration. The resin was washed according to the following procedure: the resin was filtered with 0.5:1:3.5 (*v/v/v*) dry DCM/dry MeCN/dry EtOAc (15 ml). The resin was resuspended in 0.5:1:3.5 (*v/v/v*) dry DCM/dry MeCN/dry EtOAc (6 mL), shaken, drained by filtration and the procedure was repeated six more times with fresh solvent. The filtration and the shaking procedures were repeated using dry MeCN, dry

EtOAc, dry DCM. If the following step was Fmoc-deprotection, then the resin was further washed with dry DMF otherwise the resin was stored dry at 4°C. Starting from triazolyl tripeptide tartrate-based linker resin (**26**) (≈ 35.5 μmol), the amino acids incorporated in the synthesis consisted of N^{α}-Fmoc-alanine, N^{α}-Fmoc-leucine, *N*-1-Boc-3*a*-HO-HPIC-OH, N^{α}-Fmoc-alanine.

3*a*-HO-HPIC derivatives coupling procedure:

The N^{α}-Fmoc-deprotected H-Leu^{7}-Ala^{1}-DThr^{2}(triazole-tartrate-resin)-Cys^{3}(Tr)-*cis*-4-Hyp^{4}(TBDPS)-OCAM (≈ 35.5 μmol) was resuspended in a yellow, clear, solution made of 0.04 M *N*-1-Boc-3*a*-HO-HPIC-OH in 0.5:1:3.5 (*v/v/v*) dry DCM/dry MeCN/dry EtOAc (5 eq., 0.04 M final), Oxyma Pure (5 eq.), COMU (5 eq.), DIPEA (18 eq.) at pH 10. The resin was shaken 17 h 30 min in the dark and drained by filtration. The resin was washed as for the Fmoc-protected amino acid coupling procedure to afford the *N*-1-Boc-3*a*-HO-HPIC-Leu^{7}-Ala^{1}-DThr^{2}(triazole-tartrate-resin)-Cys^{3}(Tr)-*cis*-4-Hyp^{4}(TBDPS)-OCAM (**27**) and stored dry at 4°C.

Savige-Fontana reaction (tryptathionylation) and last amino acid coupling:

The dry *N*-1-Boc-3*a*-HO-HPIC-Leu^{7}-Ala^{1}-DThr^{2}(triazole-tartrate-resin)-Cys^{3}(Tr)-*cis*-4-Hyp^{4}(TBDPS)-OCAM (**27**) (≈ 35.5 μmol), in a spin desalting column (5 mL, Pierce), was resuspended in TFA (5 mL), shaken for 5 h in dark and drained by filtration. The resin was washed according to the following procedure: the resin was filtered with 1:9 (*v/v*) Toluene/AcOH (15 ml). The resin was resuspended in 1:9 (*v/v*) Toluene/AcOH (6 mL), shaken, drained by filtration and procedure was repeated nine more times with fresh solvent. The filtration and shaking procedure was repeated using 1:1 (*v/v*) toluene/EtOAc, MeCN, 1:1 (*v/v*) EtOH/EtOAc, MeCN, EtOAc and DCM. A Kaiser test was performed on the resulting H-[2-mercapto-Trp^{6}-Lys^{7}-Ala^{1}-DThr^{2}(triazole-tartrate-resin)Cys^{3})-*cis*-4-Hyp^{4}(TBDPS)OCAM [*S*-3→6] hexapeptide resin (**28**) which was coupled to N^{α}-Fmoc-Ala-OH as described in the Fmoc-protected amino acid coupling procedure.

Deprotection and macrolactamization:

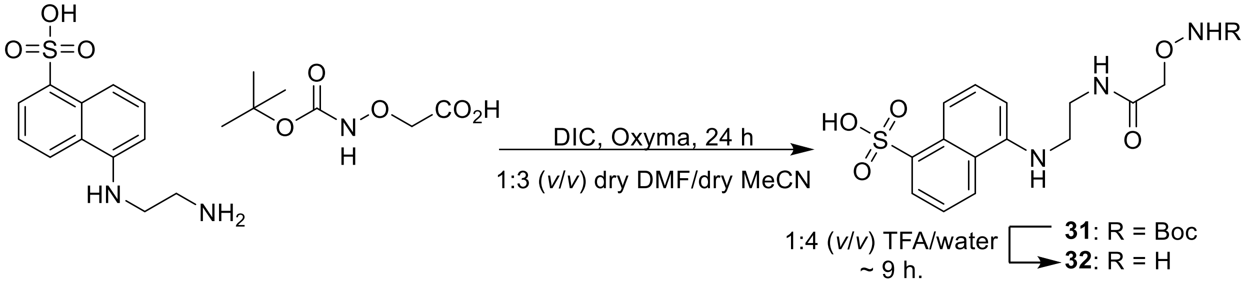
The resulting monocyclic N^{α}-Fmoc-Ala^{5}-[2-mercapto-Trp^{6}-Lys^{7}-Ala^{1}-DThr^{2}(triazole-tartrate-resin)Cys^{3})-*cis*-4-Hyp^{4}(TBDPS)OCAM [*S*-3→6] heptapeptide resin (≈ 35.5 μmol)

(**29a**) was deprotected according to the Fmoc-deprotection procedure. Then, the carboxamidomethyl ester protecting group was saponified as follows: The CAM protected monocyclic heptapeptide resin was filtered with 1:1 (*v/v*) Milli-Q water/DMF (10 mL). The resin was resuspended in 1:1 (*v/v*) Milli-Q water/DMF (6 mL), shaken for 30 minutes, drained by filtration and the procedure was repeated twice with fresh solvent. The resin was resuspended in 0.15 M Cs_2CO_3 of 1:1 (*v/v*) Milli-Q water/DMF (5 mL) pH 14, shaken in the dark for 20 h and drained by filtration. The resin was filtered with 1:1 (*v/v*) Milli-Q water/DMF (10 mL). The resin was resuspended in 1:1 (*v/v*) Milli-Q water/DMF (6 mL), shaken, drained by filtration and the washing procedure was repeated six more times with fresh solvent. The filtration and the shaking procedures were repeated using Milli-Q water, 1:4 (*v/v*) EtOH/Milli-Q water, 1:4 (*v/v*) EtOH/MeCN, MeCN, EtOAc, DCM, 0.5:1:3.5 (*v/v/v*) dry DCM/dry MeCN/dry EtOAc.

The resulting monocyclic H-Ala^5-[2-mercapto-Trp^6-Lys^7-Ala^1-DThr^2(triazole-tartrate-resin)Cys^3)-*cis*-4-Hyp^4(TBDPS)-OH [*S*-3→6] heptapeptide resin (≈ 35.5 μmol) (**29b**), in a spin desalting column (5 mL, Pierce), was shaken 3 x 20 minutes in 0.5:1:3.5 (*v/v/v*) dry DCM/dry MeCN/dry EtOAc (6 mL) and drained by filtration each time. The resin was resuspended in a clear, solution made of PyBOP (2 eq., 0.016 M), HOBt hydrate (2 eq.), DIPEA (4 eq.) in 0.5:1:3.5 (*v/v/v*) dry DCM/dry MeCN/dry EtOAc (5 mL) at pH 9.5 and shaken for 23 h 30 min. Upon completion of reaction, estimated by Kaiser test and bromocresol green, the resin was drained by filtration and washed according to the following procedure: The resin was filtered with 0.5:1:3.5 (*v/v/v*) dry DCM/dry MeCN/dry EtOAc (15 ml). The resin was resuspended in 0.5:1:3.5 (*v/v/v*) dry DCM/dry MeCN/dry EtOAc (6 mL), shaken, drained by filtration and procedure was repeated five more times with fresh solvent. The filtration and shaking procedure was repeated using MeCN, 1:1 (*v/v*) EtOH/EtOAc, MeCN, EtOAc and DCM to afford the bicyclic(Ala^1-DThr^2[O$^\beta$-[5-(1’-(18’-(4’’-bromophenyl)-16’-oxo-3’,6’,9’,12’-tetraoxa-15’-azaoctadecan-17’-yl)carbamoyl)-2,2-dimethyl-1,3-dioxolane-4-*N*-(TentaGel MB)-carboxamide)-1*H*-[1’,2’,3’]-triazol-4’-yl)methyl]]-[Cys^3-*cis*-4-Hyp^4(TBDPS)-Ala^5-2-mercapto-Trp^6]-Leu^7)[*S*-3→6] resin (**29c**). The resin was dried in vacuo and stored at 4°C.

The TBDPS protecting group was removed from the bicyclic phallotoxin resin as follows: The bicyclic(Ala^1-DThr^2[O$^\beta$-[5-(1'-(18'-(4''-bromophenyl)-16'-oxo-3',6',9',12'-tetraoxa-15'-azaoctadecan-17'-yl)carbamoyl)-2,2-dimethyl-1,3-dioxolane-4-*N*-(TentaGel MB)-carboxamide)-1*H*-[1',2',3']-triazol-4'-yl)methyl]] -[Cys^3-*cis*-4-Hyp^4(TBDPS)-Ala^5-2-mercapto-Trp^6]-Leu^7)[*S*-3→6] resin (≈ 35.5 μmol) (**29c**) in a spin desalting column (5 mL, Pierce), was shaken 3 x 15 minutes THF (6 mL) and drained by filtration each time. The resin was resuspended in 0.58 M TBAF in THF (5.5 mL), shaken overnight, drained by filtration and washed according to the following procedure: The resin was filtered with THF (10 ml). The resin was resuspended in THF (6 mL), shaken, drained by filtration and procedure was repeated five more times with fresh solvent. The filtration and shaking procedure was repeated using DMF, MeCN, 1:4 (*v/v*) EtOH/MeCN, MeCN and DCM to afford the bicyclic(Ala^1-DThr^2[O$^\beta$-[5-(1'-(18'-(4''-bromophenyl)-16'-oxo-3',6',9',12'-tetraoxa-15'-azaoctadecan-17'-yl)carbamoyl)-2,2-dimethyl-1,3-dioxolane-4-*N*-(TentaGel MB)-carboxamide)-1*H*-[1',2',3']-triazol-4'-yl)methyl]] -[Cys^3-*cis*-4-Hyp^4-Ala^5-2-mercapto-Trp^6]-Leu^7)[*S*-3→6] resin (**30**). The resin was dried in vacuo and stored at 4°C.

1.8. Fluorescent Probe preparation:

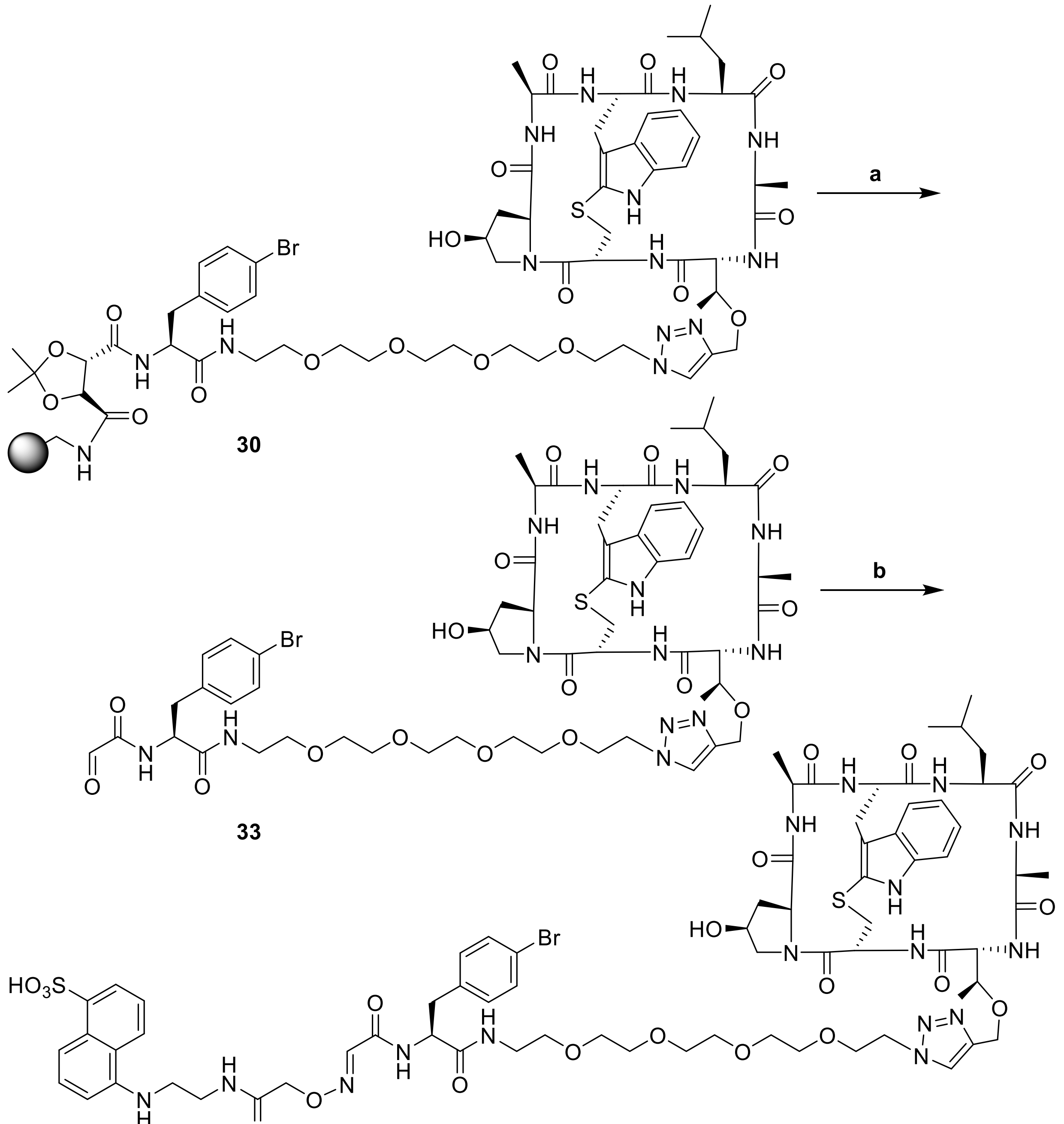


Scheme *S6* Fluorescent EDANS (**32**) preparation.

***N*-[5''-(2'-Aminoethyl)-1''-naphthalenesulfonic)-[({[(2-methyl-2-propanyl)oxy]carbonyl}amino)oxy]acetamide (31).** Under argon, a clear yellow solution of [({[(2-methyl-2-propanyl)oxy]carbonyl}amino)oxy]acetic acid (0.102 g, 0.533 mmol), 5-[(2-aminoethyl)amino]-1-naphthalenesulfonic acid (0.169 g, 0.586 mmol), Oxyma Pure (0.083 g, 0.586 mmol) and DIC (0.092 mL, 0.586 mmol) in 1:3 (v/v) dry DMF/dry MeCN (28 mL) was stirred for 24 h in the dark. The mixture was evaporated to dryness under reduced pressure to afford a crude orange solid that was resolved on silica by flash chromatography (7:3 DCM/EtOAc to 2:4:4 EtOH/DCM/EtOH), repurified by manual reverse phase column chromatography on Sep-Pak C18 gel (Waters, Delaware) as described in General Experimental Information, then repurified on silica by flash chromatography (1:5:4 EtOH/DCM/EtOAc to 3:7 EtOH/DCM) to afford *N*-[5’’-(2’-aminoethyl)-1’’-naphthalenesulfonic)-[({[(2-methyl-2-propanyl)oxy]carbonyl}amino)oxy]acetamide (**31**) (0.078 g, 0.178 mmol), as a white solid in 33% yield, R_f (**31**) = 0.10 (2:3:5 EtOH/EtOAc/DCM). RP-HPLC (C18): t_r = 20.75 min (A^{341} 100%) with gradient of 20% to 100% solvent B over 32 min at a flow rate of 1 mL/min; UV-Vis spectra acquired at three values: λ_{max} = 215, 252, 340 nm. **HR-MS** (ESI-TOF) *m/z*: calcd for $C_{19}H_{24}N_3O_7S$ [*M* - H]$^-$: 438.1335, found: 438.1333. **^{1}H-NMR** (400 MHz, MeOD + D_2O): δ 8.05 (d, *J* = 8.7 Hz, 1H), 7.99 (t, *J* = 6.8 Hz, 2H), 7.40-7.35 (m, 2H), 6.68 (d, *J* = 7.7 Hz, 1H), 3.55 (t, *J* = 5.9 Hz, 2H), 3.37 (t, *J* = 5.9 Hz, 2H), 1.24 (s, 9H). **^{13}C-NMR** (100 MHz, APT, $CDCl_3$): δ 172.95 (**C**), 145.31 (**C**), 141.08 (**C**), 131.21 (**C**), 129.23 (**C**H), 127.25 (**C**H), 126.00 (**C**), 125.90 (**C**H), 124.53 (**C**H), 116.82 (**C**H), 106.65 (**C**H), 84.59 (**C**), 76.47 (**C**), 44.81 (**C**H_2), 39.50 (**C**H_2), 28.70 (**C**H_3).

***N*-[5’’-(2’-Aminoethyl)-1’’-naphthalenesulfonic)-[(amino)oxy] acetamide (32)**. A solution of 99 mM *N*-Boc-Aoa-EDANS (**31**) (0.022 g, 0.050 mmol) in 1:4 (*v/v*) TFA/water (105 equivalents, 0.5 mL) was stirred for 9 hours in the dark. Completion of reaction was assessed by RP-HPLC and LR-MS (ESI-single quadrupole). The mixture was cooled in an ice/water bath, and basified to pH 5 following treatment with 5 M NaOH (aq) (1 mL) and AcOH (0.02 mL). The crude *N*-[5’’-(2’-Aminoethyl)-1’’-naphthalenesulfonic)-[(amino)oxy] acetamide (**32**) mixture was diluted in MeCN (2 mL) and used immediately for the oximation of glyoxamide [Leu^7, Thr^2 modified phalloidin (**33**).

1.9. Fluorescent [Leu⁷, Thr²(Oxime-EDANS)]-phalloidin (34)



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Scheme *S7* Reagents and conditions: **a**) *i*) 95:2.5:2.5 (*v/v/v*) TFA/water/TIS, 2 h; *ii*) 1:1:4 (*v/v/v*) AcOH/*t*-BuOH/water, NaIO$_4$, pH ≈ 5, 10 min; *iii*) dimethylsulfide, ≈ 5 min; 1 M aqueous sodium thiosulfate ($Na_2S_2O_3$); **b**) *i*) Freshly deprotected under argon H-Aoa-EDANS (**32**), 1,4-phenylenediamine hydrochloride, MeCN, NaOH, pH ≈ 6.5, 24 h; *ii*) Sep-Pak (C18) in 0.1% FA (v/v) water/MeCN; *iii*) RP-HPLC (C18) in 0.1% FA *(v/v)* water/MeCN.

Tartrate acetonide deprotection and oxidative cleavage:

The dry bicyclic(Ala^{1}-DThr^{2}[O^{β}-[5-(1’-(18’-(4’’-bromophenyl)-16’-oxo-3’,6’,9’,12’-tetraoxa-15’-azaoctadecan-17’-yl)carbamoyl)-2,2-dimethyl-1,3-dioxolane-4-*N*-(TentaGel MB)-carboxamide)-1*H*-[1’,2’,3’]-triazol-4’-yl)methyl]] -[Cys^{3}-*cis*-4-Hyp^{4}-Ala^{5}-2-mercapto-Trp^{6}]-Leu^{7})[*S*-3→6] resin (**30**) (≈ 8 µmole), in a mini-spin desalting column (0.5 mL, Pierce), was resuspended in 95:2.5:2.5 (*v*/*v*/*v*) TFA/ H_2O/TIS (0.5 mL), shaken for 3 hours in the dark and the solution was drained by filtration. The resin was washed according to the following procedure: the resin was filtered with AcOH (5 mL). The resin was resuspended in AcOH (0.6 mL), shaken, drained by filtration and procedure was repeated nine more times with fresh solvent. The filtration and shaking procedure was repeated using MeCN, CPME, DCM, MeCN, and 1:1:1:3 (*v*/*v*/*v*) *t*-BuOH/AcOH/MeCN/Milli-Q water.

The resulting resin was resuspended in 1:1:1:3 (*v*/*v*/*v*) *t*-BuOH/AcOH/MeCN/Milli-Q water (0.6 mL), shaken for 30 minutes, drained by filtration and procedure was repeated twice with fresh solvent. The resin was resuspended in a solution of 90 mM $NaIO_4$ (6 eq.) in 1:1:1:3 (*v*/*v*/*v*) *t*-BuOH/AcOH/MeCN/Milli-Q water, shaken for 8 minutes in the dark. The supernatant was collected by filtration. The resin was drained by filtration with 1:1:1:3 (*v*/*v*/*v*) *t*-BuOH/AcOH/MeCN/Milli-Q water (1 mL) and the filtrate was pooled with the previous filtrate. The resin was resuspended in 1:1:1:3 (*v*/*v*/*v*) *t*-BuOH/AcOH/MeCN/Milli-Q water (0.5 mL), shaken and drained by filtration where the filtrate was pooled with the previous one. The shaking procedure was repeated nine more times with fresh solvent. Then, to the pooled filtrate was added dimethyl sulfide (30 equivalents relative to $NaIO_4$) (0.112 mL) to quench excess sodium meta-periodate. The mixture then turned a clear brown. Products were further collected from the resin by repeating the filtration and shaking procedure with 2:4 (*v*/*v*) water/MeCN and MeCN. To the pooled filtrate was added a solution of 0.5 M sodium thiosulfate aqueous (6 equivalents relative to $NaIO_4$) to quench iodine by-products. The bicyclic(Ala^{1}-DThr^{2}[O^{β}-[5-(1’-(18’-(4’’-bromophenyl)-16’-oxo-3’,6’,9’,12’-tetraoxa-15’-azaoctadecan-17’-yl)-glyoxamide)-1*H*-[1’,2’,3’]-triazol-4’-yl)methyl]]-[Cys^{3}-*cis*-4-Hyp^{4}-Ala^{5}-2-mercapto-Trp^{6}]-Leu^{7})[*S*-3→6] (**33**) mixture was clear and colorless at first with the slow appearance of a white precipitate and was used directly for the oximation step.

Oximation:

To the crude bicyclic(Ala^1-DThr^2[*O*$^\beta$-[5-(1’-(18’-(4’’-bromophenyl)-16’-oxo-3’,6’,9’,12’-tetraoxa-15’-azaoctadecan-17’-yl)-glyoxamide)-1*H*-[1’,2’,3’]-triazol-4’-yl)methyl]]-[Cys^3-*cis*-4-Hyp^4-Ala^5-2-mercapto-Trp^6]-Leu^7)[*S*-3→6] (**33**) (≈ 0.8 μmol) mixture was added the freshly prepared crude *N*-[5’’-(2’-Aminoethyl)-1’’-naphthalenesulfonic)-[(amino)oxy] acetamide (**32**) mixture. Under vigorous stirring was added MeCN (3 mL), Milli-Q water (1 mL), THF (0.7 mL), 1:1:1:3 (*v*/*v*/*v*) *t*-BuOH/AcOH/MeCN/Milli-Q water (5.3 mL), 5 M NaOH (aq) (2.066 mL) and 1,4-phenylenediamine dihydrochloride (0.024 g, 0.133 mmol). The resulting turbid mixture was stirred in the dark for 15 h 30 min and evaporated under reduced pressure, to dryness, to afford the crude bicyclic(Ala^1-DThr^2[*O*$^\beta$-[5-(1’-(18’-(4’’-bromophenyl)-16’-oxo-3’,6’,9’,12’-tetraoxa-15’-azaoctadecan-17’-yl)-amido-aldoxime-EDANS)-1*H*-[1’,2’,3’]-triazol-4’-yl)methyl]] -[Cys^3-*cis*-4-Hyp^4-Ala^5-2-mercapto-Trp^6]-Leu^7)[*S*-3→6] (**34**) as a purple solid.

<u>Fluorescent [Leu^7, Thr^2(Oxime-EDANS)]-phalloidin (**34**) purification</u>:

The crude mixture was dissolved in 0.1%FA in Milli-Q water (Eluent) A and loaded onto a Sep-Pak (C18, 500 mg) column (Waters, Delaware) pre-equilibrated with Eluent A (*General experimental Information*). The desired fluorescent [Leu^7, Thr^2(Oxime-EDANS)]-phalloidin (**34**) was eluted with a 10 % (*v*/*v*) gradient of 0.1%FA in MeCN (Eluent B) per 10 mL against Eluent A (*General experimental Information*), lyophilized and further purified by RP-HPLC (C18) with a gradient of 30% to 100% of Eluent B against Eluent A (*General experimental Information*) over 18 min at 2 ml/min to afford the desired fluorescent [Leu^7, Thr^2(Oxime-EDANS)]-phalloidin (**34**) (0.18 μmol, 2% if initial loading 0.25 mmol/g) as a white powder following lyophilisation. RP-HPLC (C18): t_r = 12.81 min (A_{290} 89%), with gradient of 30% to 100% eluent B over 18 min at 2 mL/min; UV-Vis spectra with λ_{max} = 226 nm, 292 nm, 338 nm. **LR-MS** (MALDI-TOF) *m*/*z*: calcd for $C_{73}H_{94}BrN_{16}O_{20}S_2$ [*M*-H]$^-$: 1657.5: 1659.5, found 1658.0: 1660.0.

<u>EDANS-oxime-phallotoxin derivative (**34**) quantification procedure.</u>

All pipetmen were calibrated by gravimetry using water. A standard (calibration) curve was prepared by measuring the absorbance at 340 nm of solutions of various, but exact, concentrations of EDANS (λ_{max} = 252, 335 nm) in 0.1% FA 4:6 (*v*/*v*) MeCN/Milli-Q water. All volumes and absorbance measurements were done in duplicate to give a linear regression curve of $A_{340} = 4728.4x - 0.0134$ with $R^2 = 0.998$ where A_{340} is the absorbance measured at 340 nm and x is the corresponding molar concentration according to the Beer-Lambert law (l = 1 cm). Solutions of fluorescent [Leu^7, Thr^2(Oxime-EDANS)]-phalloidin (**34**) were prepared in 0.1% FA in 4:6 (*v*/*v*) MeCN/water, samples were taken, diluted in 0.1% FA 4:6 (*v*/*v*) MeCN/Milli-Q water and absorbance at 340 nm was measured. All volumes and absorbance measurements were done in duplicate and the linear regression formulas were used to deduce the amount of fluorescent [Leu^7, Thr^2(Oxime-EDANS)]-phalloidin (**34**).

1.10. *In vitro* F-actin polymerization on Leu^7, Thr^2 modified phalloidin resin (30).

The G-actin from rabbit skeletal muscle was purified and stored according to the standard protocol.[9] Otherwise, it was reconstituted with 0.22 µm filtered Milli-Q water from a commercial lyophilized mixture (Cytoskeleton, Inc) of 10 mg/mL rabbit skeletal muscle G-actin, 5 mM Tris-HCl pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ATP, 5% (*w*/*v*) sucrose and 1% (*w*/*v*) dextran. Buffer A was prepared fresh from 5 mM Tris-HCl pH 8.0, 0.2 mM $CaCl_2$, 0.5 mM DL-1,4-dithiothreitol (DTT) DNAse, RNAse and Protease free, 0.2 mM ATP and filtered through a 0.22 µm filter. Buffer A was used to prepare a fresh solution of 0.1% (*w*/*v*) bovine serum albumin (BSA) protease and enzyme-free, then filtered through a 0.22 µm filter. The 10X Polymerization Buffer was prepared fresh from 500 mM KCl, 20 mM $MgCl_2$ and 10 mM ATP in 100 mM Tris-HCl, pH 7.5, filtered through a 0.22 µm filter. The resin was filtered with 70% (*v*/*v*) ethanol in Milli-Q water (5 mL). The Leu^7, Thr^2 modified phalloidin resin (**30**) was resuspended in 70% (*v*/*v*) ethanol in Milli-Q water (5 mL), shaken, drained by filtration and the procedure was repeated four more times. The procedure was reapplied to the resin with sterile Milli-Q water (5 mL) and sterile Buffer A (5 mL). The resin was resuspended in 0.1% (*w*/*v*) BSA in Buffer A and swollen for 2 h. Meanwhile, a solution of fluorescent “doped” G-actin was prepared from 0.4 mg/mL rabbit skeletal muscle G-actin, 0.06 mg/mL rabbit skeletal muscle G-actin Alexa Fluor™ 647 conjugate (Thermo Fisher Sci.) and 0.1% BSA in Buffer A, mixed and left on ice for 1 h in the dark. The swollen resin was distributed in four wells of a 96-wells glass flat bottom plate. To each well was poured various amount of: 0.1% BSA in Buffer A and fluorescent “doped” G-actin solution for a total of 180 µL. To each well was added the 10X Polymerization Buffer (20 µL), mixed and left on ice in dark for 1 hour. The solution was withdrawn from the wells, replaced by fresh 0.1% BSA in Buffer A (200 µL) and the procedure was repeated once. The beads were injected between a microscopy slide and a coverslip previously mounted. The beads were immediately imaged with a Leica SP5 X Laser Scanning Confocal Microscope (inverted), objective HC PL APO 10x/0.40 CS oo/0.17 /A, excitation laser 554 nm with emission filter (band of collection) from 555-650 nm.

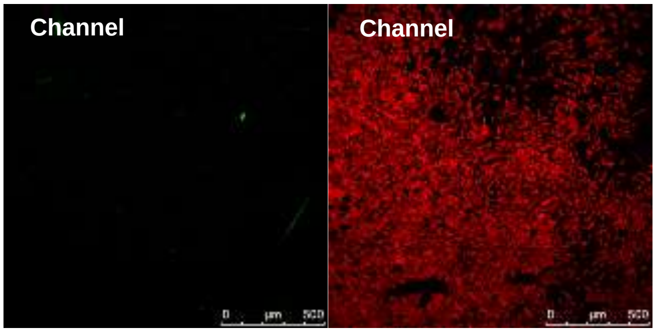
1.11. Bio-assay of Fluorescent [Leu^7, Thr^2(Oxime-EDANS)]-phalloidin (34)

Cell culture. CHO cells were provided by the Biological Services Laboratory (Dr. Elena Polishchuk), Chemistry dept. UBC. All manipulations were carried out in a sterile and cell culture grade environment including equipment, chemicals, solutions, media and supplements. Cells were cultured in α-MEM medium supplemented with 10% (*v*/*v*) FBS and 100 units/mL of penicillin as well as 100 µg/mL of streptomycin under incubation in 95% air: 5% CO_2 at 37^{o}C. Medium was replaced every 5 days. Sub-culturing was achieved as follows: From 80% confluent cells in 75 cm^2 flask, the medium was removed and discarded. The cell layer was briefly rinsed with phosphate buffer saline (PBS (1X)) pH 7.4, then 0.25% (*w*/*v*) Trypsin-0.53 mM EDTA solution (1 to 2 mL) was added and incubated in 95% air: 5% CO_2 at 37^{o}C until cell layer dispersed (minimum exposure time, usually within 5 to 15 minutes). To the flask was added 6.0 to 8.0 mL of complete growth medium and the cell suspension was transferred to a centrifuge tube by gentle pipetting. They were spun at approximately 125 x g for 5 to 10 minutes; the supernatant was discarded and the cell pellet was re-suspended in a known volume of freshly made growth medium. The concentration of cells was determined and distributed in a new flask with a sub-cultivation ratio of 1:3 to 1:4.

Fluorescent binding assay with CHO cells. Prior to the staining experiment, the CHO cells were detached from culture dishes with trypsin/EDTA as previously described but dispersed in 10^5 cells/well of LabTek 8-chamber slides (Nunc Inc. Rochester, NY), working volume of 400 µL and incubated overnight in α-MEM medium supplemented with 10% (*v*/*v*) FBS and 100 units/mL of penicillin as well as 100 µg/mL of streptomycin in 95% air: 5% CO_2 at 37^{o}C. Stock labeling solutions were made from lyophilized EDANS-oxime-phallotoxin derivative (**34**) (0.148 µmole) dissolved in 791 µL of sterile PBS (1X) and sterile DMSO (2 µL) to give clear solutions of 187 µM and used immediately. The stock blocking solution was prepared from commercially available phalloidin-TRITC (0.076

µmole) dissolved in 50 µL EtOH and 75 µL MeOH resulting in a pink solution of 613 µM phalloidin-TRITC. Then, to each well of LabTek 8-chamber slides containing CHO cells, cell medium was replaced by sterile PBS 1X (500 µL), discarded and procedure was repeated once more. The CHO cells were fixed with 4% (*w*/*v*) paraformaldehyde in PBS (1X) (400 µL) for 15 minutes. The paraformaldehyde solution was replaced by sterile PBS (1X) (500 µL), discarded and procedure was repeated once more. The CHO cells were permeabilized with 0.1% (*v*/*v*) Triton X-100 in PBS (1X) (400 µL) for 10 minutes. The Triton X-100 solution was replaced by sterile PBS (1X) (500 µL), discarded and procedure was repeated three additional times. CHO cells were exposed in dark for 21 min to 100 µM of EDANS-oxime-phallotoxin derivative (**34**) in sterile PBS (1X) (300 µL), or only sterile PBS (300 µL) or 613 µM stock phalloidin-TRITC (49 µL) diluted in sterile PBS (1X) (91 µL) resulting in 214 µM of phalloidin-TRITC in 140 µL, or 613 µM stock phalloidin-TRITC (5 µL) diluted in sterile PBS (1X) (295 µL) resulting in 10 µM of phalloidin-TRITC in 300 µL. Then, to the CHO cells, already exposed to 214 µM of phalloidin-TRITC, was added 160 µL of 187 µM EDANS-oxime-phallotoxin derivative (**34**) in sterile PBS (1X). All the cell assays were further exposed in the dark for 15 minutes, and all solutions were discarded. From each well of the LabTek 8-chamber slides containing CHO cells, the solution was replaced by sterile PBS (500 µL), gently shaken and solution was discarded. The washing procedure was repeated twice and each well of the LabTek 8-chamber slides containing Ar42J cells was filled with PBS (1X) (300 µL). The cells were immediately imaged with a Leica SP5 X Laser Scanning Confocal Microscope (inverted), objective HC PL APO 10x/0.40 CS oo/0.17 /A, excitation laser of 405 nm, emission filter Long Pass (415 nm-690 nm) and excitation laser 554 nm with emission filter (band of collection) from 555-650 nm.

1.12. Negative control for bio-assay of Fluorescent [Leu^7, Thr^2(Oxime-EDANS)]-phalloidin (34)



Confocal microscopy (Obj 10x) of CHO cells exposed to fluorescent phalloidin (PHD) derivatives. Fixed and permeabilized CHO cells were blocked with 214 µM [HyLeu^7(TRITC)]-PHD prior exposure to 100 µM [D-Thr^2(EDANS), Leu^7]-PHD (**34**). Signal was detected from [HyLeu^7(TRITC)]-PHD (Channel B, 545 nm excitation) but not from [D-Thr^2(EDANS), Leu^7]-PHD (**34**) (Channel A, 405 nm excitation).

2. NMR and UV-Vis spectra with RP-HPLC chromatograms

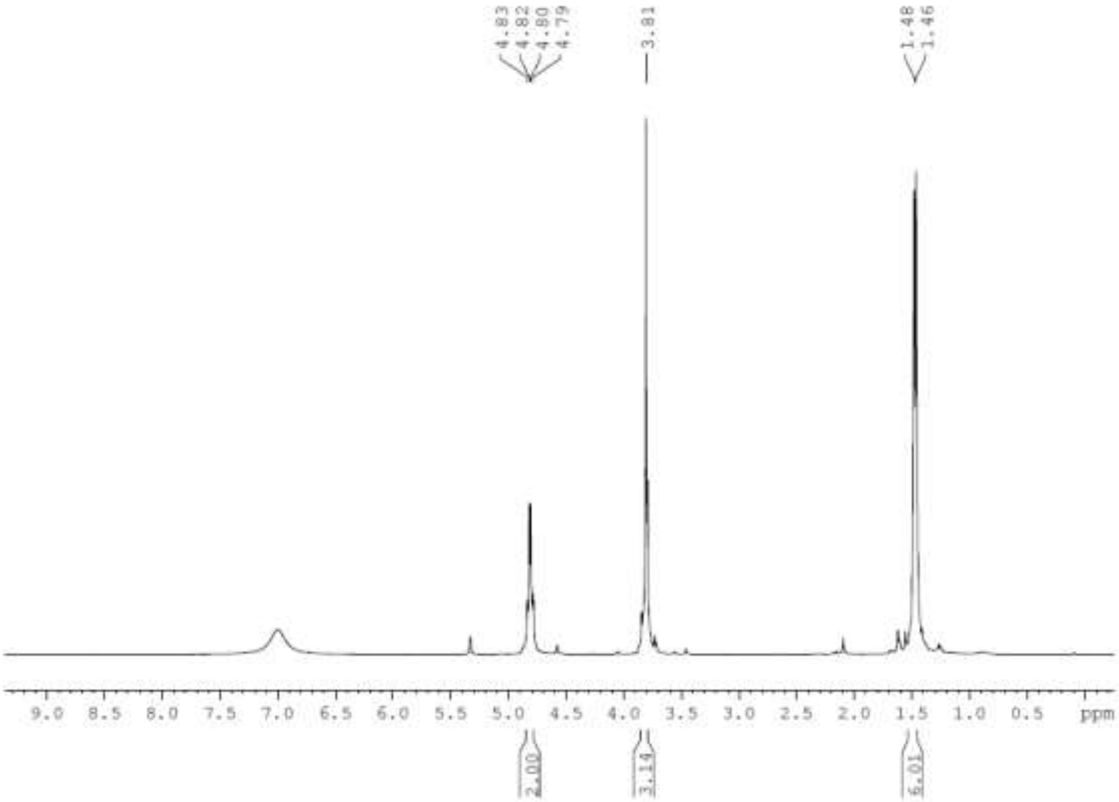


Figure *S1* ^{1}H-NMR spectra (300 MHz, CD_2Cl_2) of (4*S*,5*S*)-,2,2-dimethyl-1,3-dioxolane-4,5-dicarboxylate-4-methyl ester (**1**).

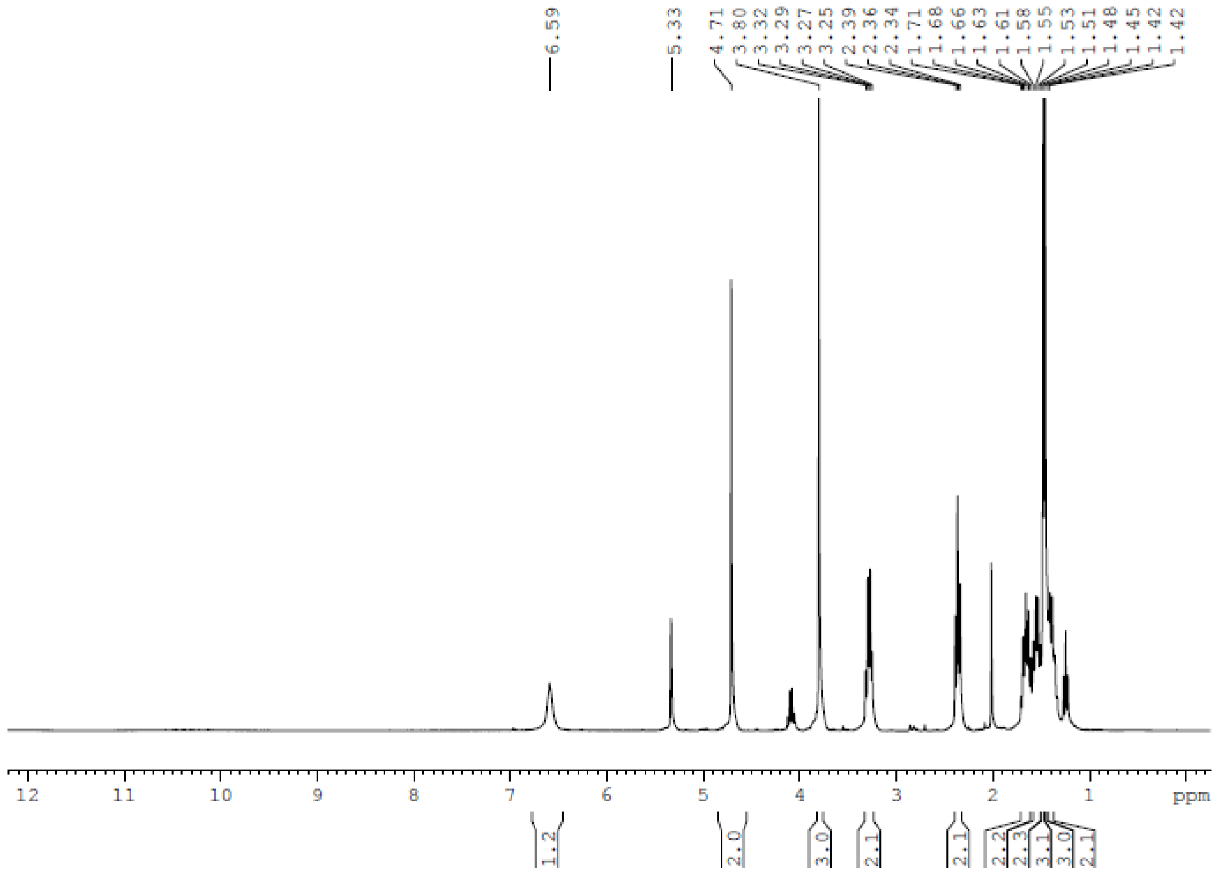


Figure *S2* ^{1}H-NMR spectra (300 MHz, CD_2Cl_2) of methyl-(4*S*,5*S*)-2,2-dimethyl-1,3-dioxolane-4-carboxylate-5-*N*-(hexanoic acid)-carboxamide (**2**).

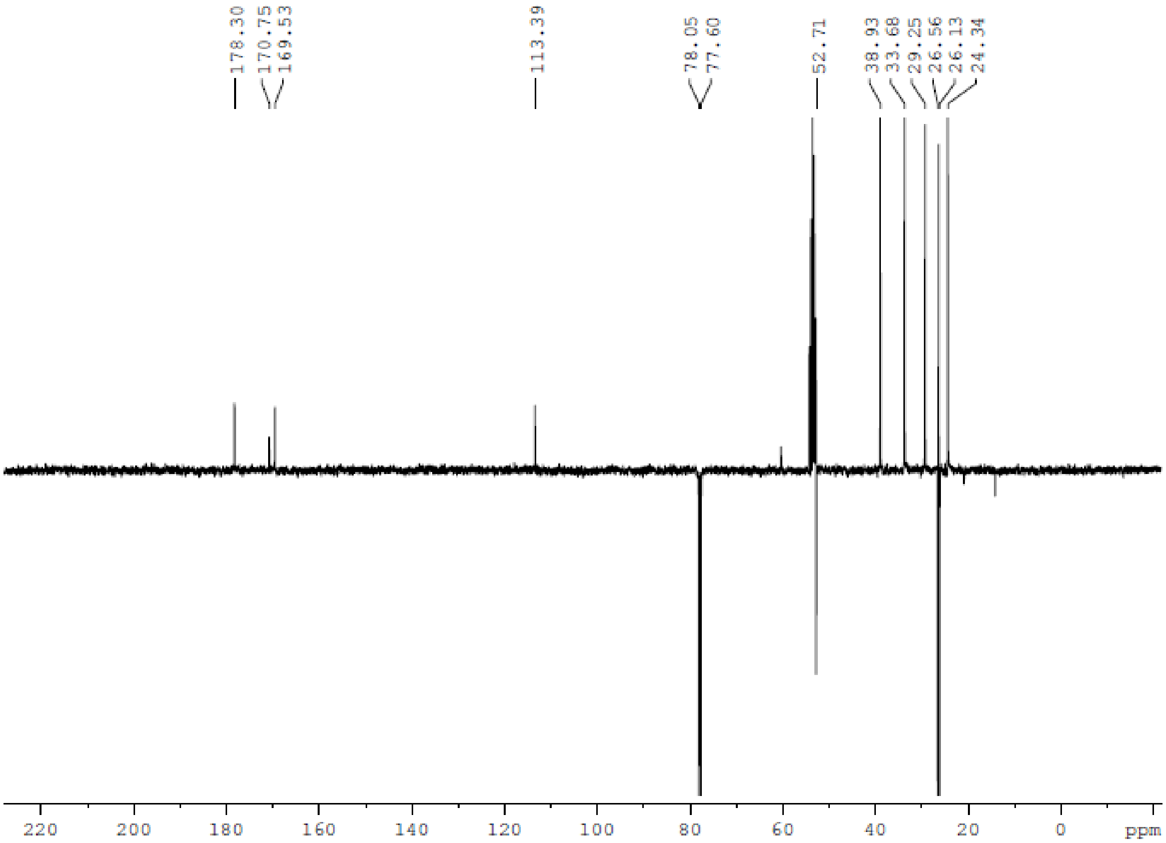


Figure *S3* ^{13}C-NMR spectra (75 MHz, APT, CD_2Cl_2) of methyl-(4*S*,5*S*)-2,2-dimethyl-1,3-dioxolane-4-carboxylate-5-*N*-(hexanoic acid)-carboxamide (**2**).

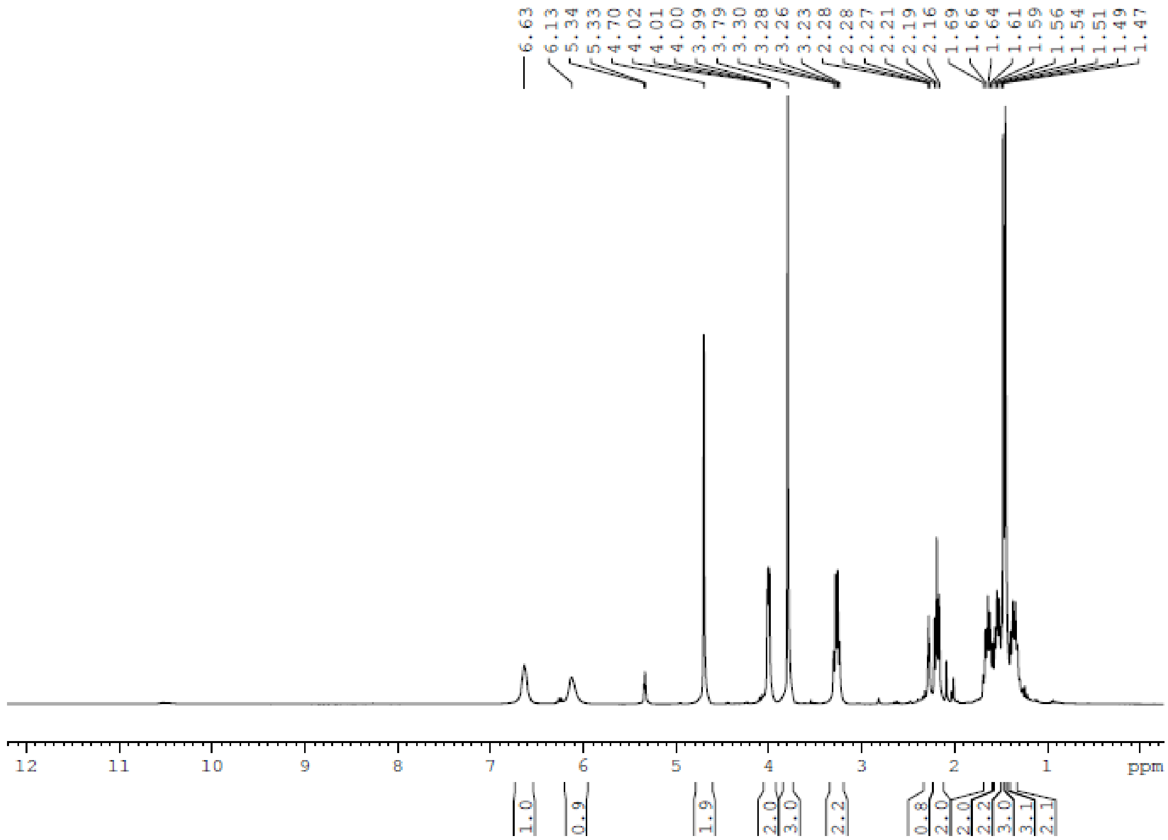


Figure *S4* ^{1}H-NMR spectra (300 MHz, CD_2Cl_2) of methyl-(4*S*,5*S*)-2,2-dimethyl-1,3-dioxolane-4-carboxylate-5-*N*-(1'-*N'*-[2''-propyn-1''-yl]-hexanamide-6'-yl)-carboxamide (**3**).

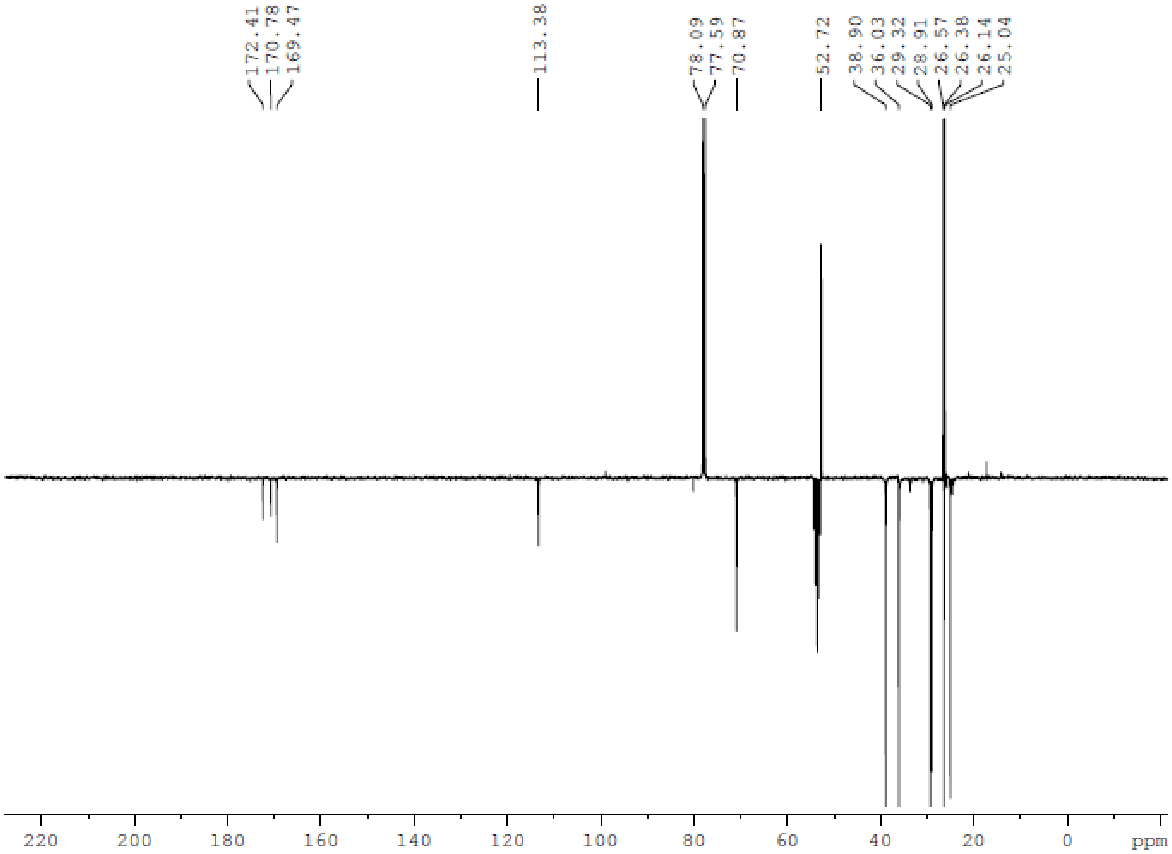


Figure *S5* ^{13}C-NMR spectra (75 MHz, APT, CD_2Cl_2) of methyl-(4*S*,5*S*)-2,2-dimethyl-1,3-dioxolane-4-carboxylate-5-*N*-(1’-*N’*-[2’’-propyn-1’’-yl]-hexanamide-6’-yl)-carboxamide (**3**).

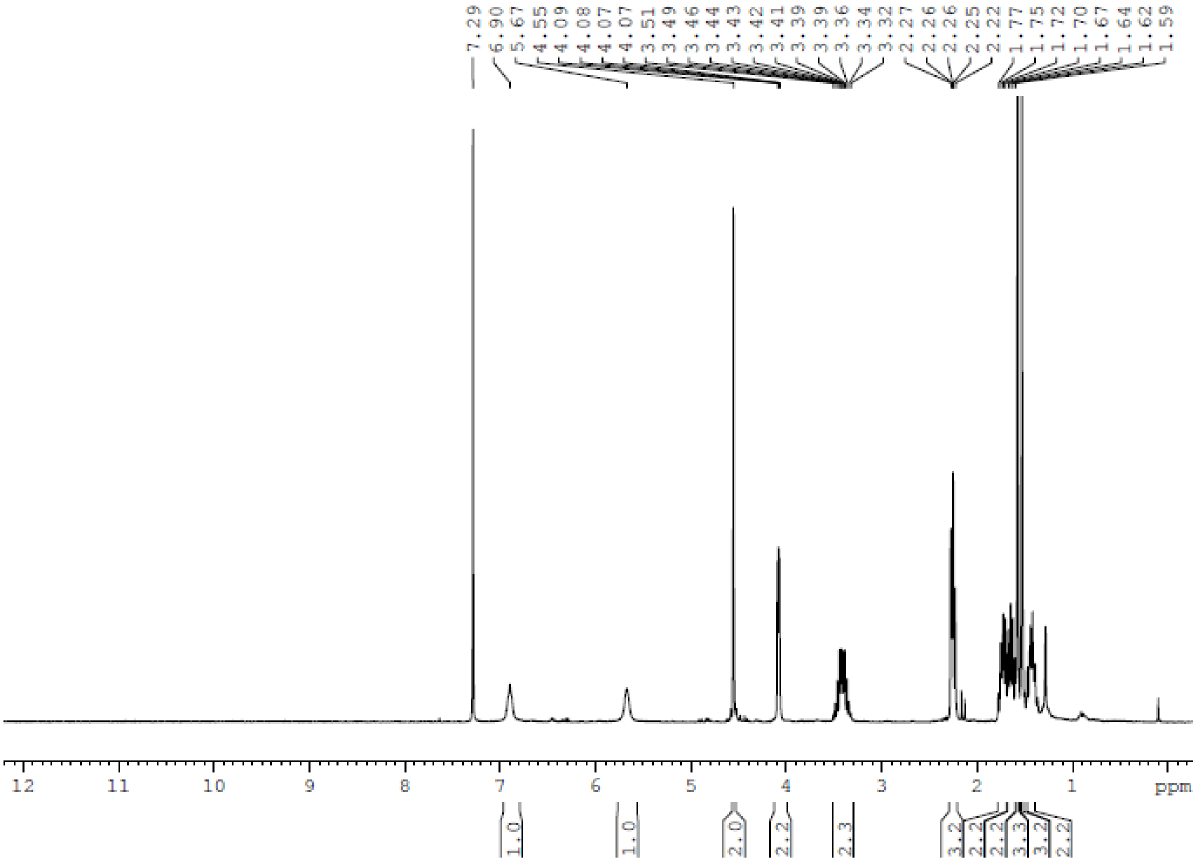


Figure *S6* ^{1}H-NMR spectra (300 MHz, $CDCl_3$) of (4*S*,5*S*)-2,2-dimethyl-1,3-dioxolane-4-carboxylic acid-5-*N*-(1'-*N'*-[2''-propyn-1''-yl]-hexanamide-6'-yl)-carboxamide (**4**).

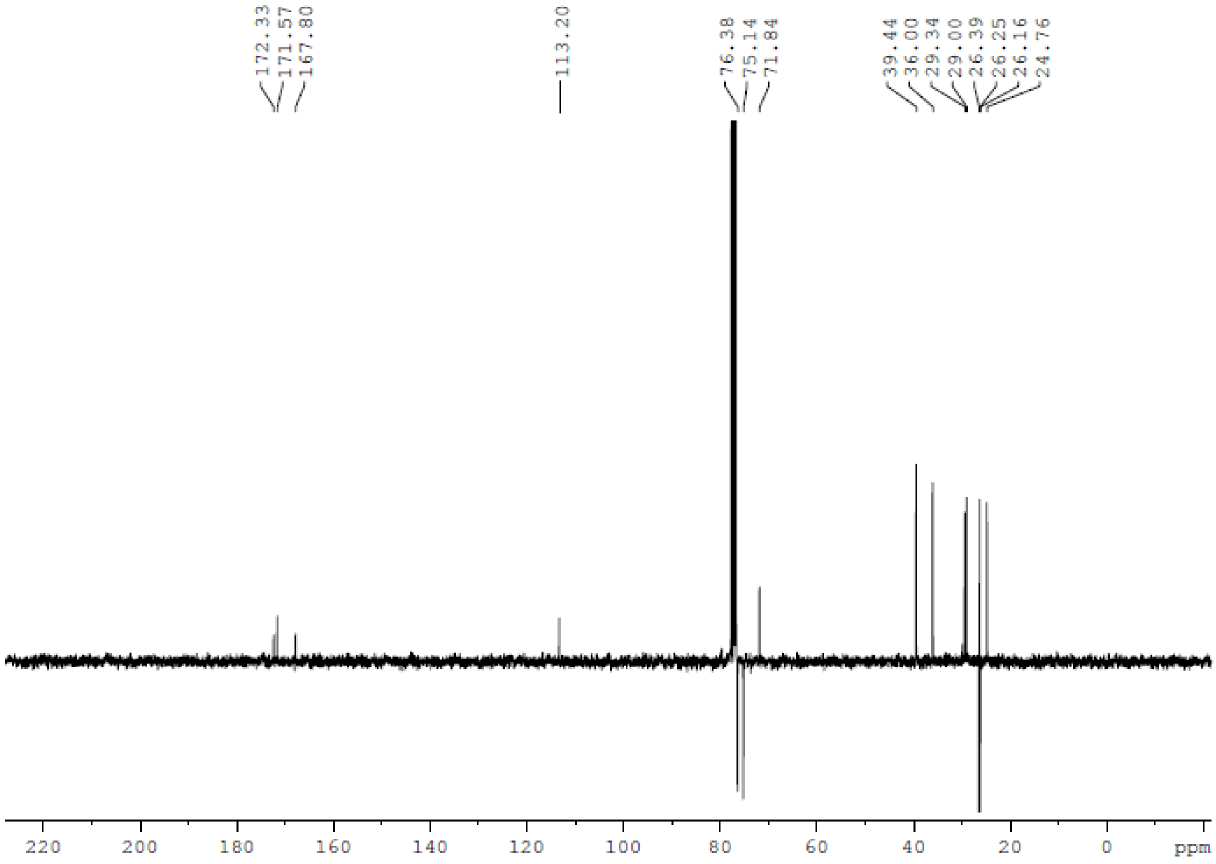


Figure *S7* ^{13}C-NMR spectra (75 MHz, APT, $CDCl_3$) of (4*S*,5*S*)-2,2-dimethyl-1,3-dioxolane-4-carboxylic acid-5-*N*-(1'-*N'*-[2''-propyn-1''-yl]-hexanamide-6'-yl)-carboxamide (**4**).

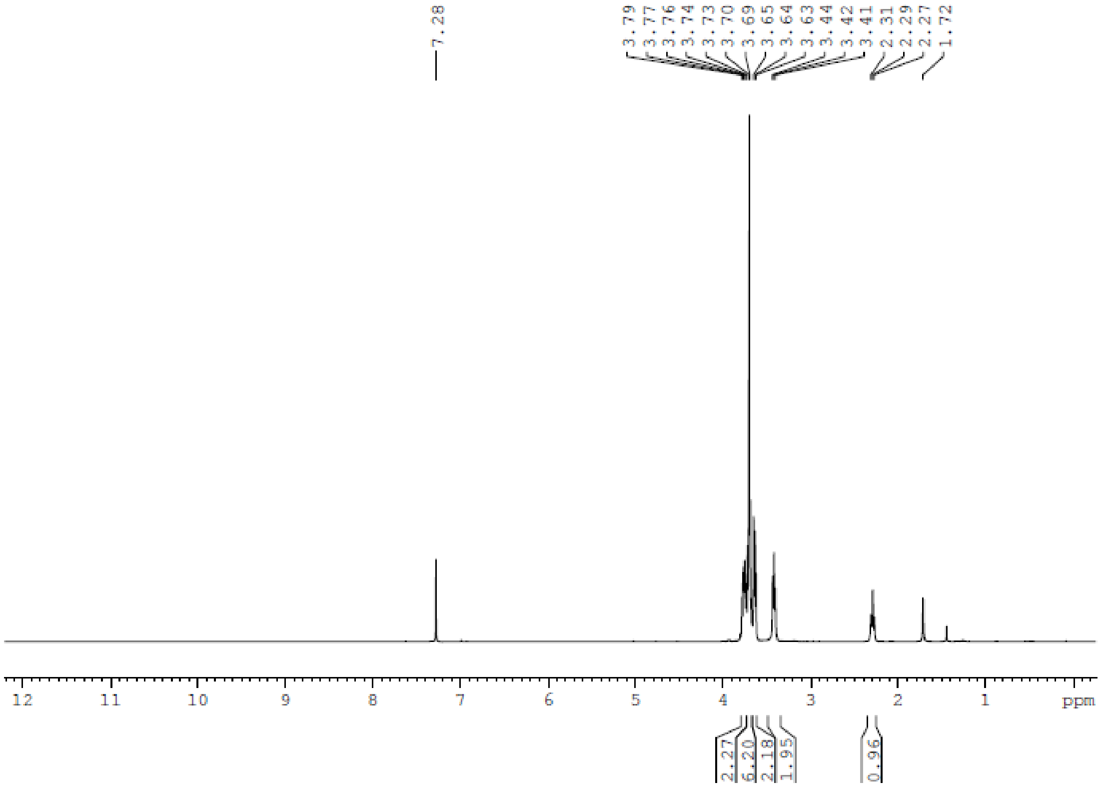


Figure *S8* ^{1}H-NMR spectra (300 MHz, $CDCl_3$) of 8-azido-3,6-dioxa-1-octanol (**7**).

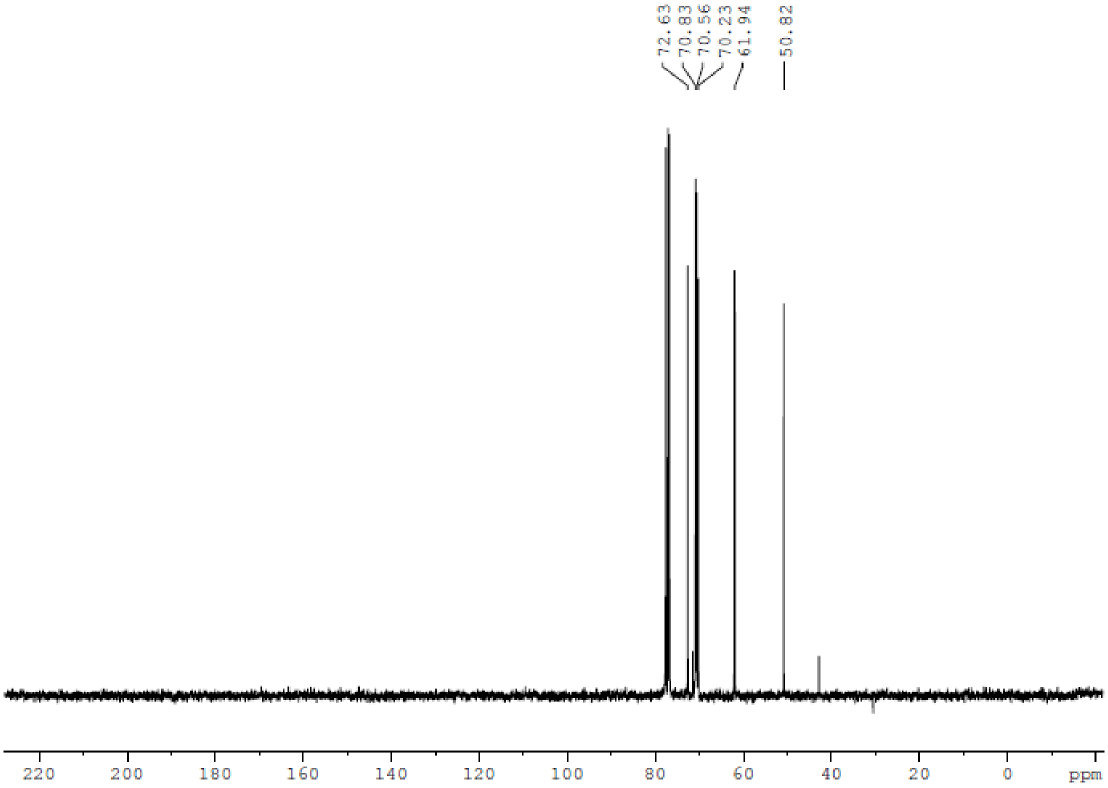


Figure *S9* ^{13}C-NMR spectra (75 MHz, APT, $CDCl_3$) of 8-azido-3,6-dioxa-1-octanol (**7**).

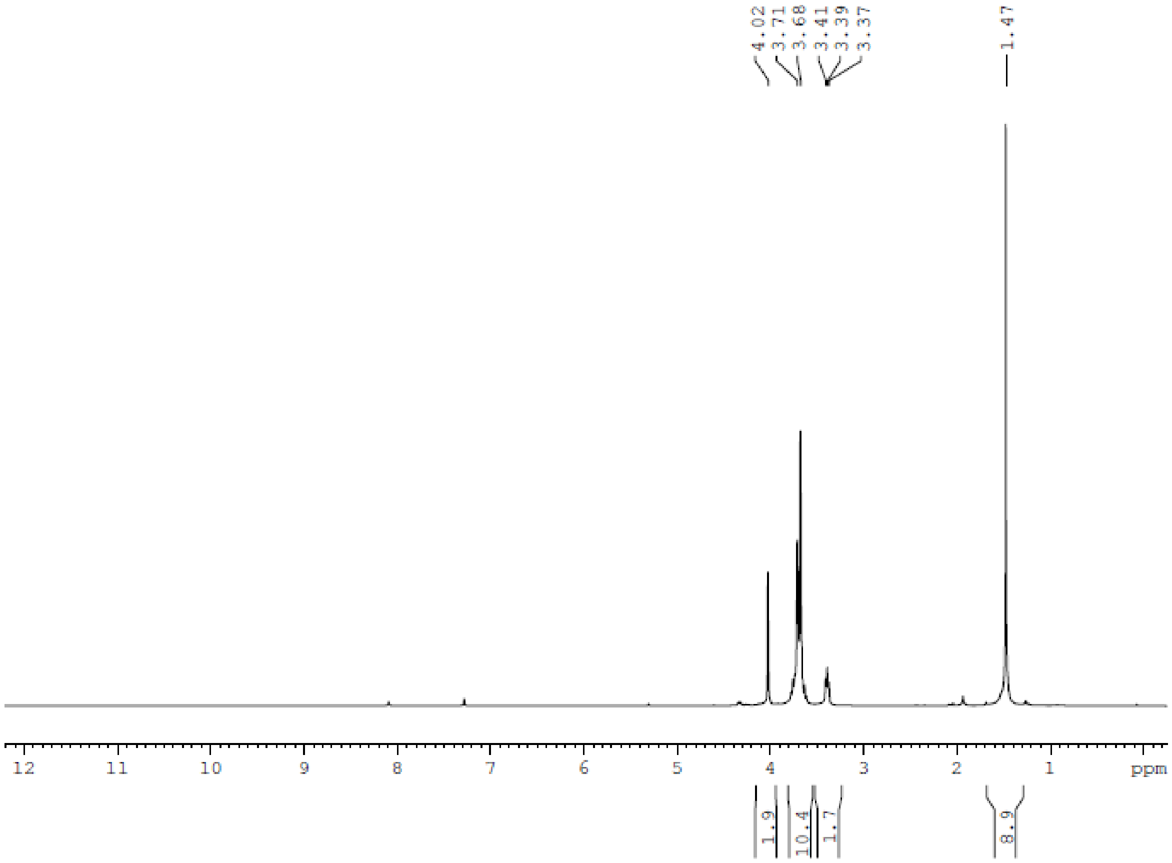


Figure *S10* ^{1}H-NMR spectra (300 MHz, $CDCl_3$) of 11-azido-3,6,9-trioxa-1-*tert*-butyl-undecanoate (**8**).

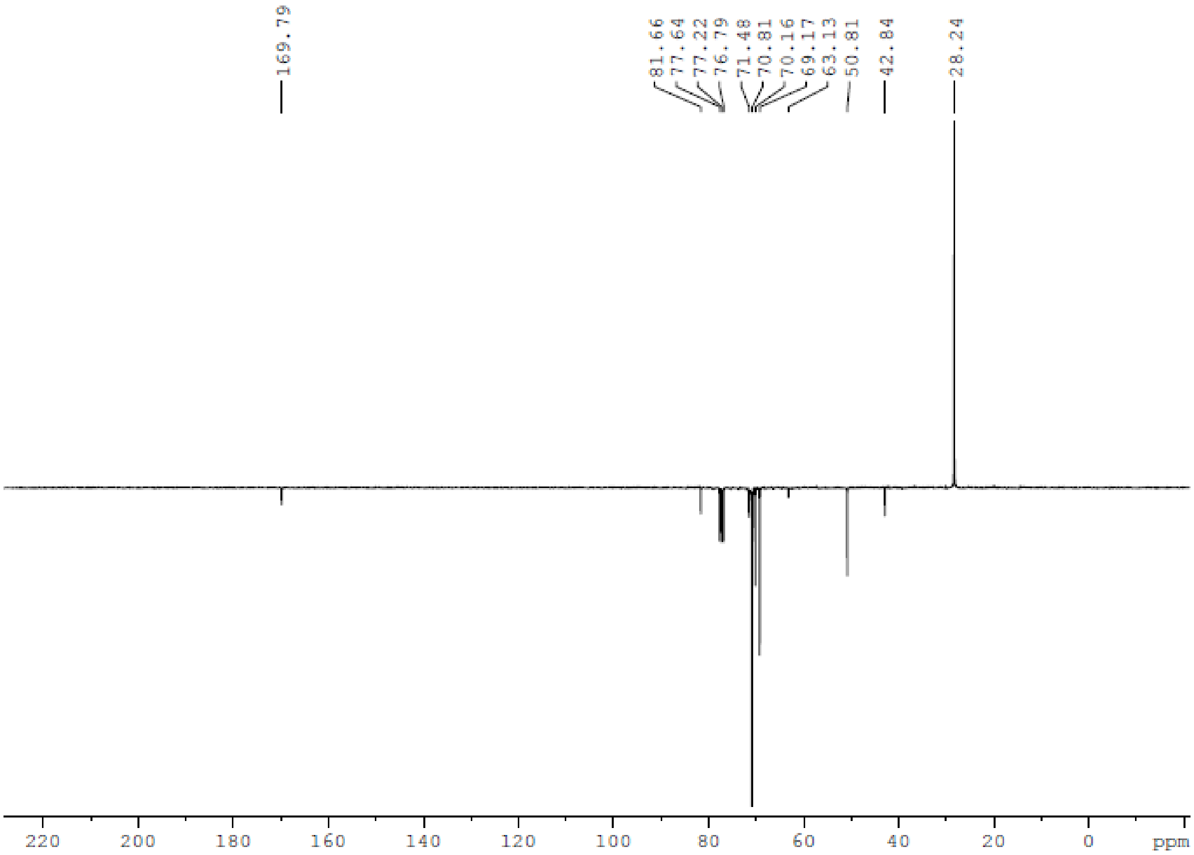


Figure *S11* ^{13}C-NMR spectra (75 MHz, APT, $CDCl_3$) of 11-azido-3,6,9-trioxa-1-*tert*-butyl-undecanoate (**8**).

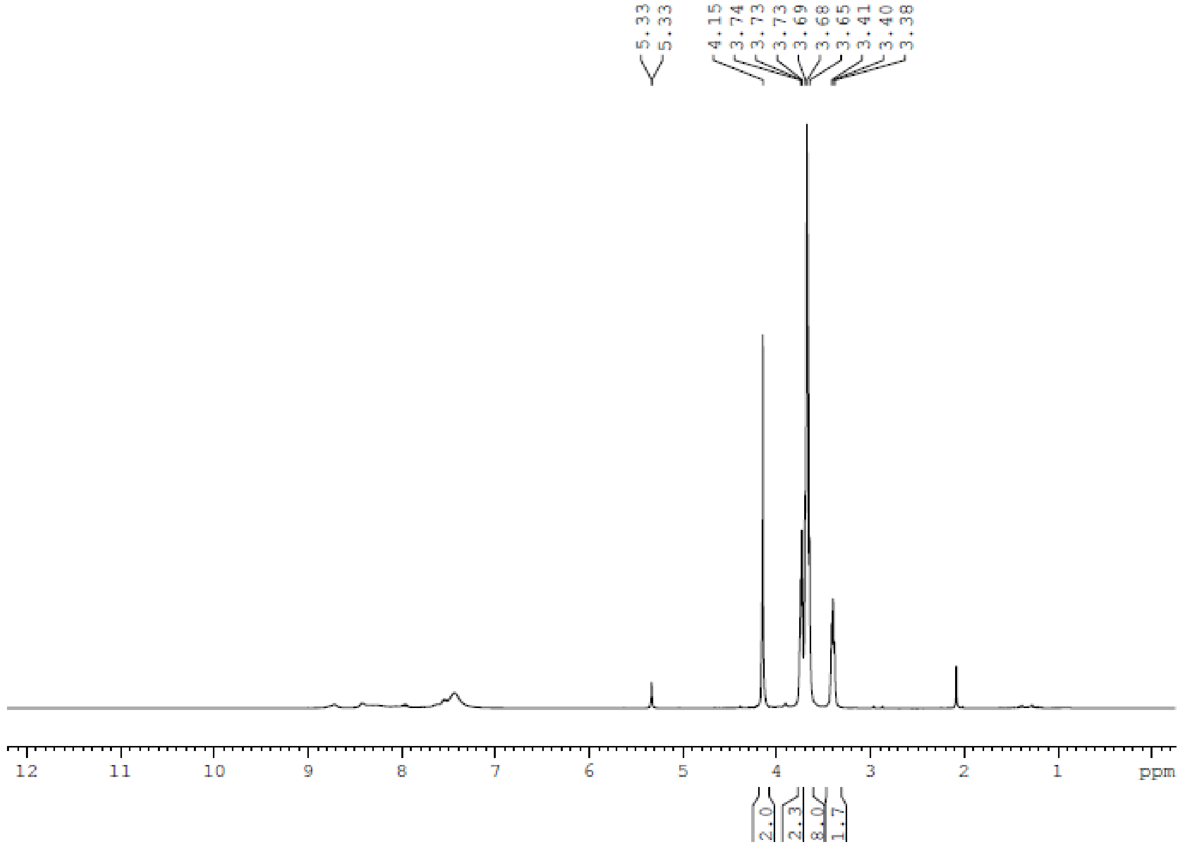


Figure *S12* ^{1}H-NMR spectra (300 MHz, CD_2Cl_2) of 11-azido-3,6,9-trioxa-1-undecanoic acid (**9**).

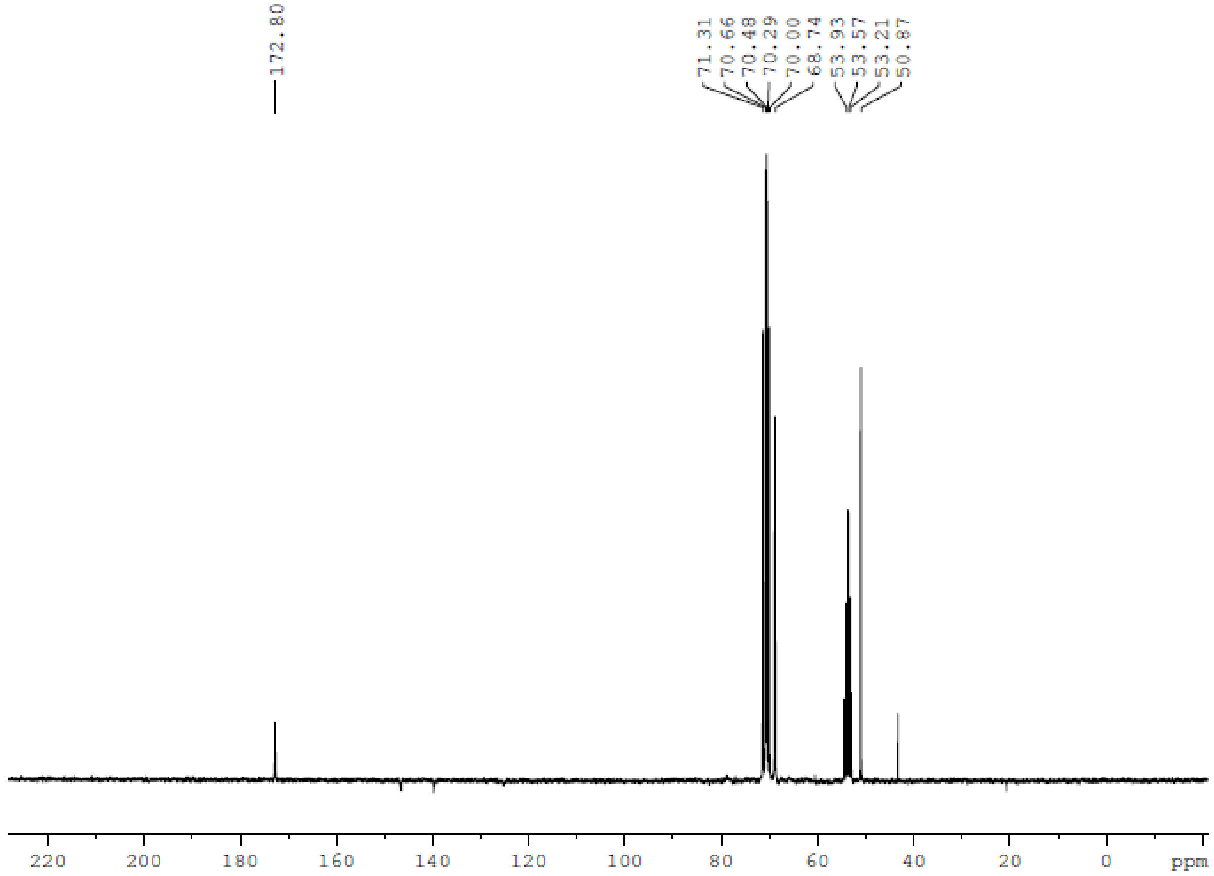


Figure *S13* ^{13}C-NMR spectra (75 MHz, APT, CD_2Cl_2) of 11-azido-3,6,9-trioxa-1-undecanoic acid (**9**).

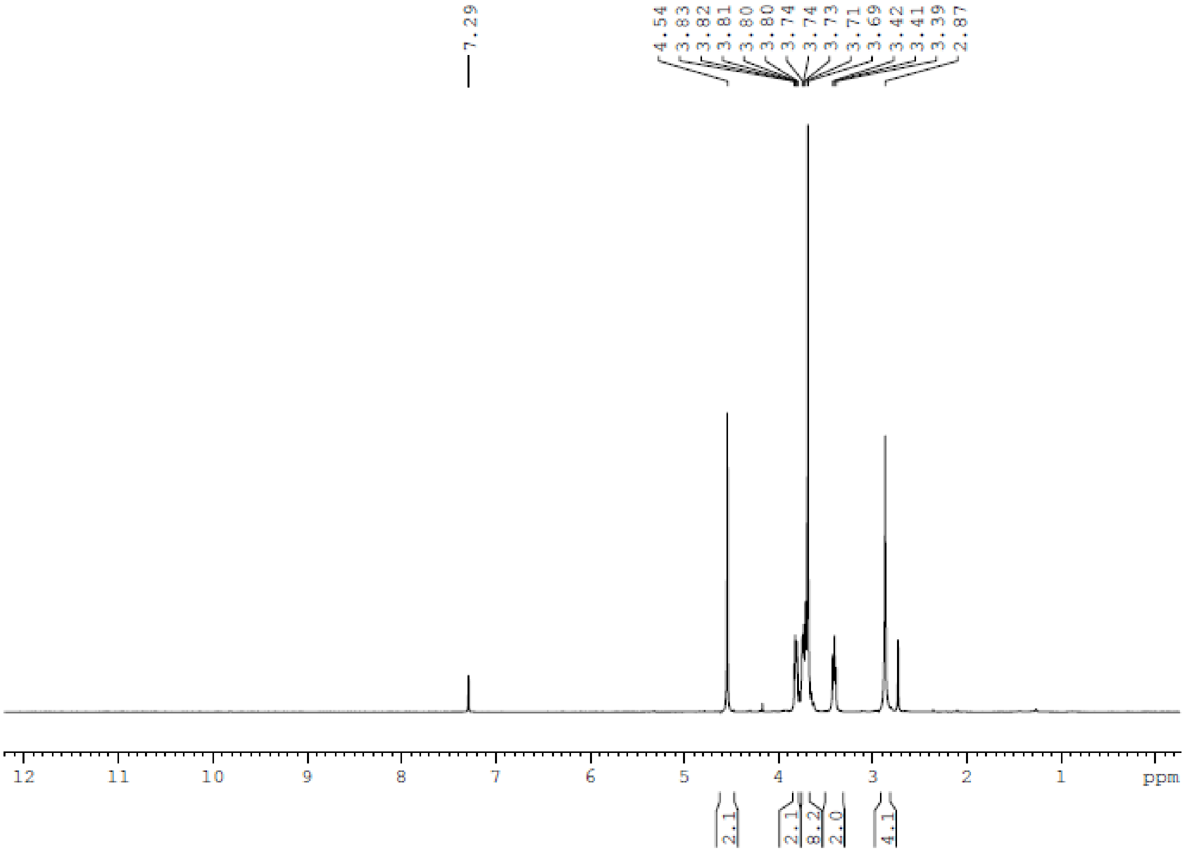


Figure *S14* ^{1}H-NMR spectra (300 MHz, $CDCl_3$) of 11-azido-3,6,9-trioxa-1-*N*-hydroxysuccinamide-undecanoate (**10**).

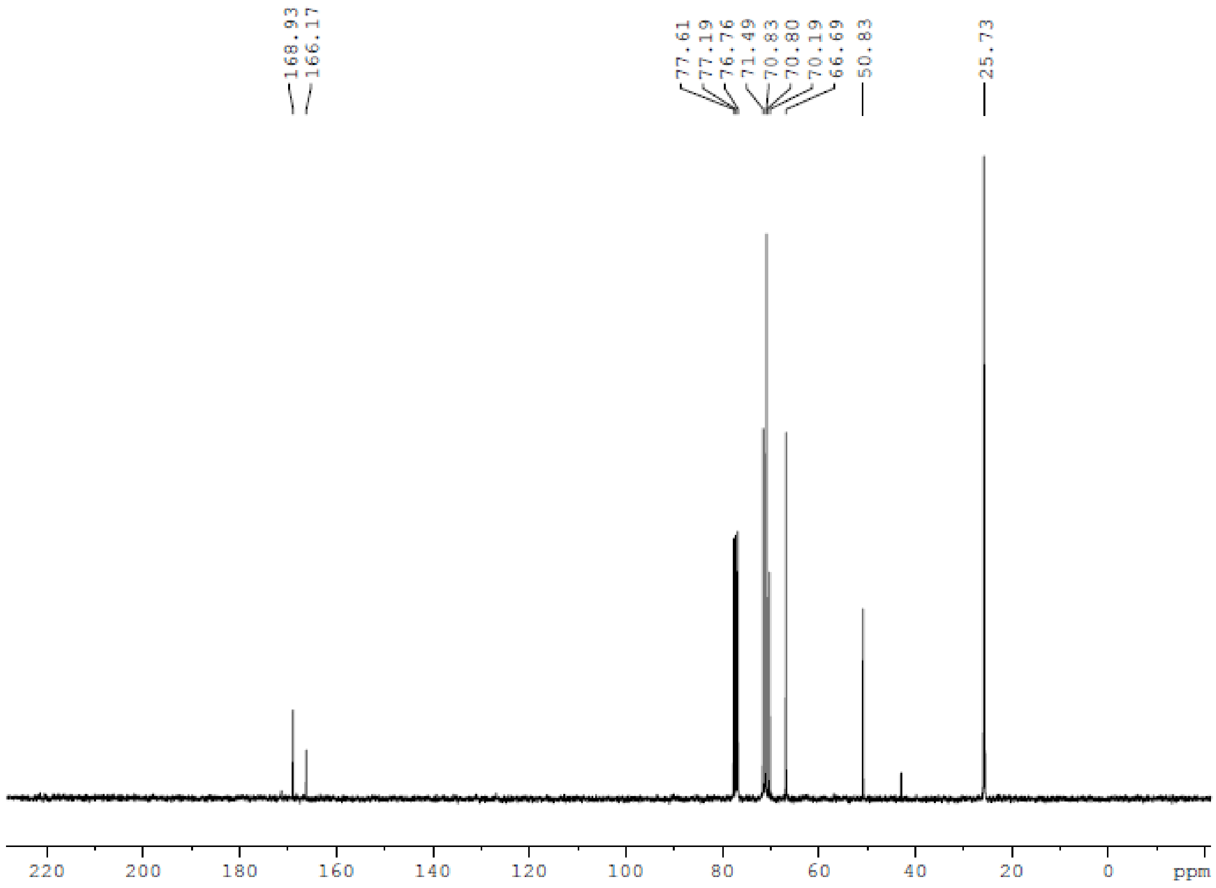


Figure *S15* ^{13}C-NMR spectra (75 MHz, APT, $CDCl_3$) of 11-azido-3,6,9-trioxa-1-*N*-hydroxysuccinamide-undecanoate (**10**).

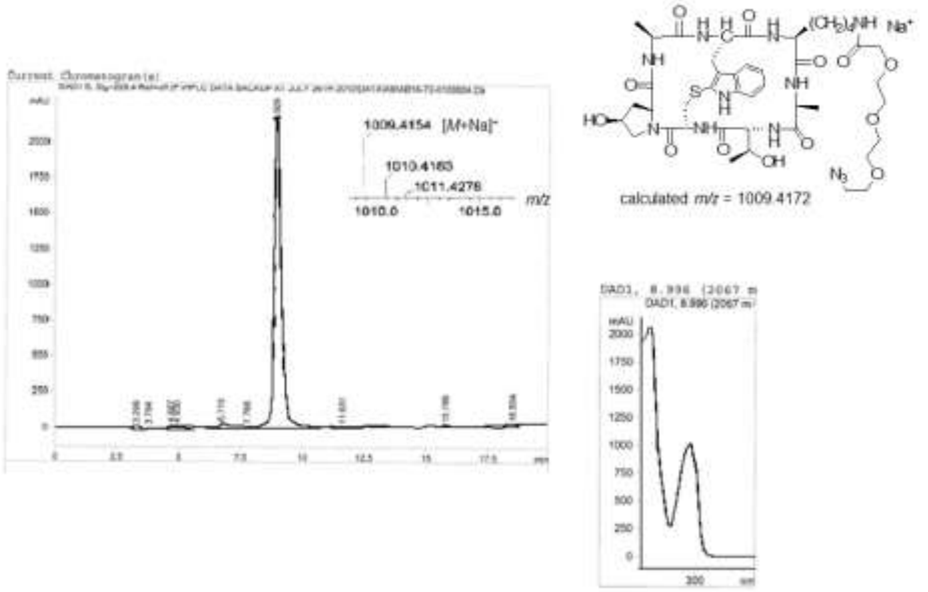


Figure ***S16*** Bicyclic(Ala-DThr-[Cys-*cis*-4-Hyp-Ala-2-mercapto-Trp]-Lys{N^{ε}-11’-azido-3’,6’,9’-trioxa-1’-undecanamide})[S-3→6] (**11**).

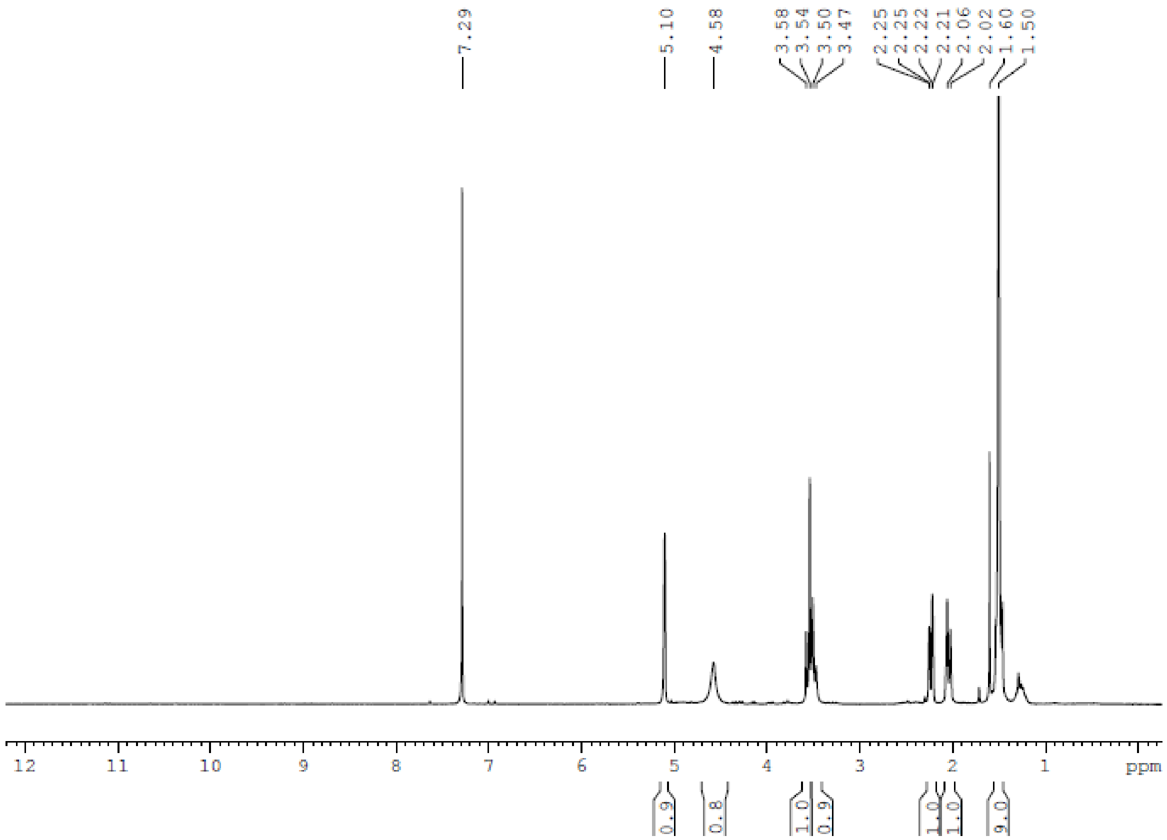


Figure *S17* ^{1}H-NMR spectra (300 MHz, $CDCl_3$) of 2’-methyl-2’-propanyl (1*S*,4*S*)-3-oxo-2-oxa-5-azabicyclo [2.2.1] heptane-5-carboxylate (**13**).

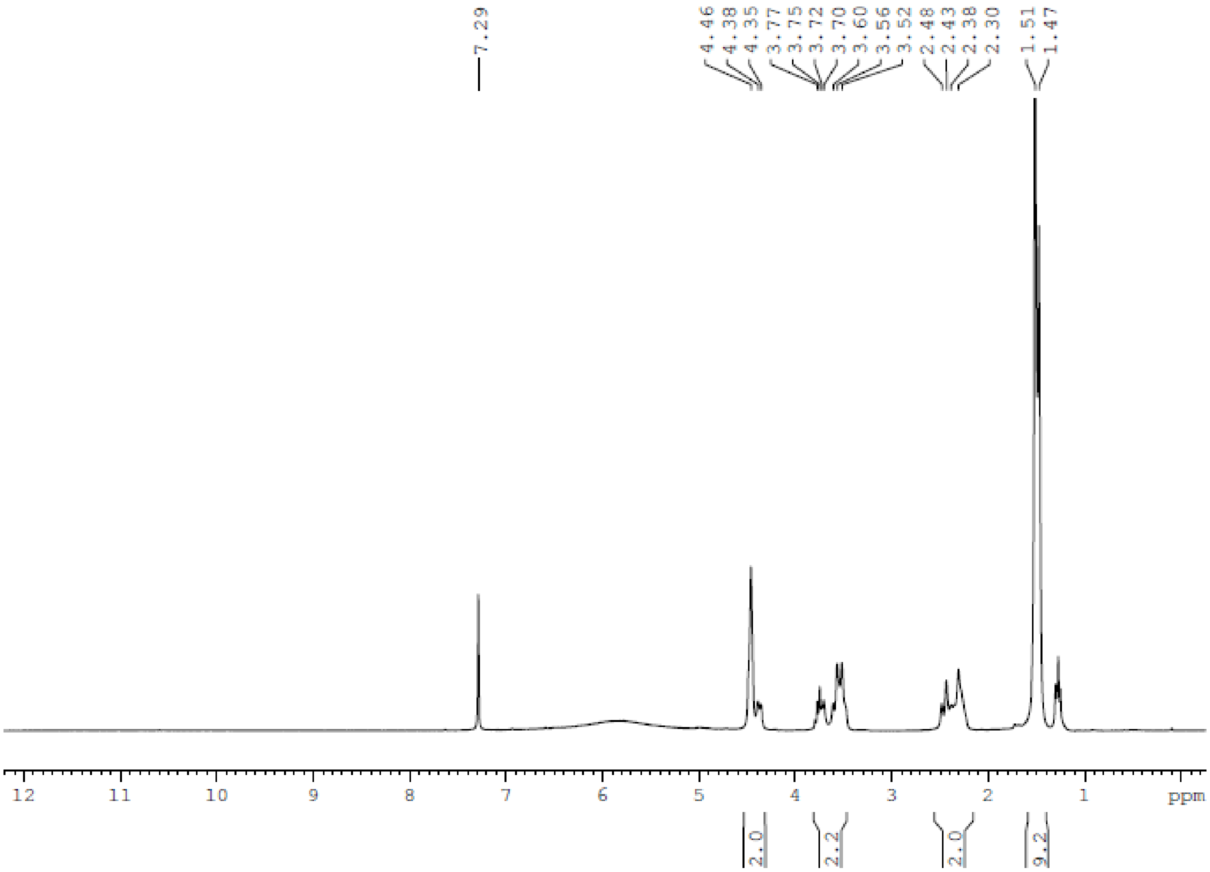


Figure *S18* ^{1}H-NMR spectra (300 MHz, $CDCl_3$) of (2S,4S)-4-Hydroxy-1-[(2’-methylpropan-2’-yl)oxycarbonyl]-pyrrolidine-2-carboxylic acid (**14**).

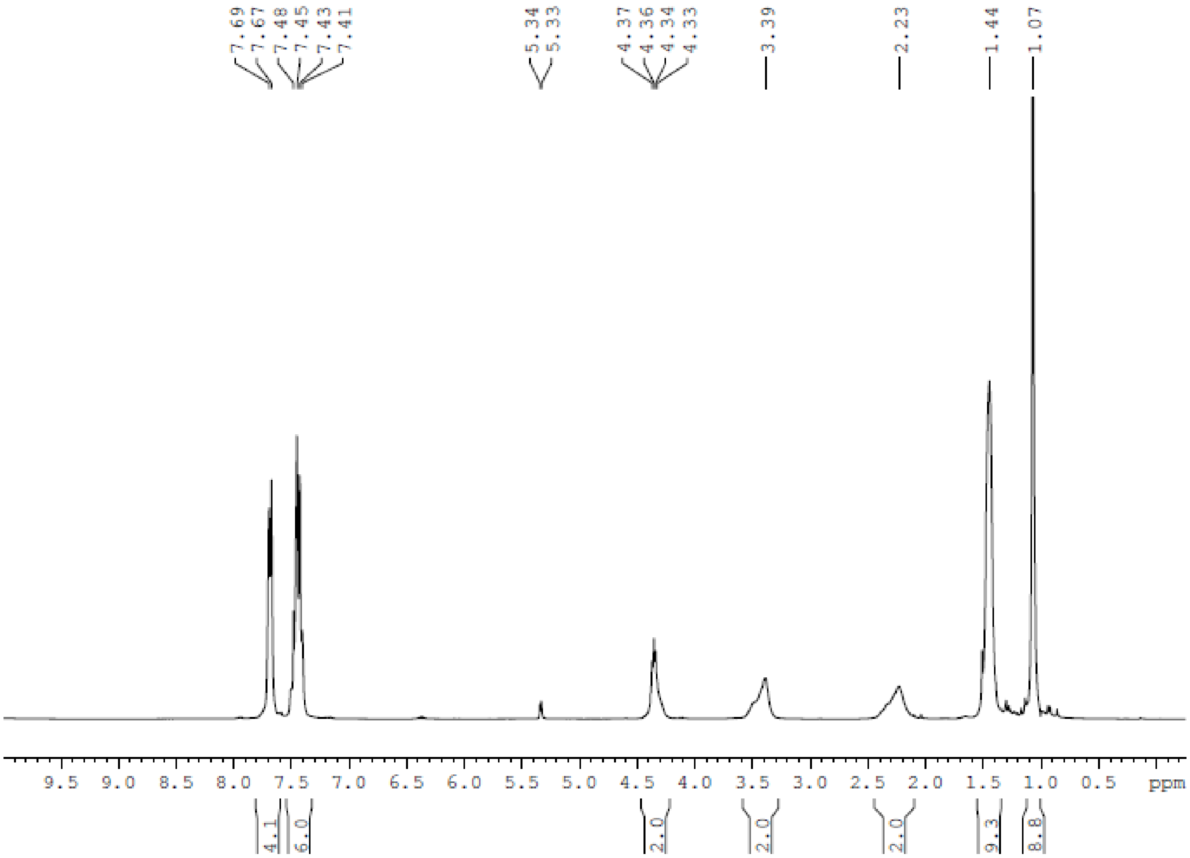


Figure *S19* ^{1}H-NMR spectra (300 MHz, CD_2Cl_2) of (2*S*,4*S*)-4-[*tert*-butyldiphenylsilyl]oxy-1-[(2'-methylpropan-2'-yl)oxycarbonyl]-pyrrolidine-2-carboxylic acid (**15**).

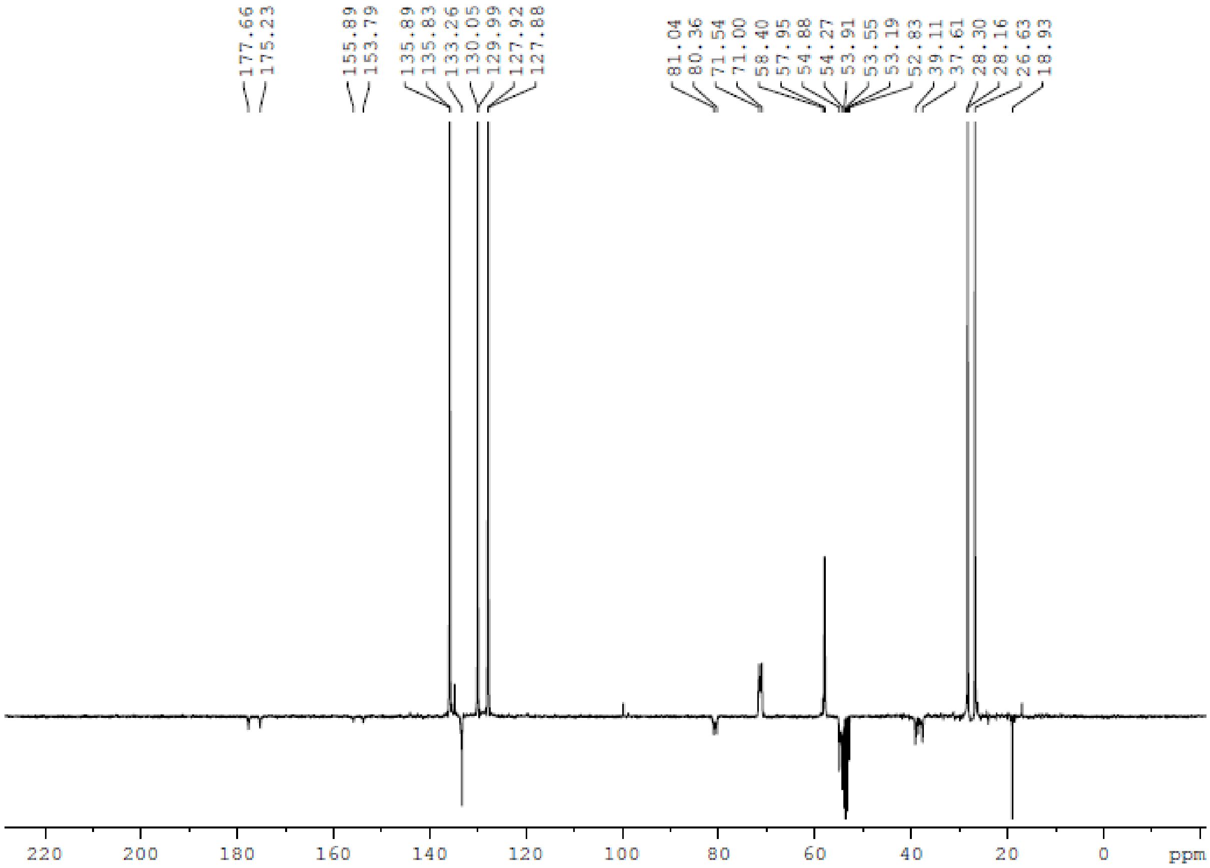


Figure ***S20*** ^{13}C-NMR spectra (75 MHz, APT, CD_2Cl_2) of (2*S*,4*S*)-4-[*tert*-butyldiphenylsilyl]oxy-1-[(2’-methylpropan-2’-yl)oxycarbonyl]-pyrrolidine-2-carboxylic acid (**15**).

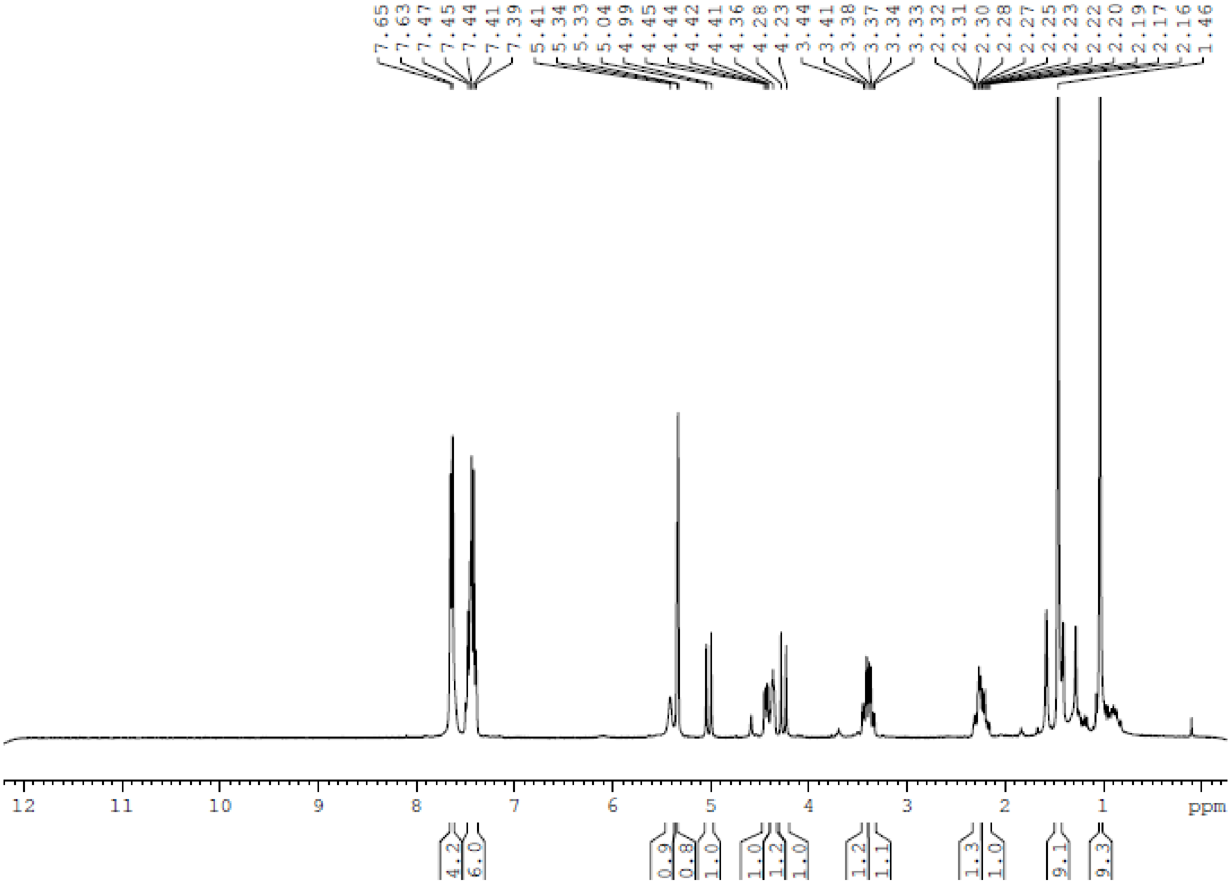


Figure *S21* ^{1}H-NMR spectra (300 MHz, CD_2Cl_2) of (2*S*,4*S*)-4-[*tert*-butyldiphenylsilyl]oxy-1-[(2’-methylpropan-2’-yl)oxycarbonyl]-pyrrolidine-2-(ethanamide-2’’–yl-)-carboxylate (**16**).

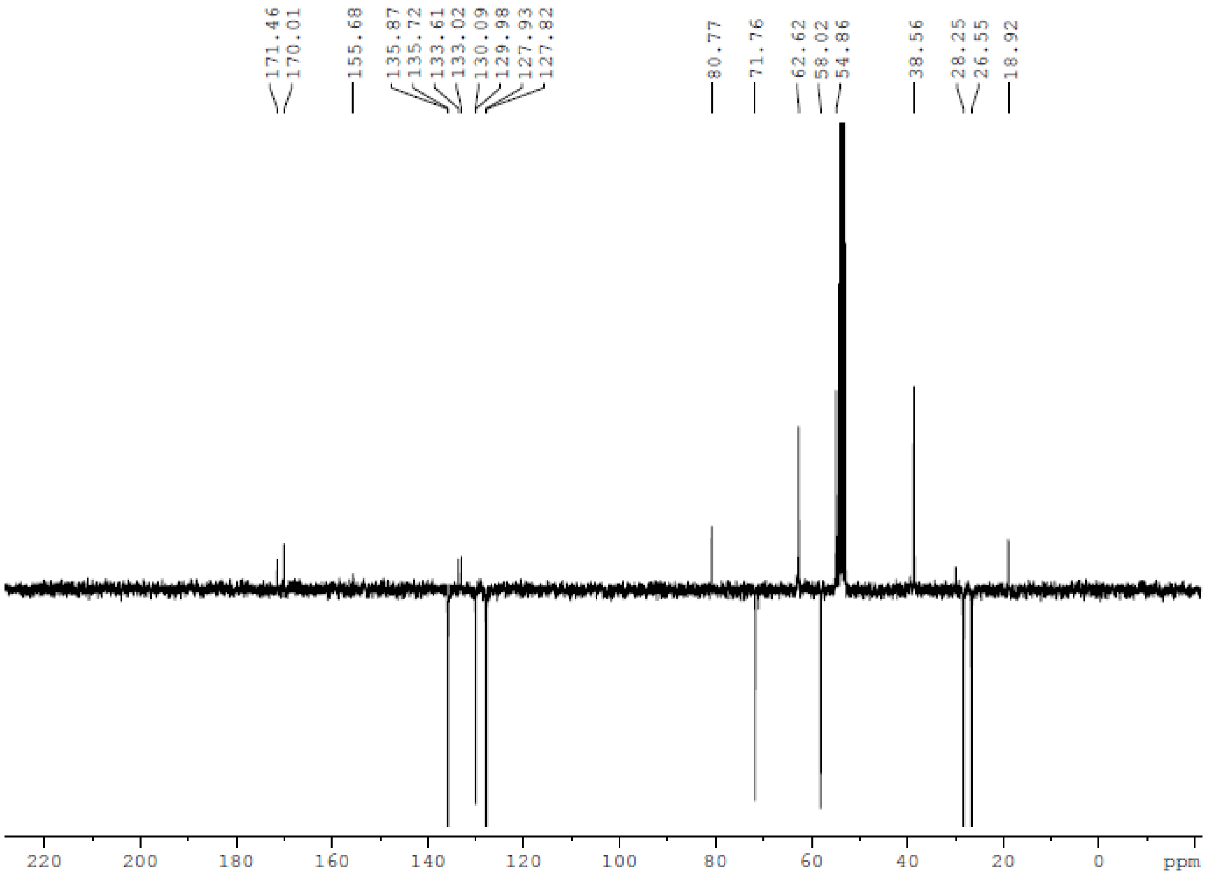


Figure *S22* ^{13}C-NMR spectra (75 MHz, APT, CD2Cl2) of (2S,4S)-4-[*tert*-butyldiphenylsilyl]oxy-1-[(2’-methylpropan-2’-yl)oxycarbonyl]-pyrrolidine-2-(ethanamide-2’’–yl-)-carboxylate (**16**).

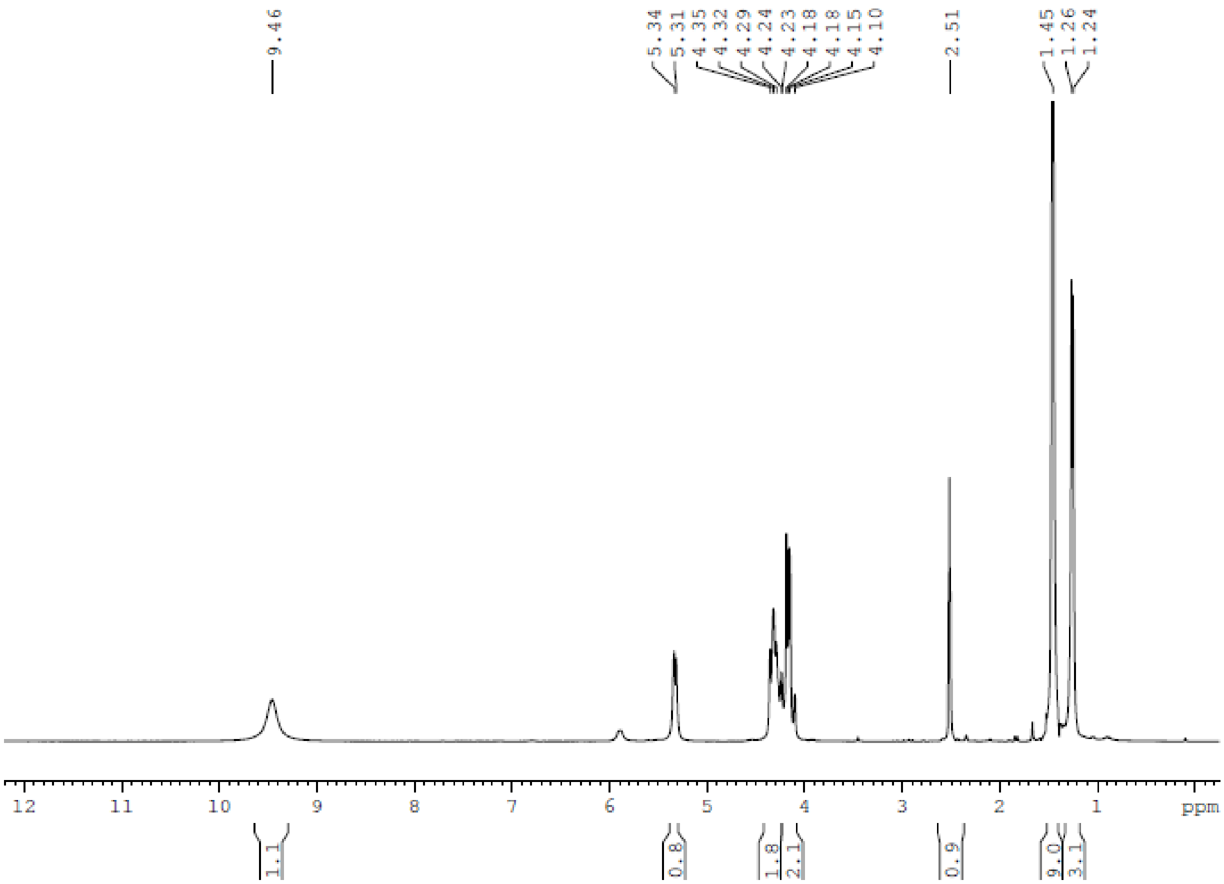


Figure *S23* ^{1}H-NMR spectra (300 MHz, CD_2Cl_2) of (2*R*,3*S*)- 2-(([(2’-methyl-2’-propanyl)oxy]carbonyl)amino)-3-(prop-2’’-yn-1’’-yloxy)-butanoic acid (**18**).

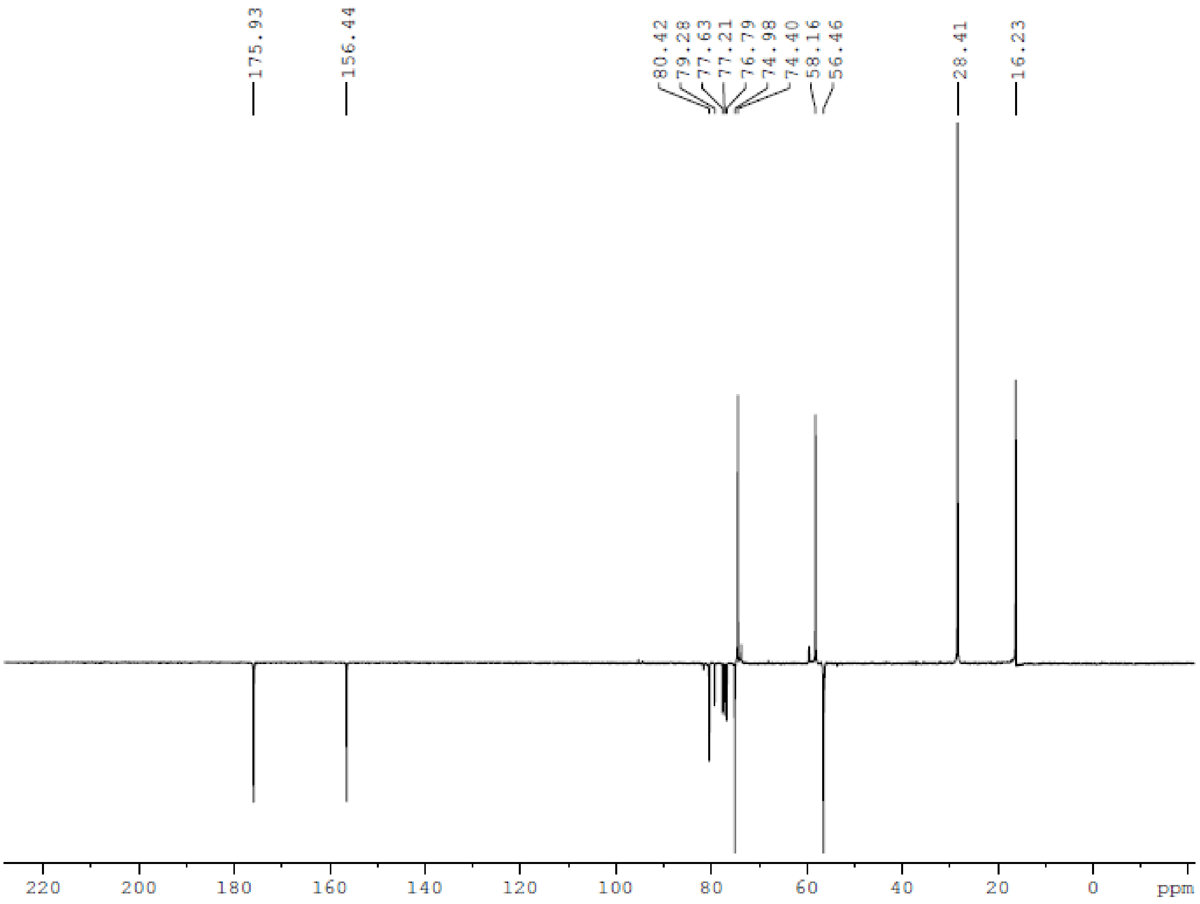


Figure *S24* ^{13}C-NMR spectra (75 MHz, APT, CD_2Cl_2) of (2R,3S)- 2-(([(2’-methyl-2’-propanyl)oxy]carbonyl)amino)-3-(prop-2’’-yn-1’’-yloxy)-butanoic acid (**18**).

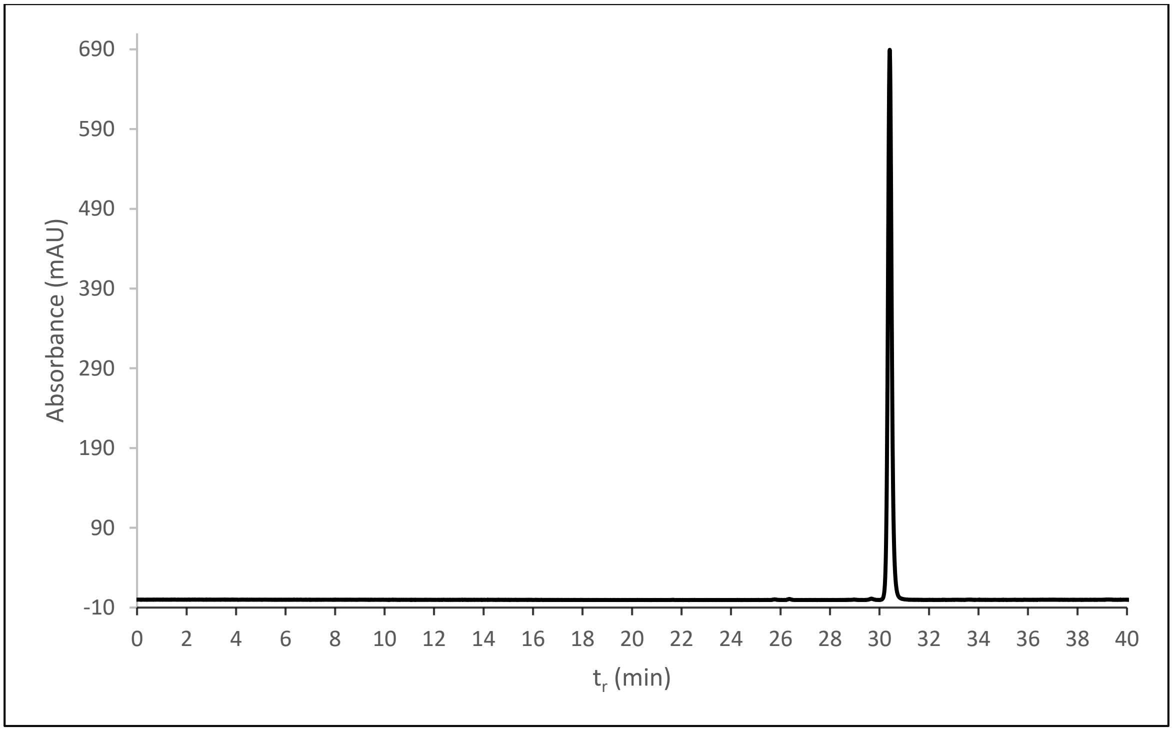


Figure *S25* RP-HPLC (C18) chromatogram of (2*R*,3*S*)- 2-(*N*-(9*H*-fluoren-9’-ylmethoxycarbonyl)amino)-3-(prop-2’’-yn-1’’-yloxy)-butanoic acid (**20**). t_r = 30.41 min (A_{301} 99%) with gradient of 20% to 95% solvent B over 25 min, 95% to 100% solvent B over 5 min at a flow rate of 1 mL/min.

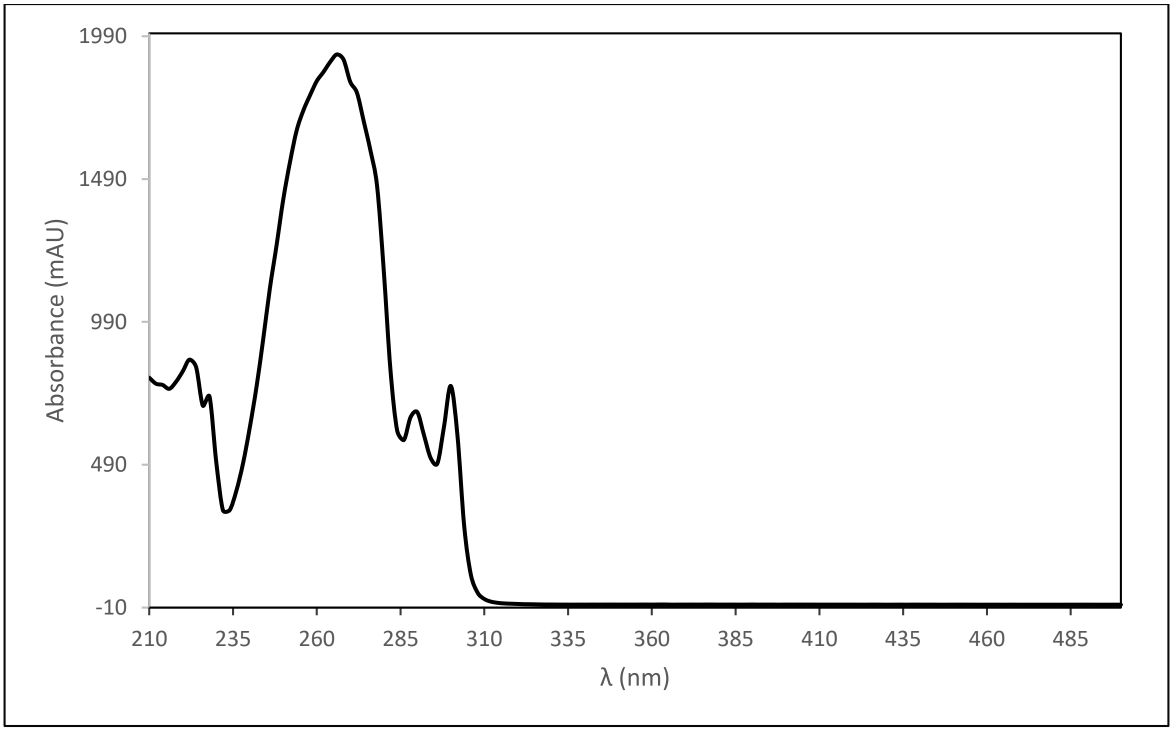


Figure *S26* UV-Vis spectra of (2*R*,3*S*)- 2-(*N*-(9*H*-fluoren-9’-ylmethoxycarbonyl)amino)-3-(prop-2’’-yn-1’’-yloxy)-butanoic acid (**20**). UV-Vis spectra were taken with the RP-HPLC diode array detectors directly in the RP-HPLC flow cells at t_r = 30.41 min.

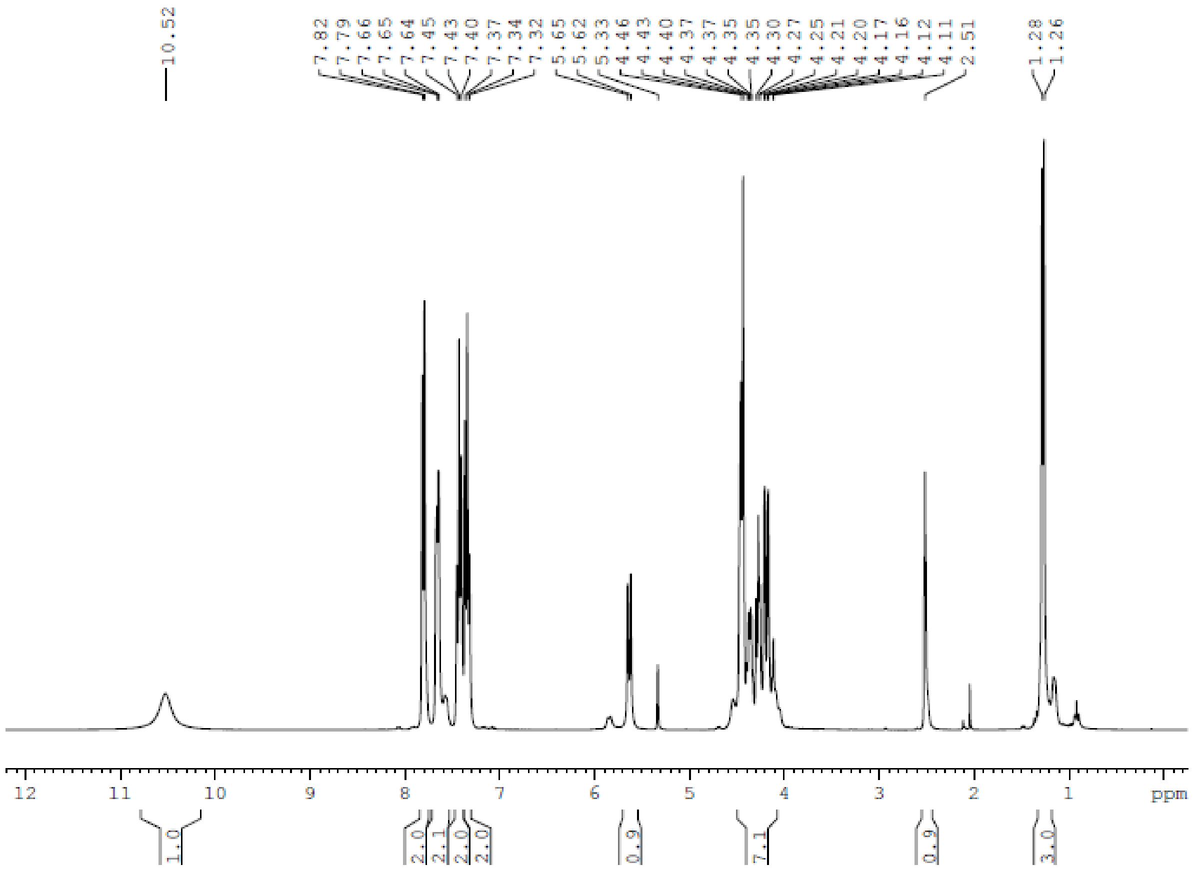


Figure *S27* ^{1}H-NMR spectra (300 MHz, CD_2Cl_2) of (2*R*,3*S*)- 2-(*N*-(9*H*-fluoren-9'-ylmethoxycarbonyl)amino)-3-(prop-2''-yn-1''-yloxy)-butanoic acid (**20**).

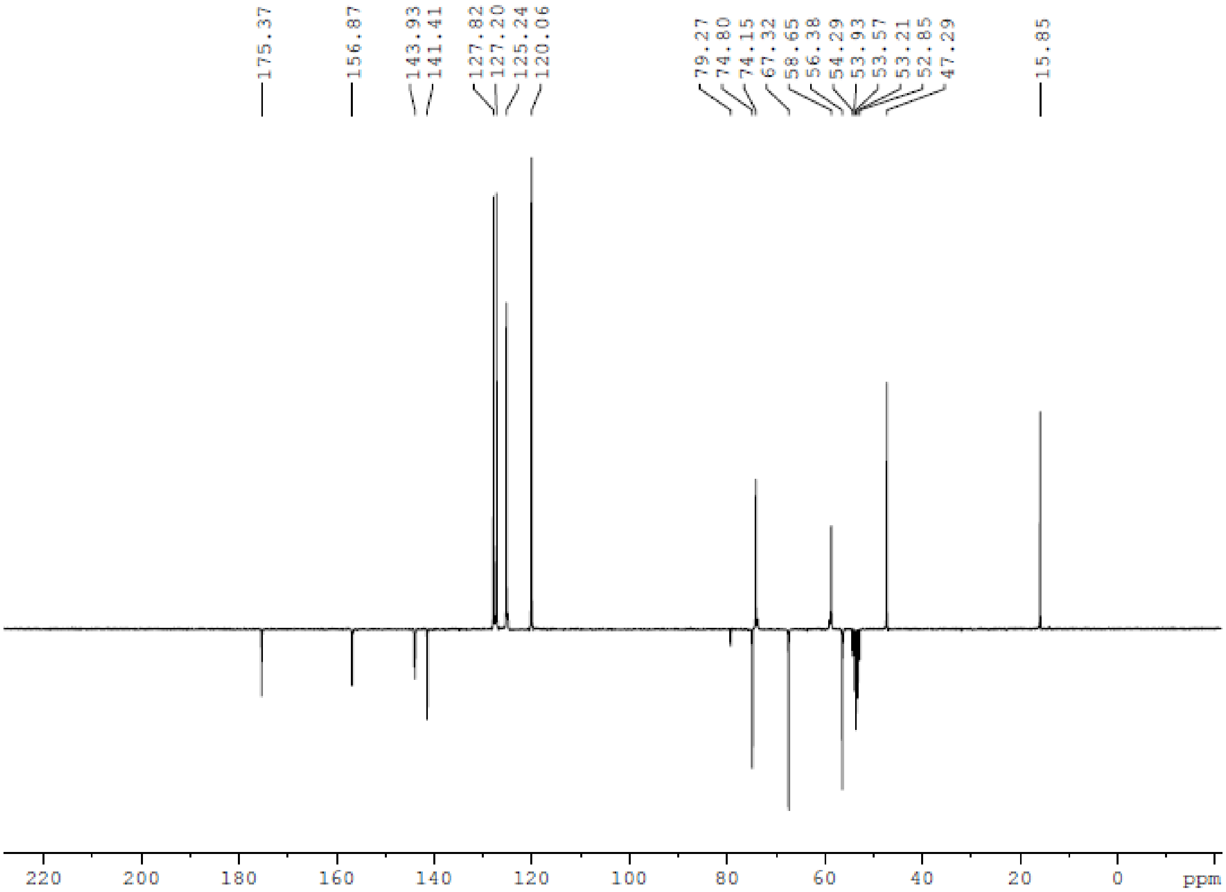


Figure *S28* ^{13}C-NMR spectra (75 MHz, APT, CD_2Cl_2) of (2*R*,3*S*)- 2-(*N*-(9H-fluoren-9’-ylmethoxycarbonyl)amino)-3-(prop-2’’-yn-1’’-yloxy)-butanoic acid (**20**).

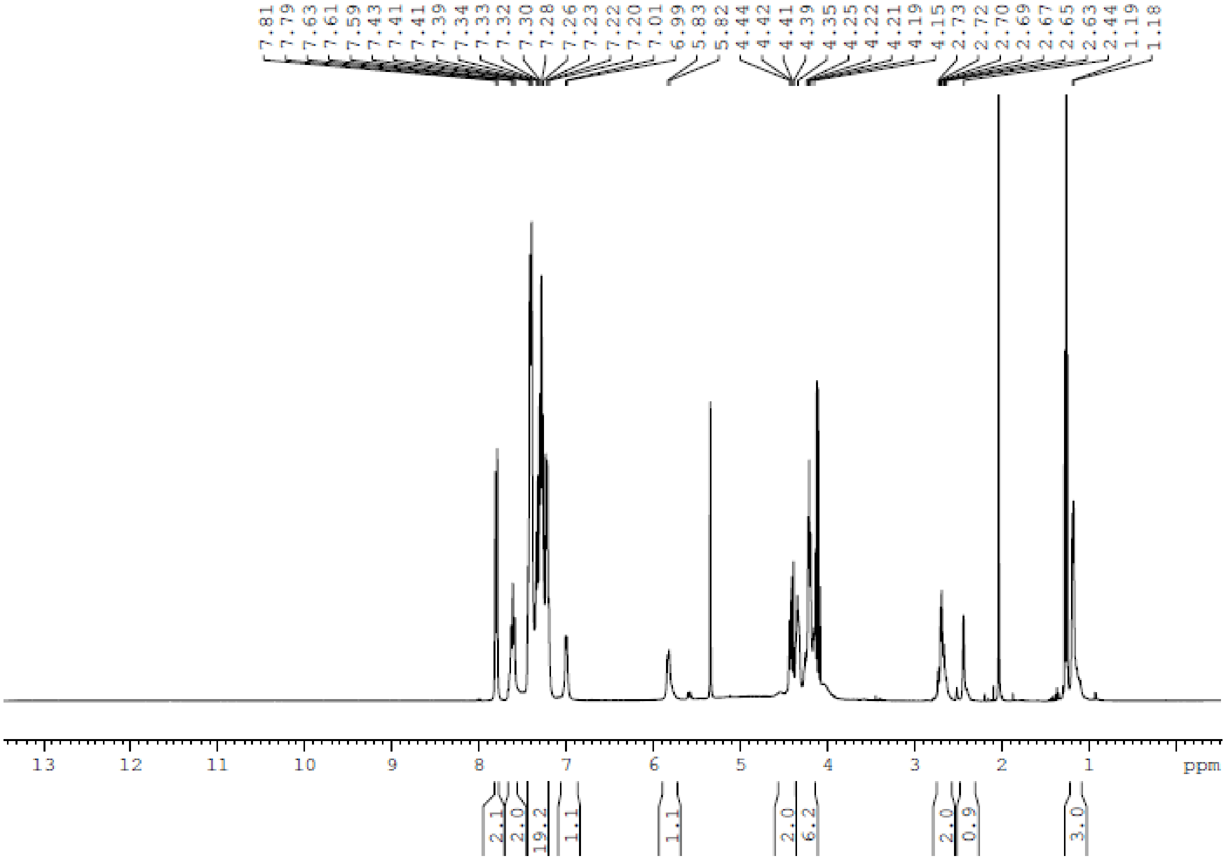


Figure *S29* ^{1}H-NMR spectra (400 MHz, CD_2Cl_2) of *N*$^\alpha$-Fmoc-*O*$^\beta$-propargyl-D-threonyl-*S*$^\beta$-trityl-cysteine (**22**).

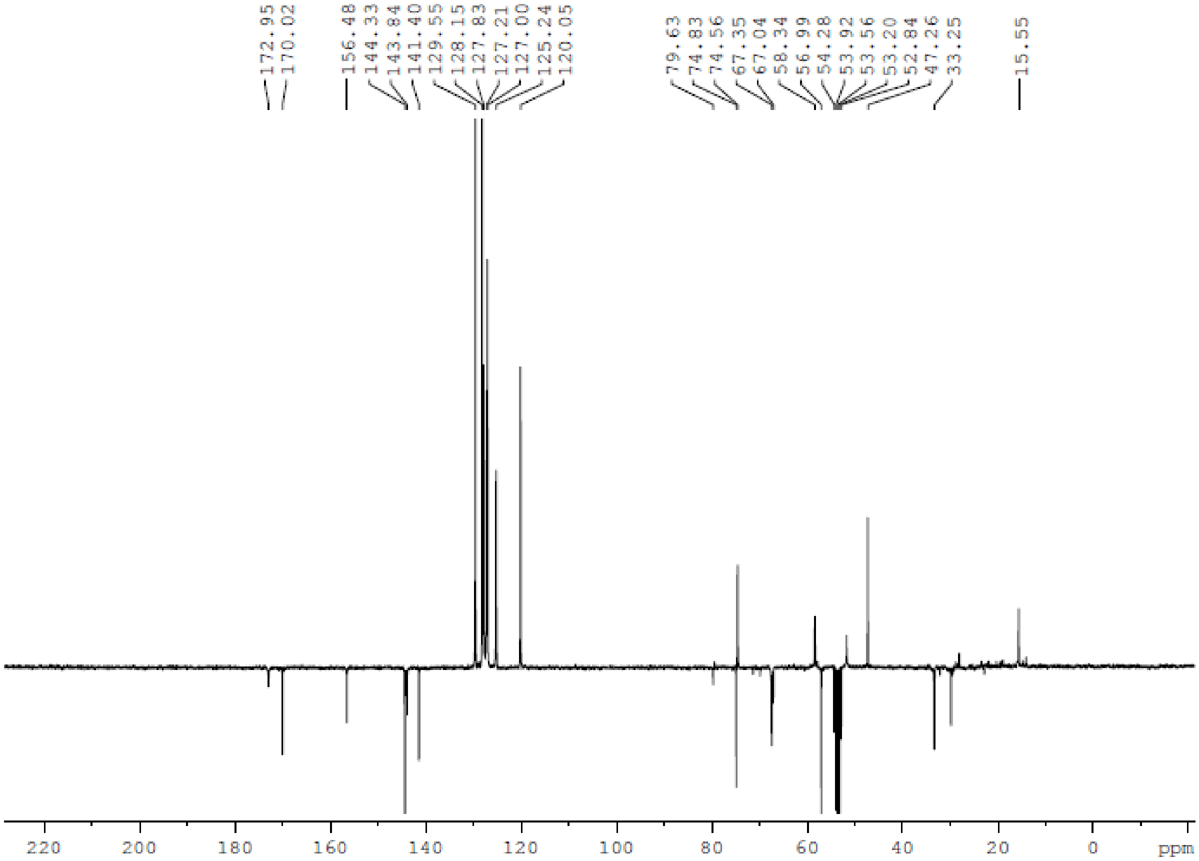


Figure *S30* ^{13}C-NMR spectra (75 MHz, APT, CD_2Cl_2) of *N*$^{\alpha}$-Fmoc-*O*$^{\beta}$-propargyl-D-threonyl-*S*$^{\beta}$-trityl-cysteine (**22**).

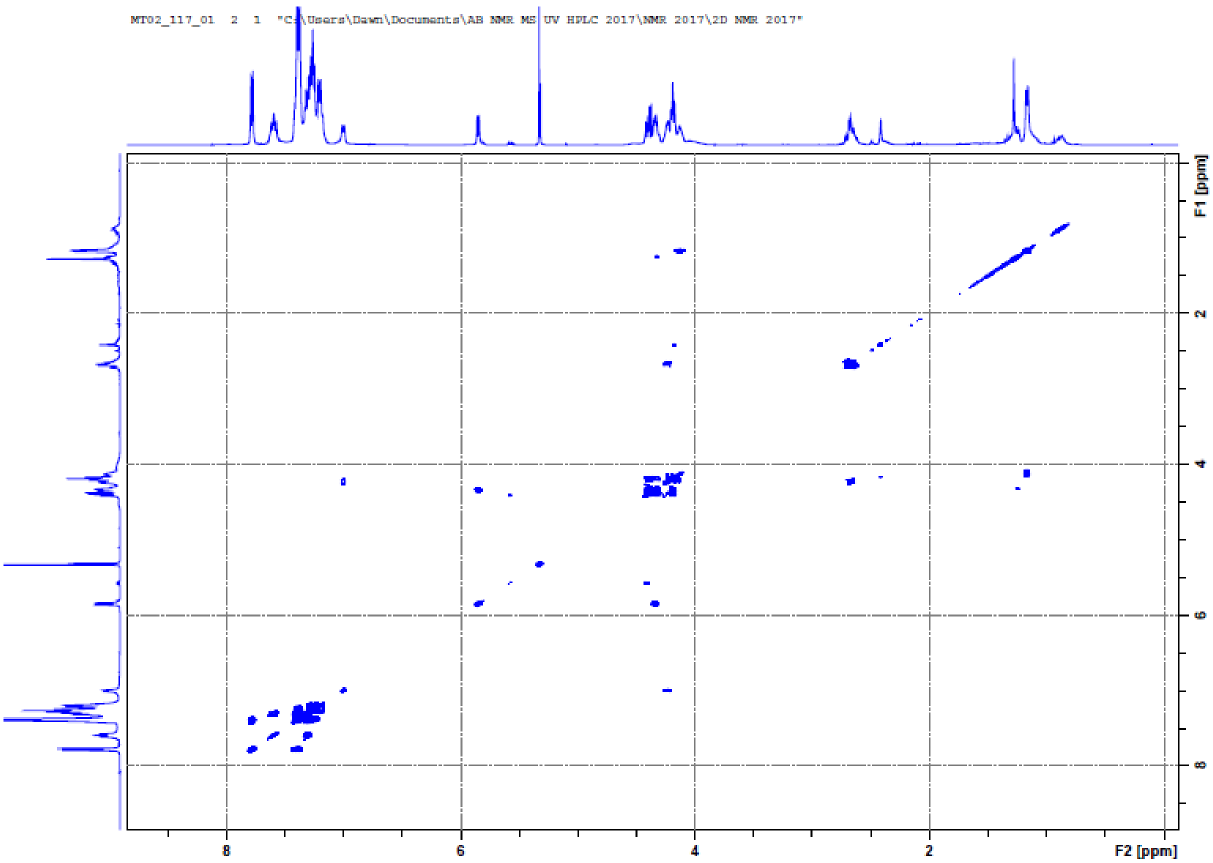


Figure *S31* COSY-NMR spectra (400 MHz, CD_2Cl_2) of *N*$^{\alpha}$-Fmoc-*O*$^{\beta}$-propargyl-D-threonyl-*S*$^{\beta}$-trityl-cysteine (**22**).

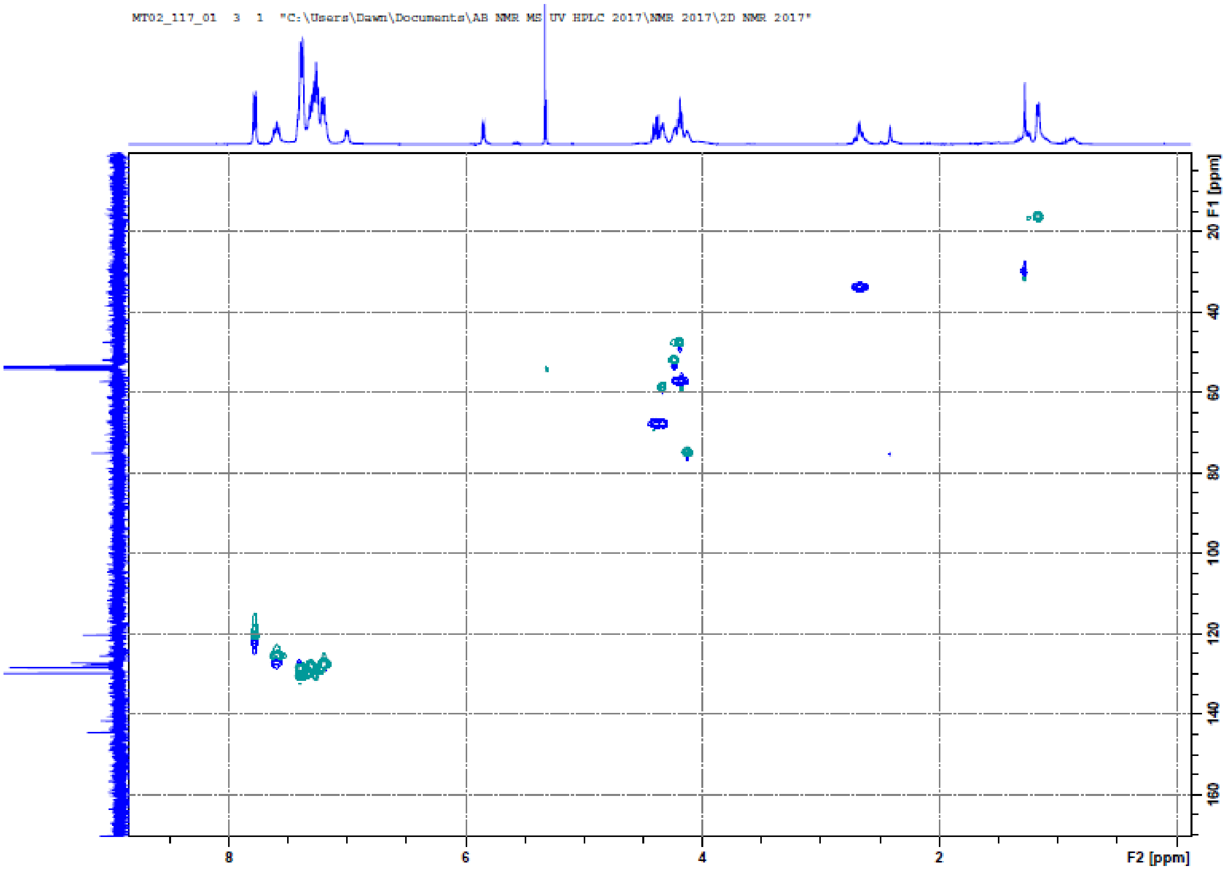


Figure *S32* ^{1}H-^{13}C HSQC-NMR spectra (400 MHz, CD_2Cl_2) of *N*$^{\alpha}$-Fmoc-*O*$^{\beta}$-propargyl-D-threonyl-*S*$^{\beta}$-trityl-cysteine (**22**).

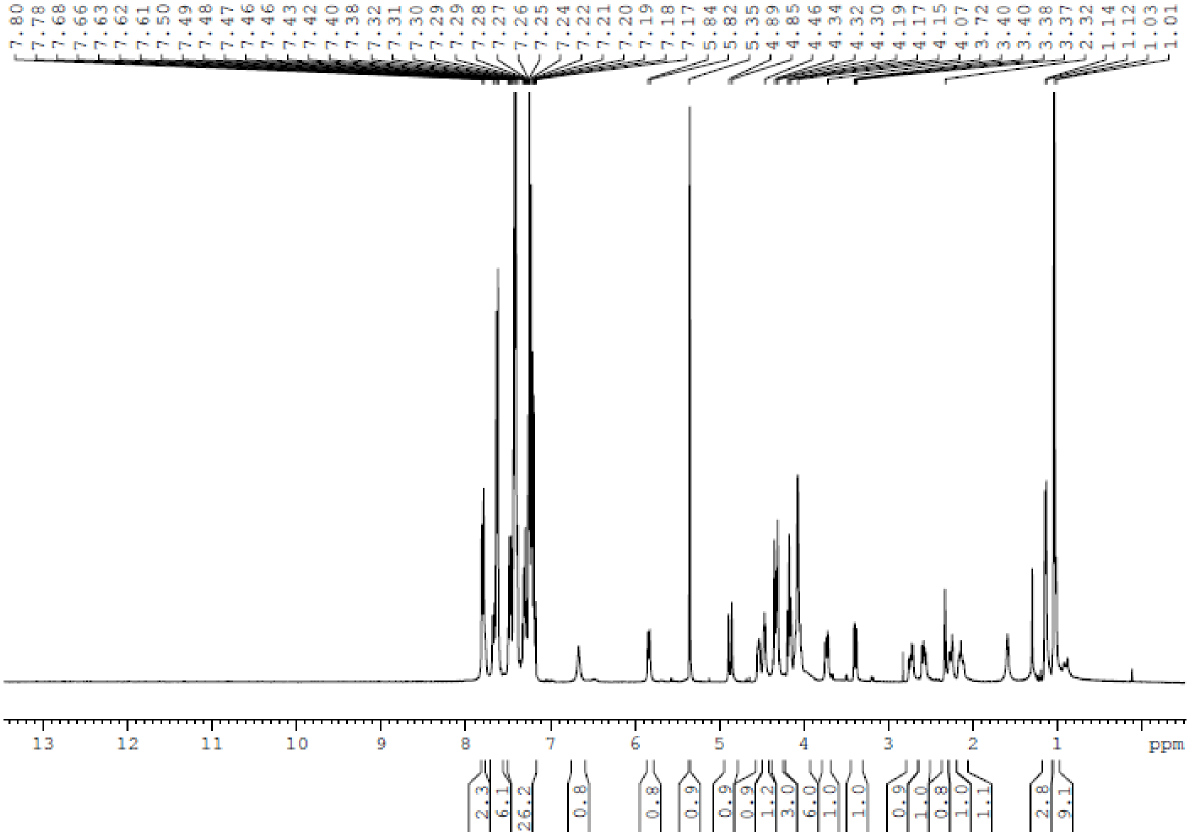


Figure *S33* ^{1}H-NMR spectra (400 MHz, CD_2Cl_2) of N^{α}-Fmoc-O^{β}-propargyl-D-threonyl-S^{β}-trityl-cysteinyl-(1-carbamoylmethyl)-O^4-(*tert*-butyldiphenylsilyl)-*cis*-hydroxyprolinate ester (**23**).

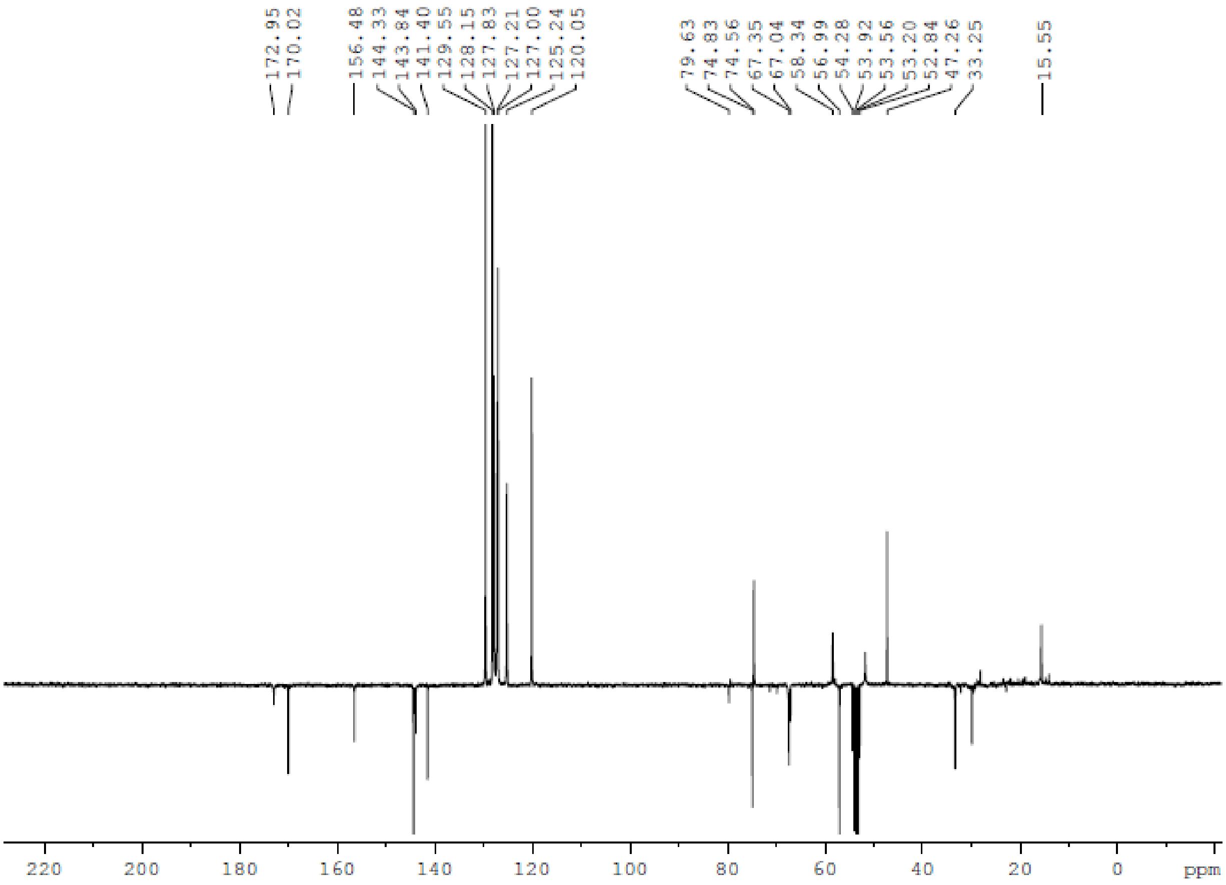


Figure *S34* ^{13}C-NMR spectra (75 MHz, CD_2Cl_2) of N^{α}-Fmoc-O^{β}-propargyl-D-threonyl-S^{β}-trityl-cysteinyl-(1-carbamoylmethyl)-O^{4}-(*tert*-butyldiphenylsilyl)-*cis*-hydroxyprolinate ester (**23**).

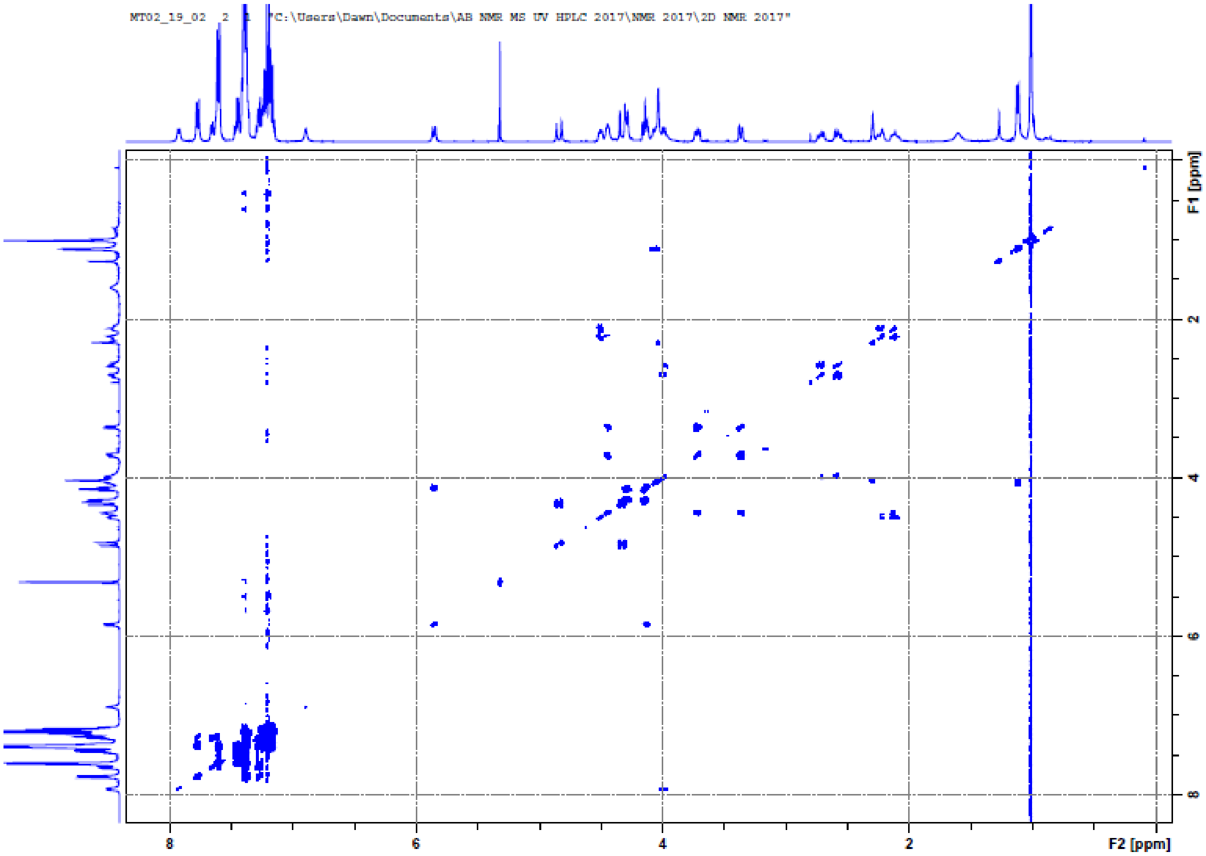


Figure *S35* COSY-NMR spectra (400 MHz, CD_2Cl_2) of N^{α}-Fmoc-O^{β}-propargyl-D-threonyl-S^{β}-trityl-cysteinyl-(1-carbamoylmethyl)-O^{4}-(*tert*-butyldiphenylsilyl)-*cis*-hydroxyprolinate ester (**23**).

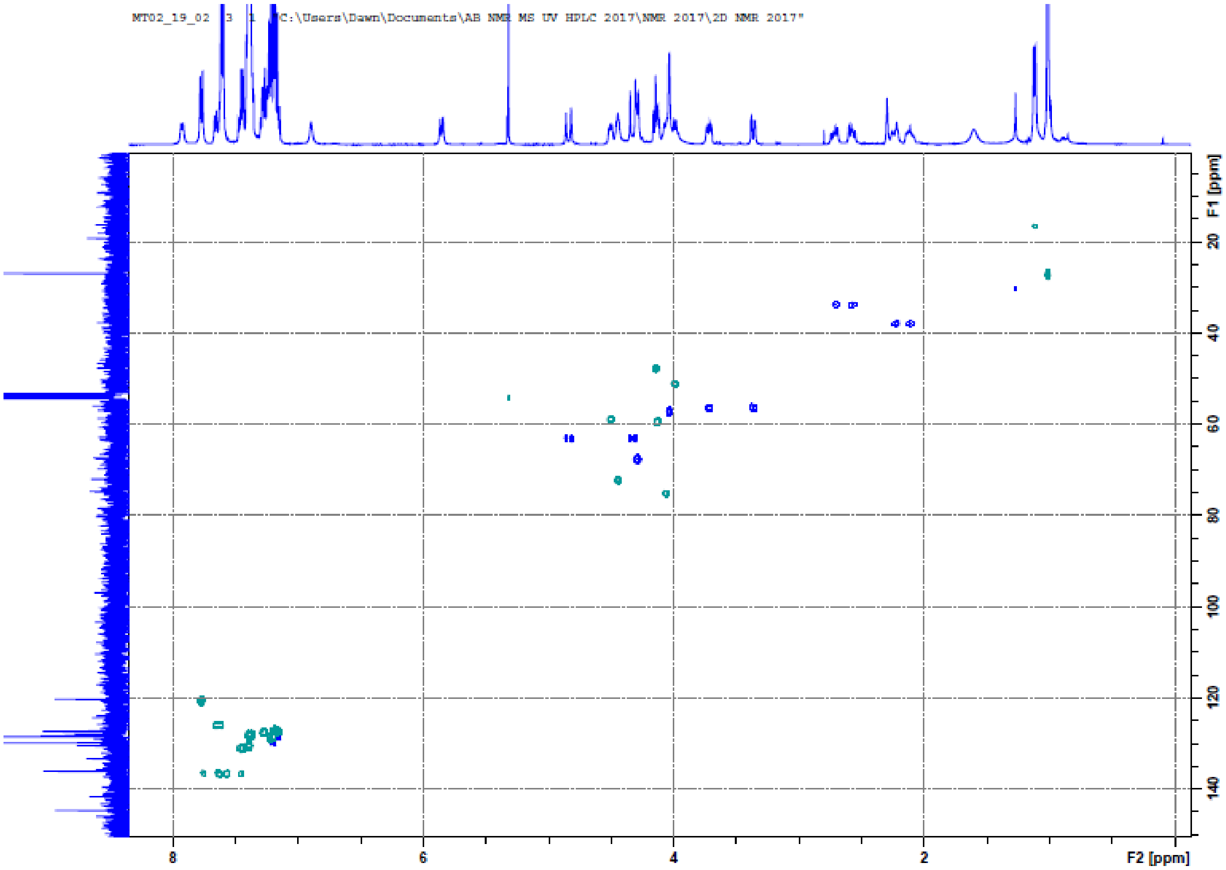


Figure *S36* HSQC-NMR spectra (400 MHz, CD_2Cl_2) of *N*$^{\alpha}$-Fmoc-*O*$^{\beta}$-propargyl-D-threonyl-*S*$^{\beta}$-trityl-cysteinyl-(1-carbamoylmethyl)-*O*^{4}-(*tert*-butyldiphenylsilyl)-*cis*-hydroxyprolinate ester (**23**).

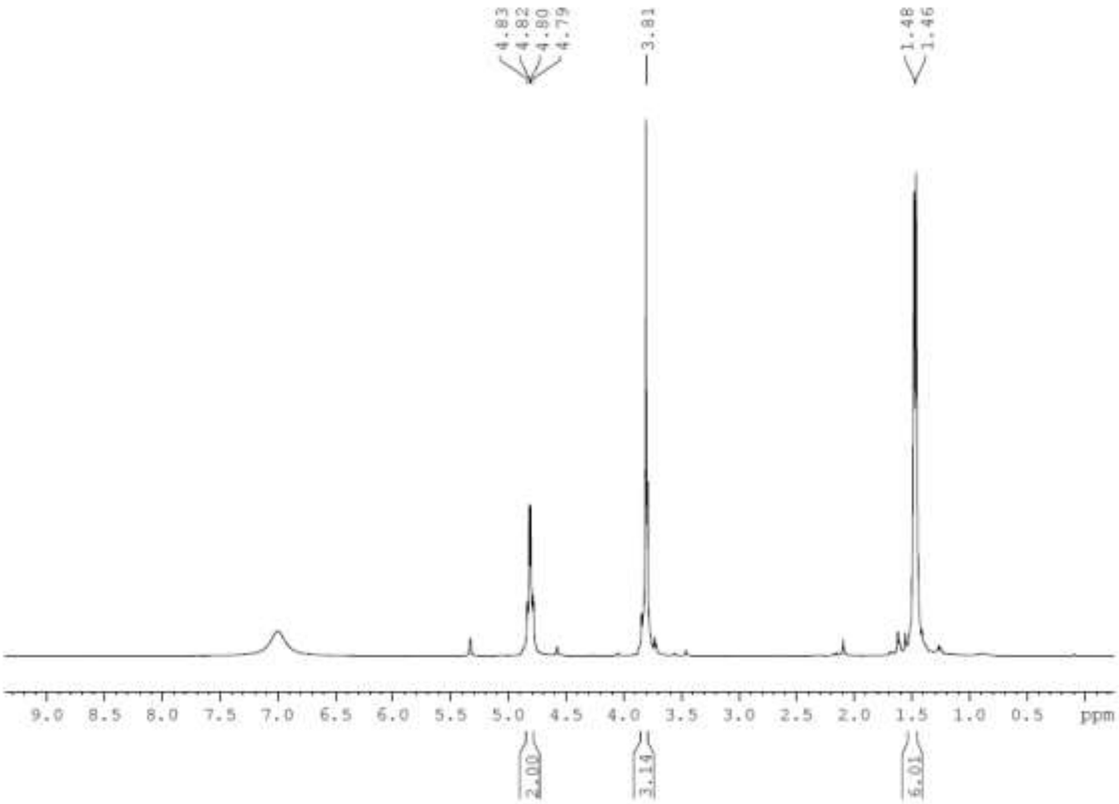


Figure ***S37*** ^{1}H-NMR spectra (300 MHz, CD_2Cl_2) of 1-methyl-2,3-*O*-isopropylidene-D-tartrate monoester (**1**).

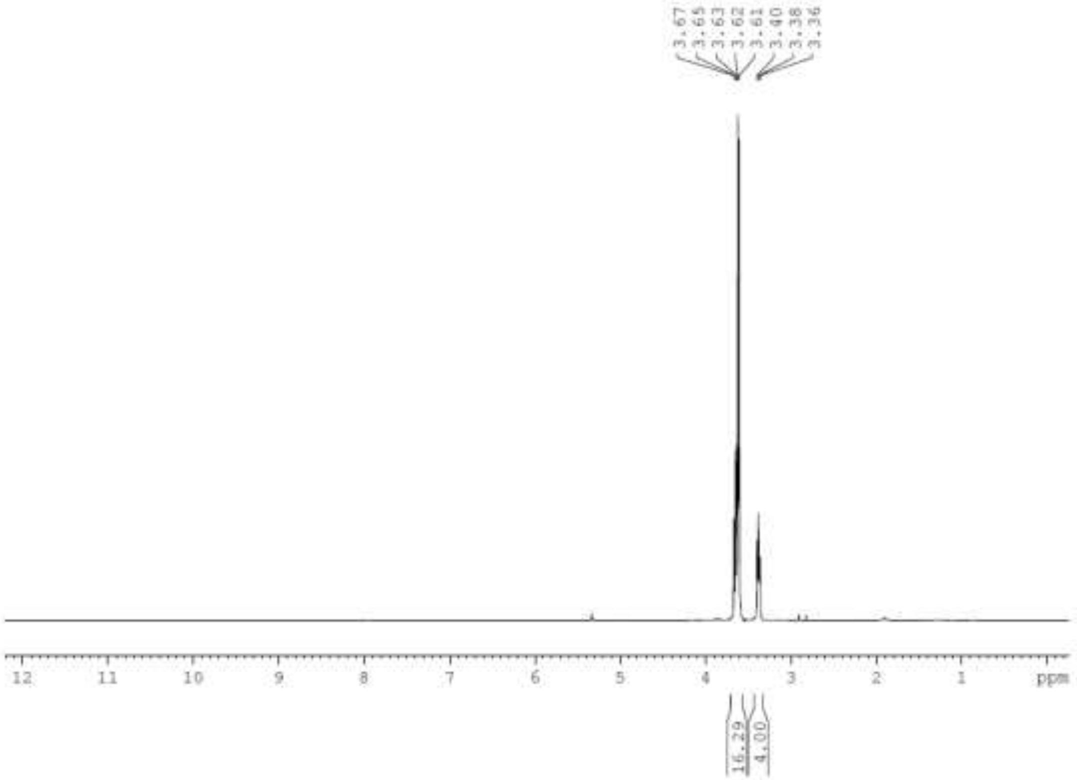


Figure ***S38*** ^{1}H-NMR spectra (300 MHz, CD_2Cl_2) of 1,14-diazido-3,6,9,12-tetraoxatetradecane (**24a**).

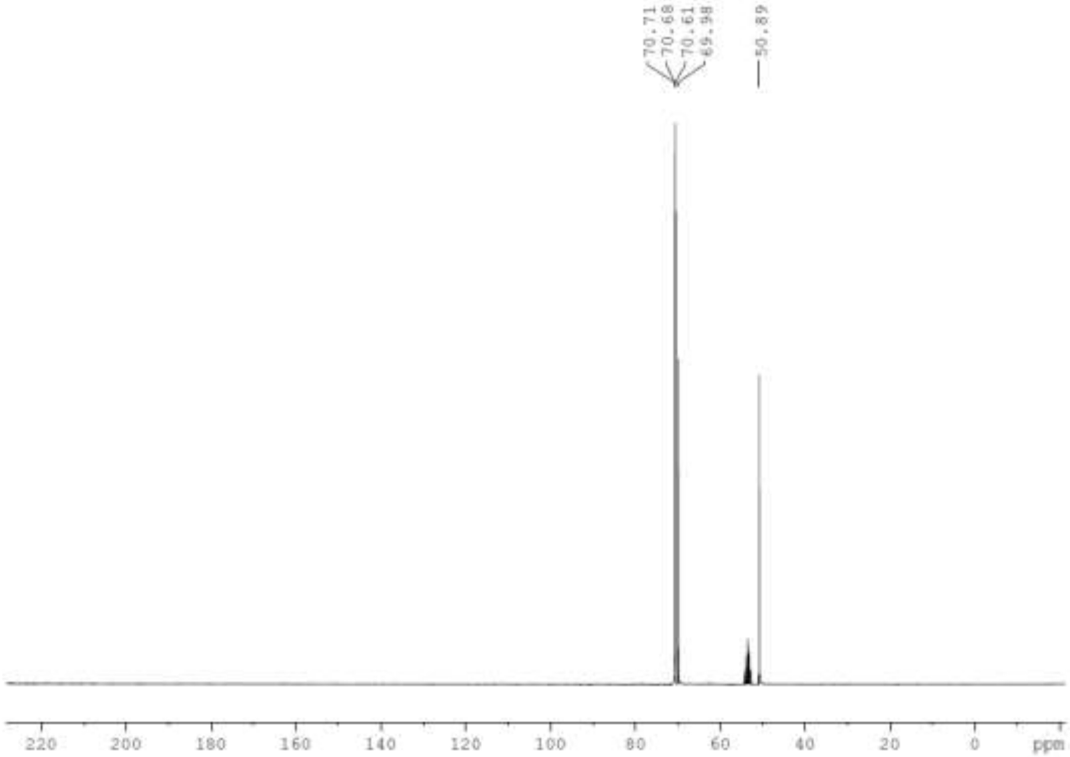


Figure *S39* ^{13}C-NMR spectra (75 MHz, APT, CD_2Cl_2) of 1,14-diazido-3,6,9,12-tetraoxatetradecane (**24a**).

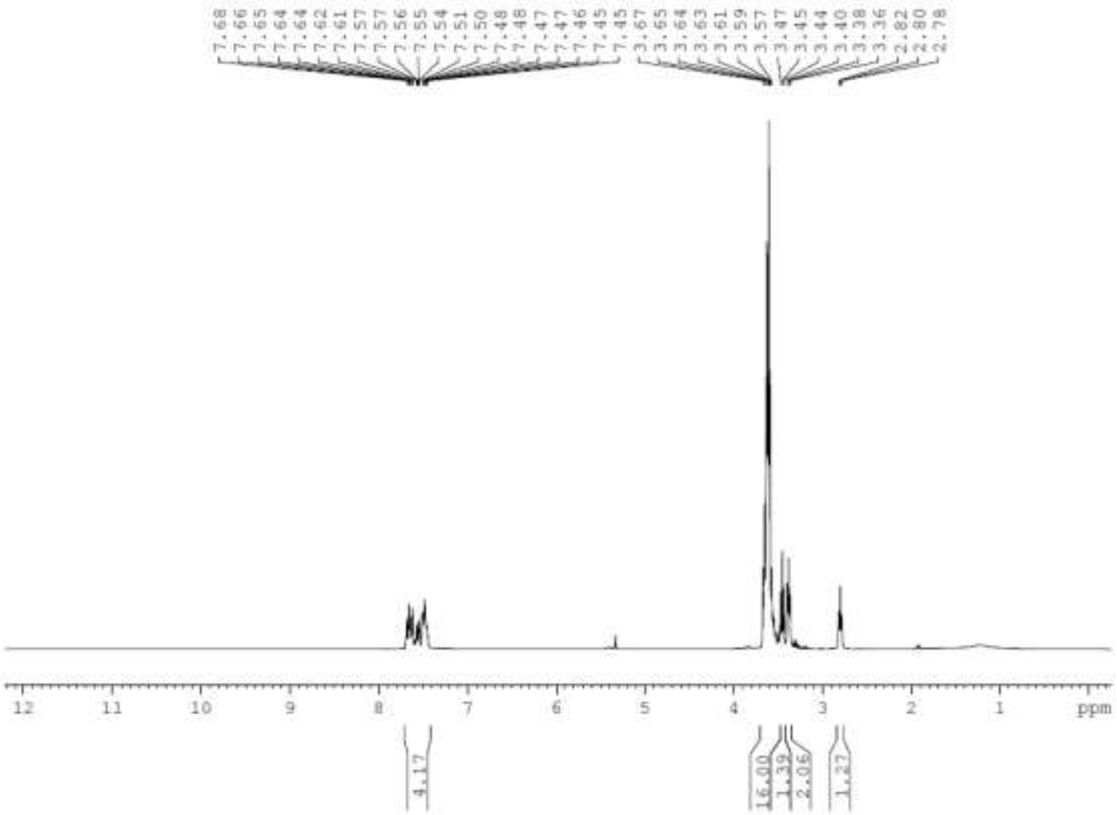


Figure *S40* ^{1}H-NMR spectra (300 MHz, CD_2Cl_2) of 14-azido-3,6,9,12-tetraoxatetradecane-1-amine (**24b**).

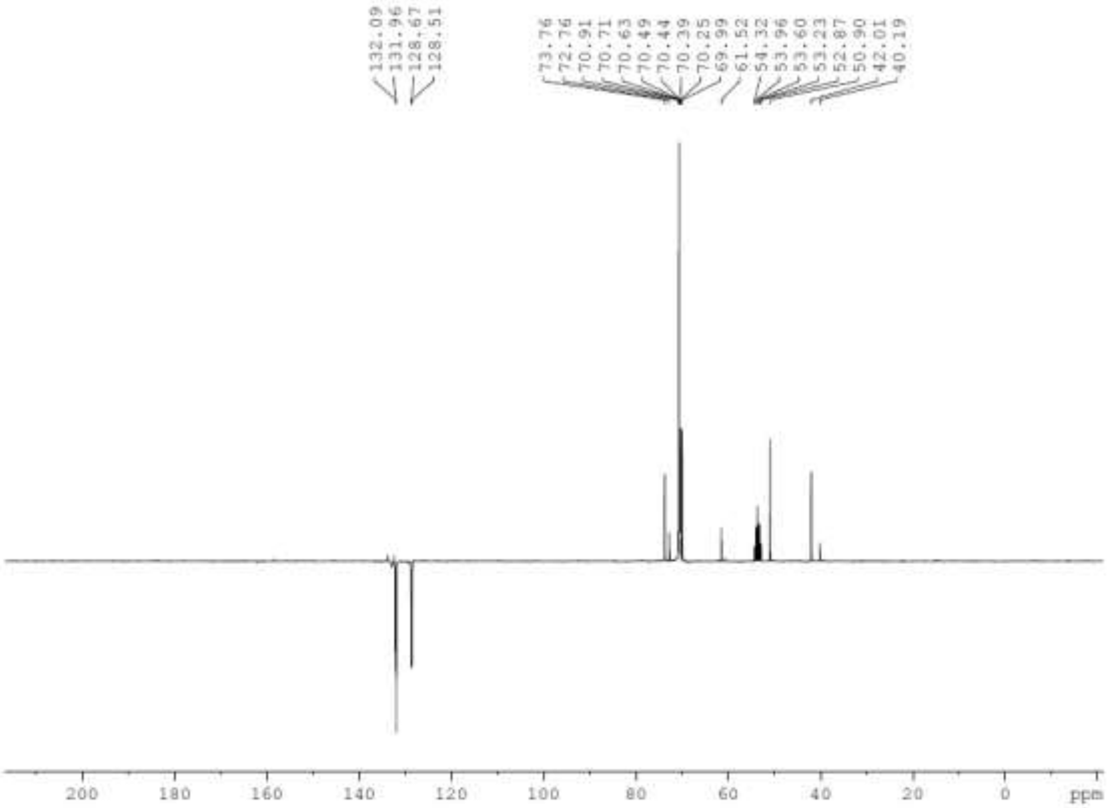


Figure *S41* ^{13}C-NMR spectra (75 MHz, APT, CD_2Cl_2) of 14-azido-3,6,9,12-tetraoxatetradecane-1-amine (**24b**).

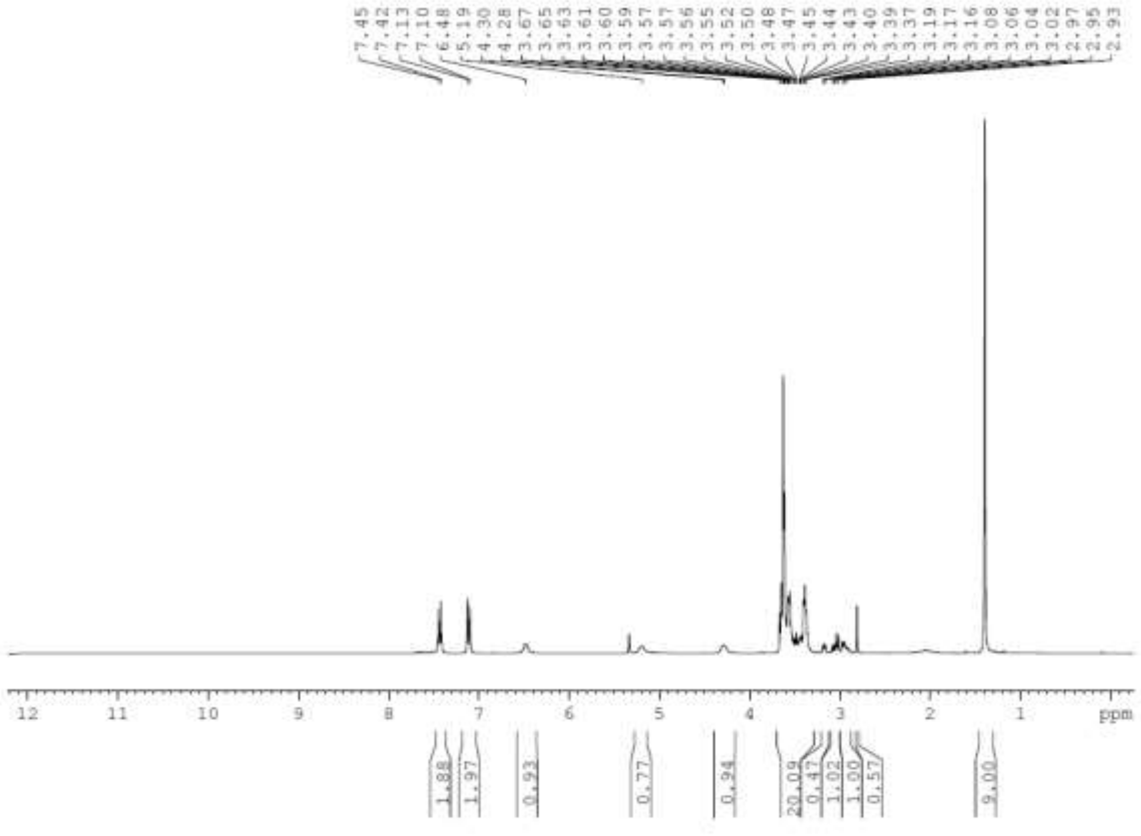


Figure ***S42*** ^{1}H-NMR spectra (300 MHz, CD_2Cl_2) of (2*S*)-2-([(2’-methyl-2’-propanyl)oxy]carbonylamino)-3-(4-bromophenyl)propanoate-1’-amido-(14’-azido-3’,6’,9’,12’-tetraoxatetradecane) (**24c**).

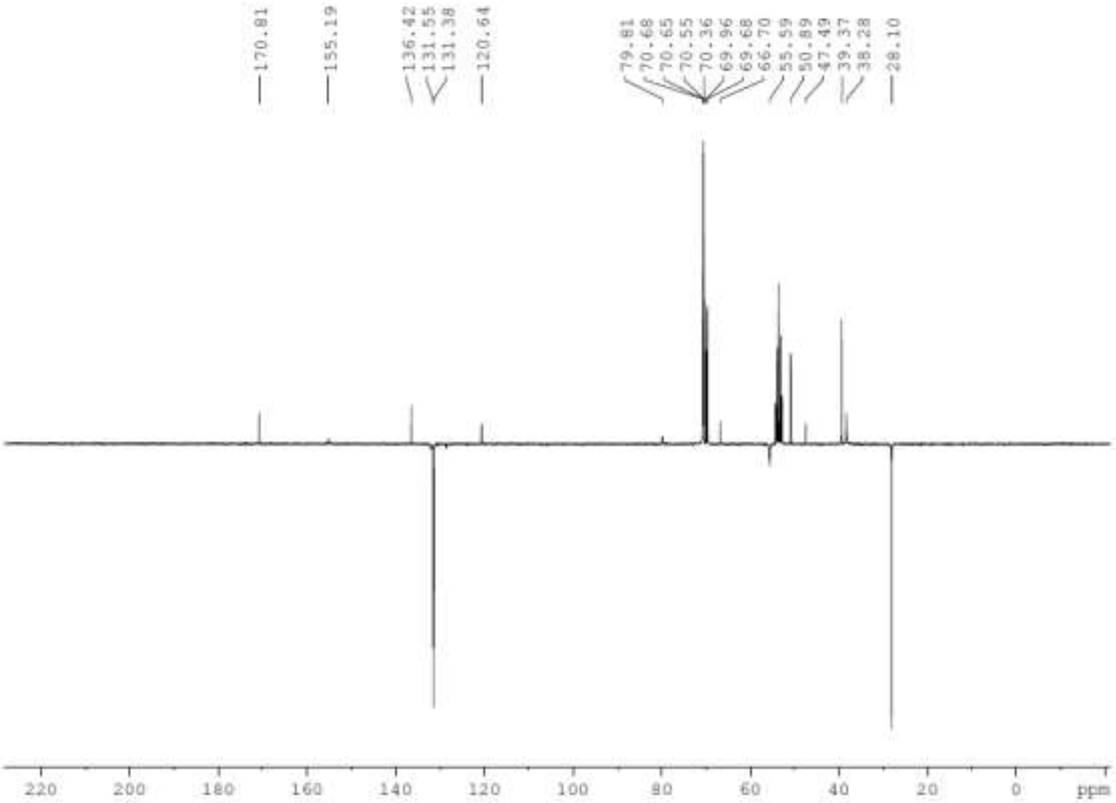


Figure *S43* ^{13}C-NMR spectra (75 MHz, APT, CD_2Cl_2) of (2*S*)-2-([(2'-methyl-2'-propanyl)oxy]carbonylamino)-3-(4-bromophenyl)propanoate-1'-amido-(14'-azido-3',6',9',12'-tetraoxatetradecane) (**24c**).

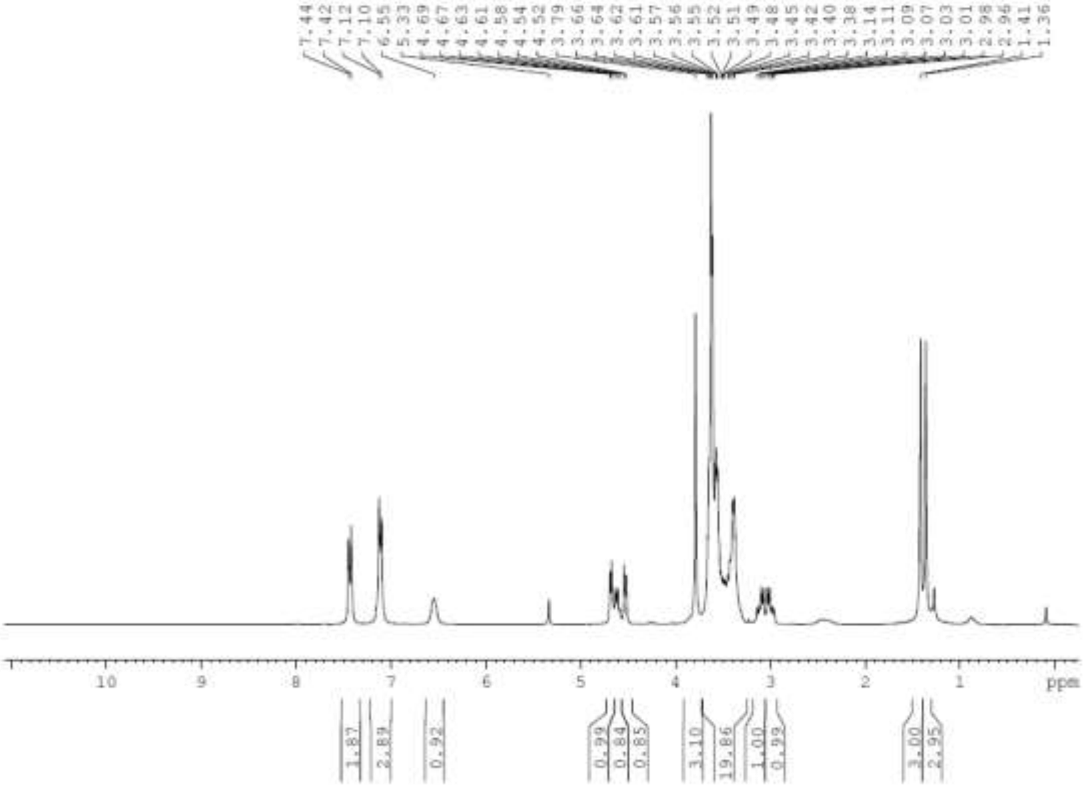


Figure *S44* ^{1}H-NMR spectra (300 MHz, CD_2Cl_2) of 2-[(1'-methyl ester-2',3'-*O*-isopropylidene-D-tartrate)- (2*S*)-2-amido)-3-(4'-bromophenyl)propanoate]-1'-amido-(14'-azido-3',6',9',12'-tetraoxatetradecane) (**24d**).

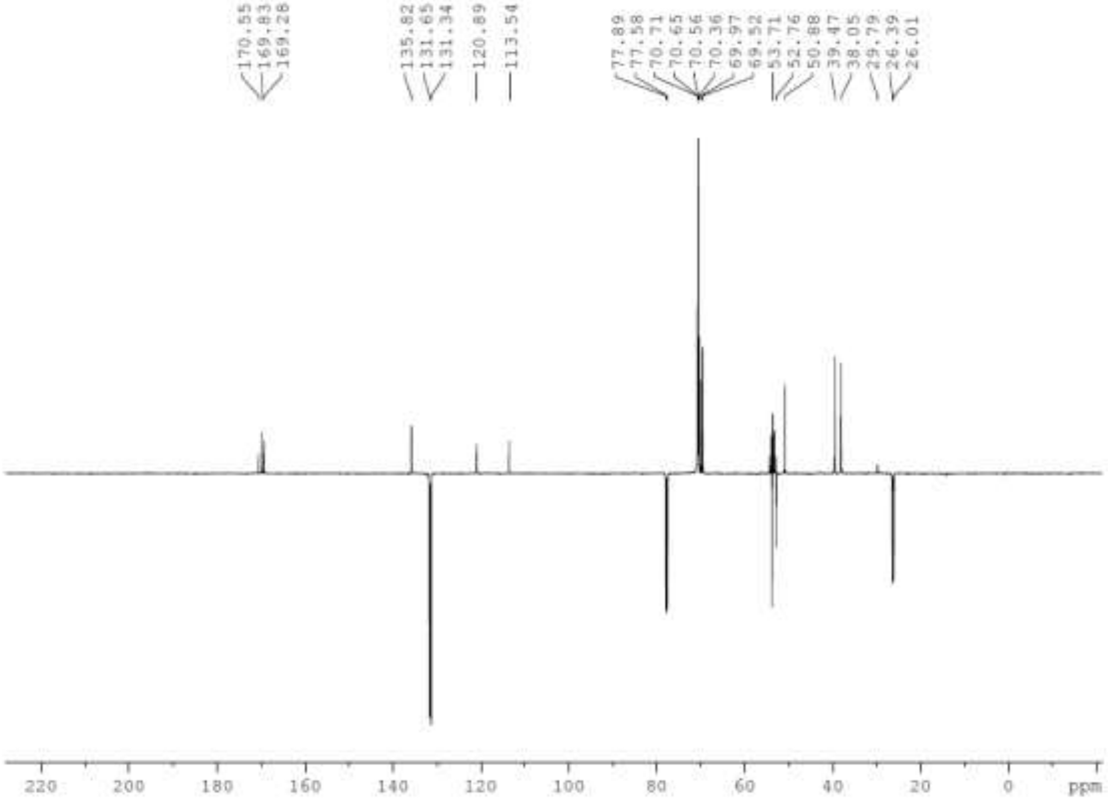


Figure *S45* ^{13}C-NMR spectra (75 MHz, APT, CD_2Cl_2) of 2-[(1'-methyl ester-2',3'-*O*-isopropylidene-D-tartrate)- (2*S*)-2-amido)-3-(4'-bromophenyl)propanoate]-1'-amido-(14'-azido-3',6',9',12'-tetraoxatetradecane) (**24d**).

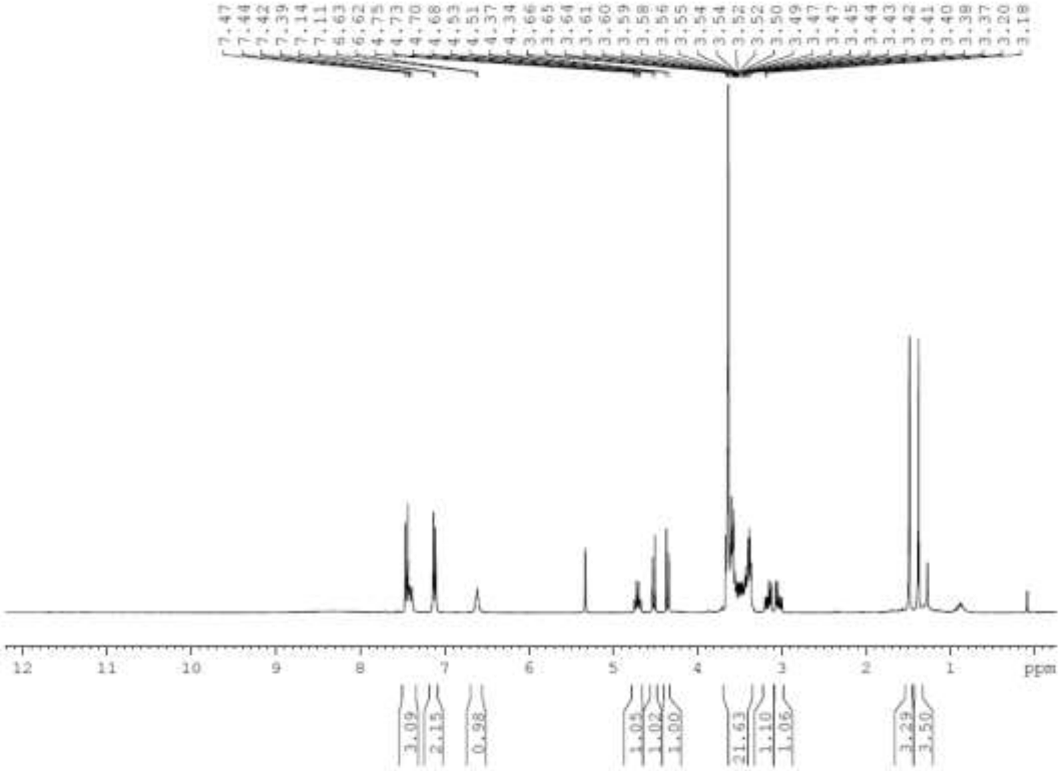


Figure *S46* ^{1}H-NMR spectra (300 MHz, CD_2Cl_2) of 2-[(2’,3’-*O*-isopropylidene-D-tartrate)-(2*S*)-2-amido)-3-(4’-bromophenyl)propanoate]-1’-amido-(14’-azido-3’,6’,9’,12’-tetraoxatetradecane) (**24**).

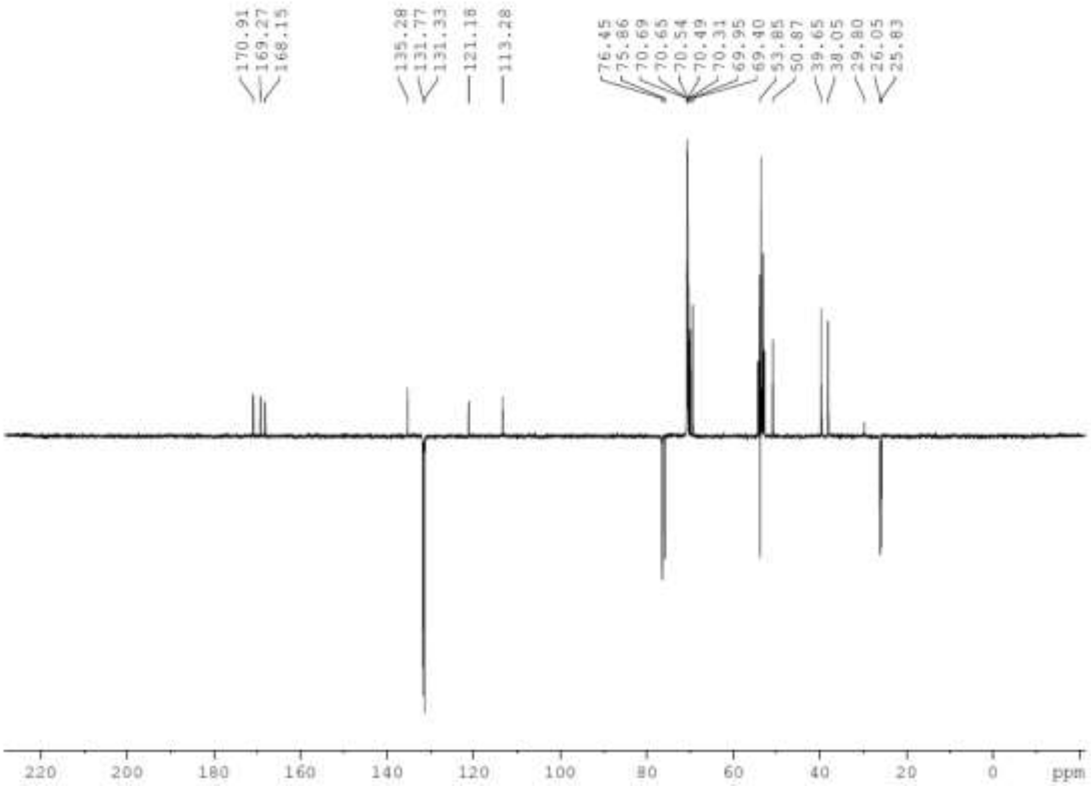


Figure *S47* ^{13}C-NMR spectra (75 MHz, CD_2Cl_2) of 2-[(2’,3’-*O*-isopropylidene-D-tartrate)-(2*S*)-2-amido)-3-(4’-bromophenyl)propanoate]-1’-amido-(14’-azido-3’,6’,9’,12’-tetraoxatetradecane) (**24**).

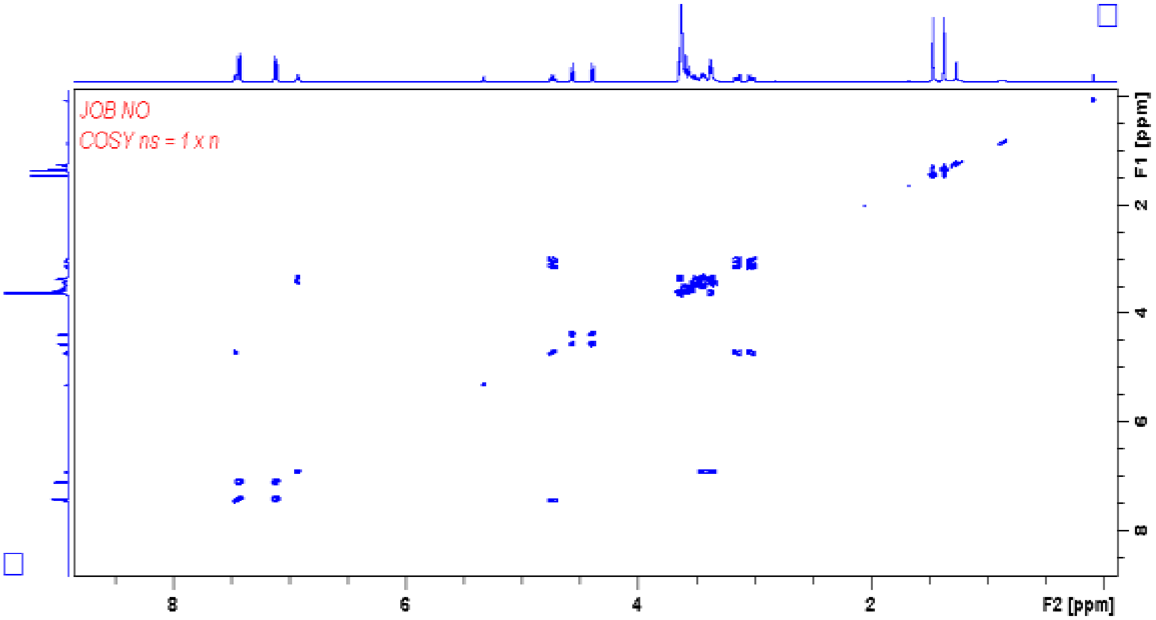


Figure *S48* COSY-NMR spectra (400 MHz, CD$_2$Cl$_2$) of 2-[(2’,3’-*O*-isopropylidene-D-tartrate)- (2*S*)-2-amido)-3-(4’-bromophenyl)propanoate]-1’-amido-(14’-azido-3’,6’,9’,12’-tetraoxatetradecane) (**24**).

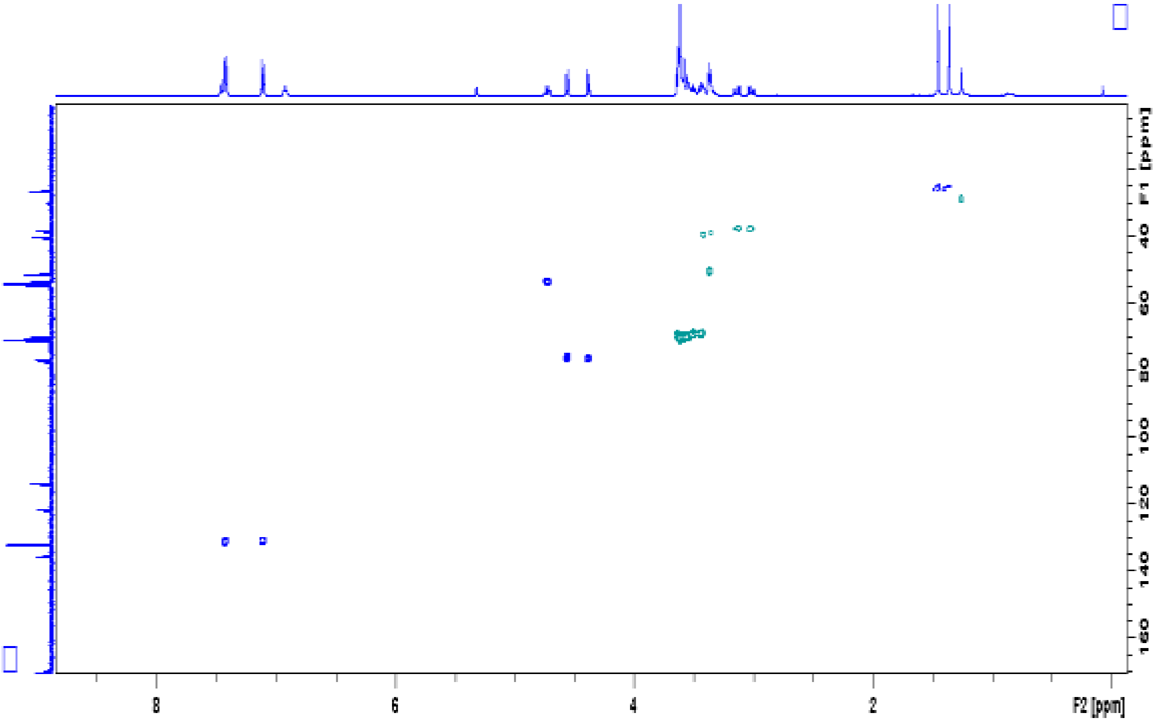


Figure *S49* HSQC-NMR spectra (400 MHz, CD_2Cl_2) of 2-[(2’,3’-*O*-isopropylidene-D-tartrate)- (2*S*)-2-amido)-3-(4’-bromophenyl)propanoate]-1’-amido-(14’-azido-3’,6’,9’,12’-tetraoxatetradecane) (**24**).

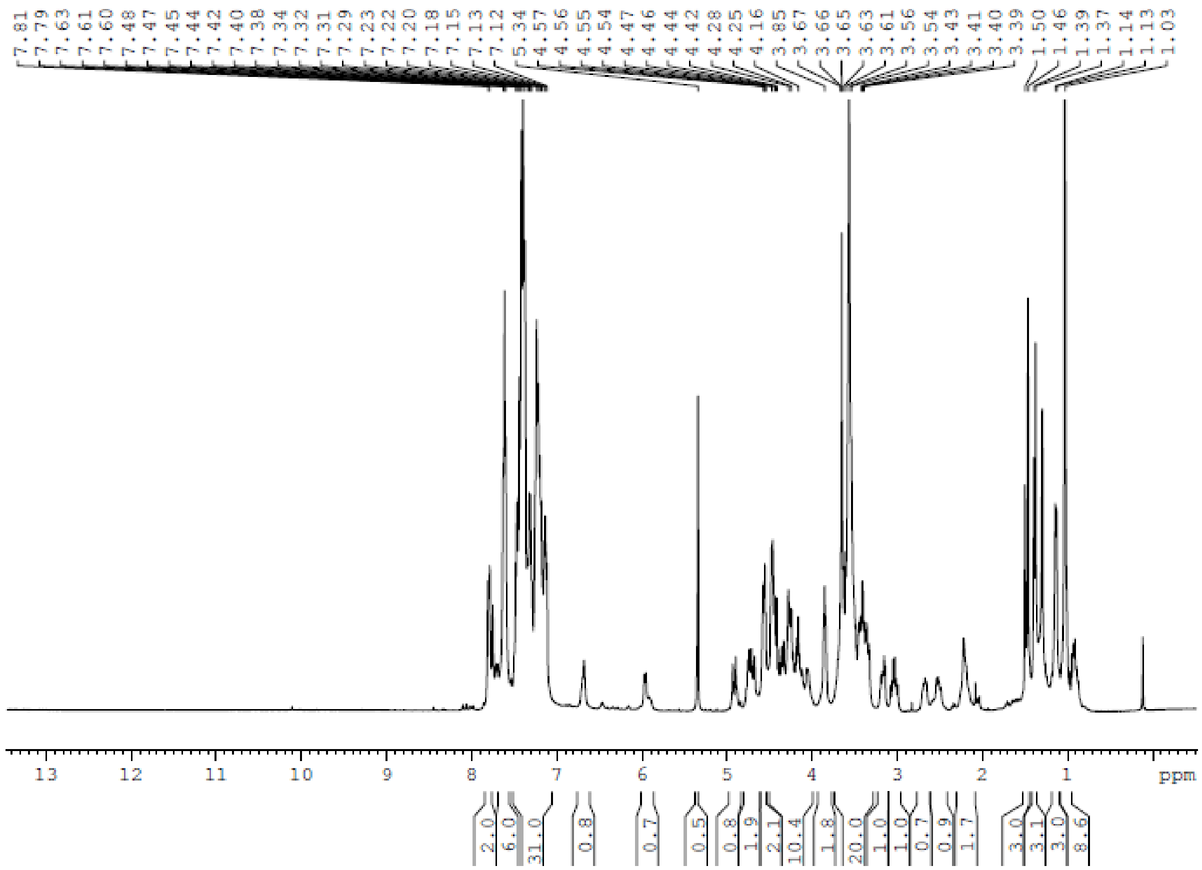


Figure *S50* ^{1}H-NMR spectra (400 MHz, CD_2Cl_2) of *N*$^{\alpha}$-Fmoc-*O*$^{\beta}$-[5-(1’-(18’-(4’’-bromophenyl)-16’-oxo-3’,6’,9’,12’-tetraoxa-15’-azaoctadecan-17’-yl)carbamoyl)-2,2-dimethyl-1,3-dioxolane-4-carboxylic acid)-1*H*-[1’,2’,3’]-triazol-4’-yl)methyl]-D-threonyl-*S*$^{\beta}$-trityl-cysteinyl-1’’’-(carbamoylmethyl)-4’’’-(*tert*-butyldiphenylsilyl)oxy-*cis*-hydroxyprolinate (**25**).

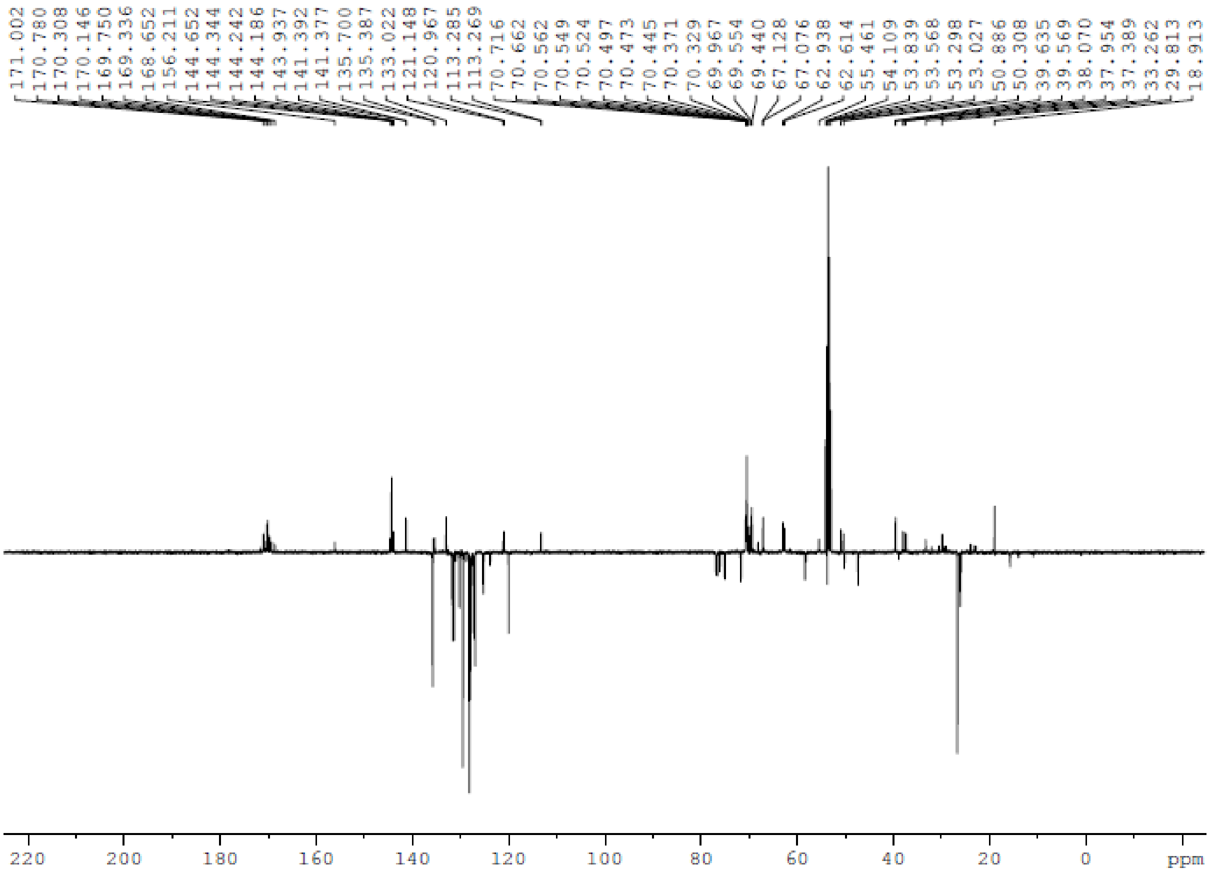


Figure *S51* ^{13}C-NMR spectra (100 MHz, CD$_2$Cl$_2$) of *N*$^{\alpha}$-Fmoc-*O*$^{\beta}$-[5-(1’-(18’-(4’’-bromophenyl)-16’-oxo-3’,6’,9’,12’-tetraoxa-15’-azaoctadecan-17’-yl)carbamoyl)-2,2-dimethyl-1,3-dioxolane-4-carboxylic acid)-1*H*-[1’,2’,3’]-triazol-4’-yl)methyl]-D-threonyl-*S*$^{\beta}$-trityl-cysteinyl-1’’’-(carbamoylmethyl)-4’’’-(*tert*-butyldiphenylsilyl)oxy-*cis*-hydroxyprolinate (**25**).

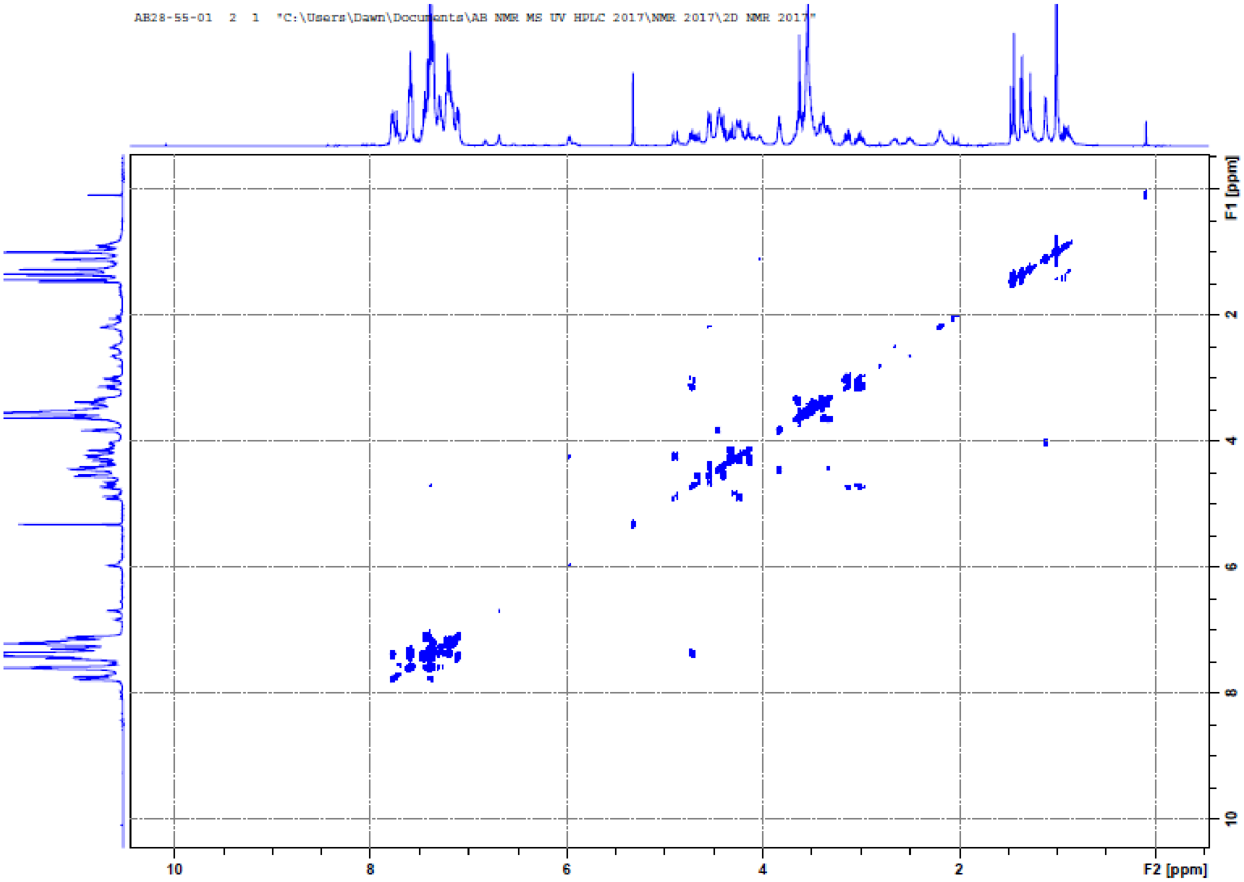


Figure *S52* COSY-NMR spectra (400 MHz, CD_2Cl_2) of *N*$^{\alpha}$-Fmoc-*O*$^{\beta}$-[5-(1'-(18'-(4''-bromophenyl)-16'-oxo-3',6',9',12'-tetraoxa-15'-azaoctadecan-17'-yl)carbamoyl)-2,2-dimethyl-1,3-dioxolane-4-carboxylic acid)-1*H*-[1',2',3']-triazol-4'-yl)methyl]-D-threonyl-*S*$^{\beta}$-trityl-cysteinyl-1'''-(carbamoylmethyl)-4'''-(*tert*-butyldiphenylsilyl)oxy-*cis*-hydroxyprolinate (**25**).

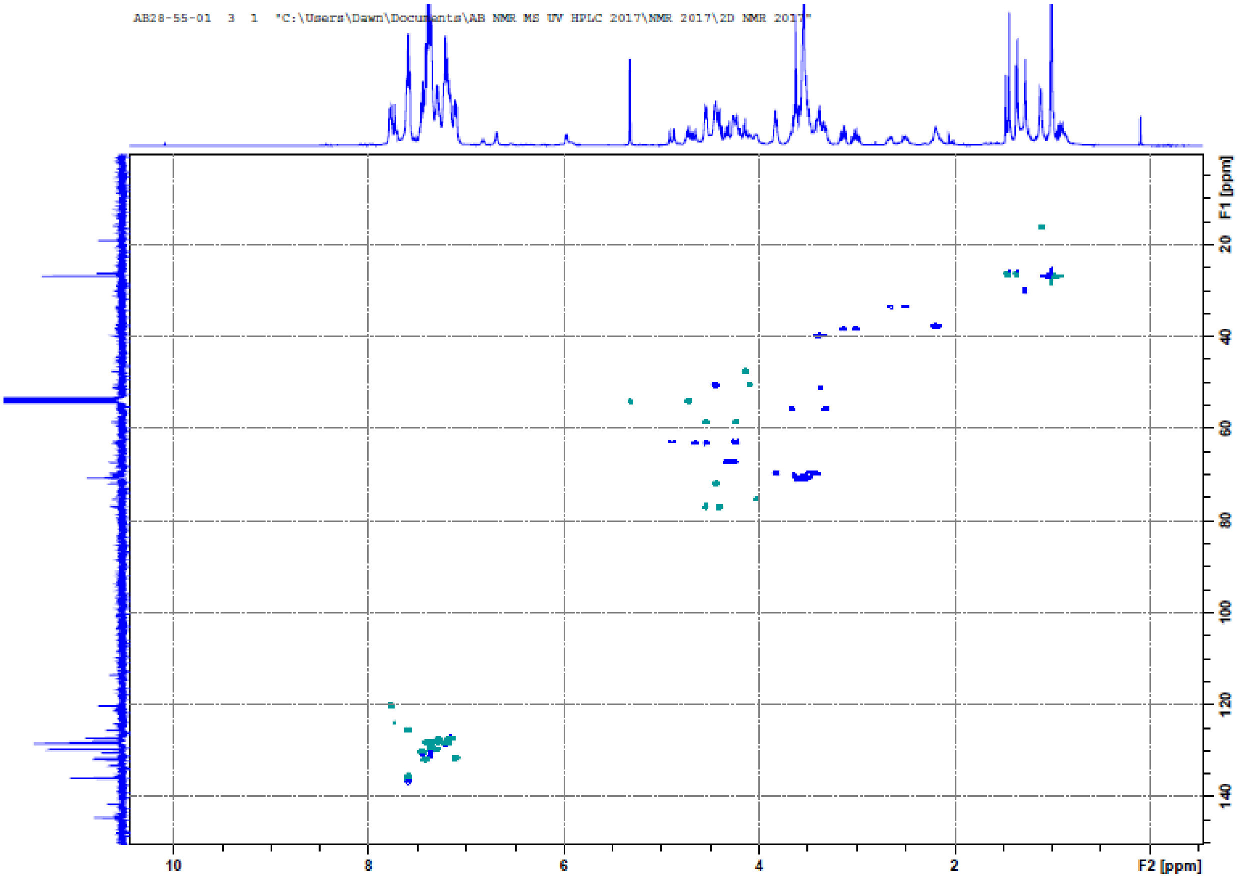


Figure *S53* HSQC-NMR spectra (400 MHz, CD_2Cl_2) of *N*$^{\alpha}$-Fmoc-*O*$^{\beta}$-[5-(1'-(18'-(4''-bromophenyl)-16'-oxo-3',6',9',12'-tetraoxa-15'-azaoctadecan-17'-yl)carbamoyl)-2,2-dimethyl-1,3-dioxolane-4-carboxylic acid)-1*H*-[1',2',3']-triazol-4'-yl)methyl]-D-threonyl-*S*$^{\beta}$-trityl-cysteinyl-1'''-(carbamoylmethyl)-4'''-(*tert*-butyldiphenylsilyl)oxy-*cis*-hydroxyprolinate (**25**).

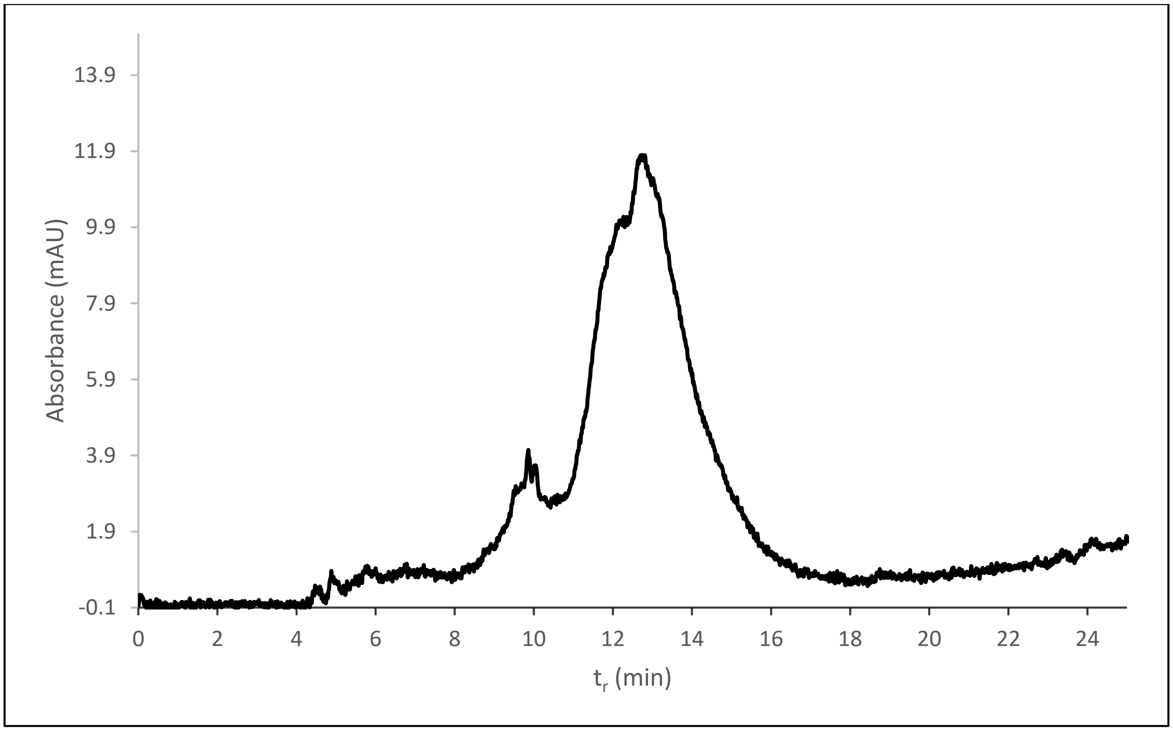


Figure *S54* RP-HPLC (C18) chromatogram of fluorescent [Leu^7, Thr^2(Oxime-EDANS)]-phalloidin (**34**). t_r = 12.81 min (A_{290} 89%) with gradient of 30% to 100% solvent B over 18 min at a flow rate of 2 mL/min.

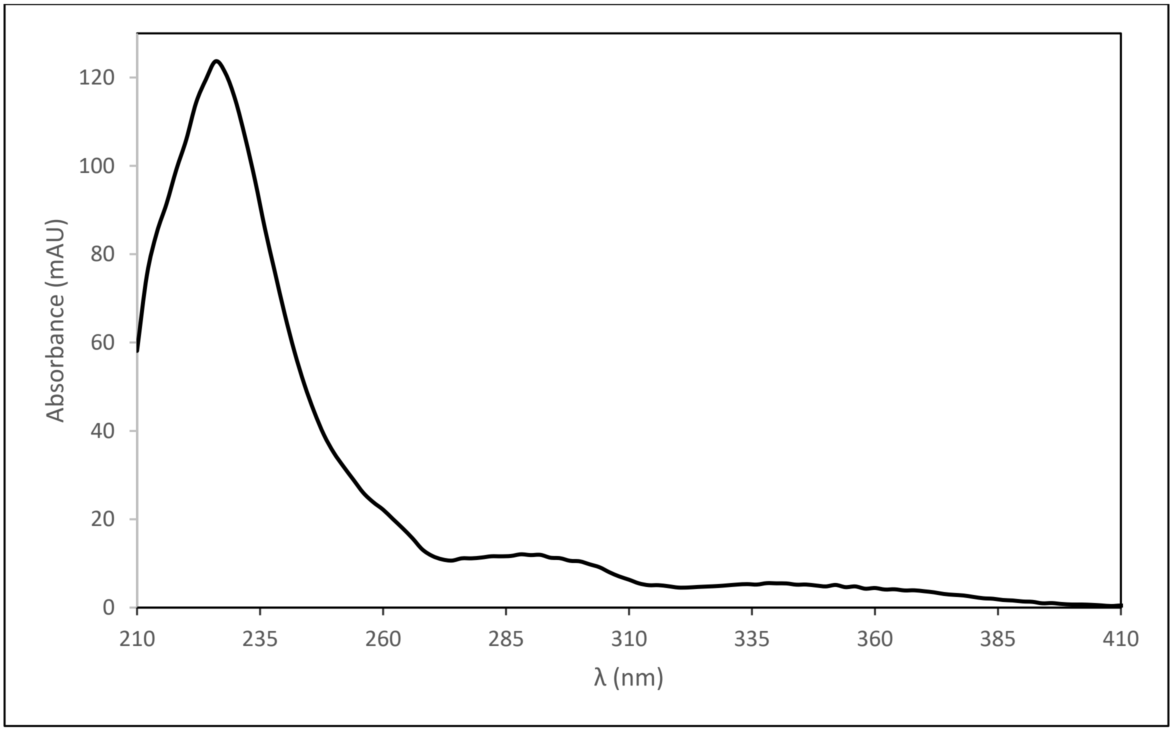


Figure *S55* UV-Vis spectra of fluorescent [Leu^7, Thr^2(Oxime-EDANS)]-phalloidin (**34**). UV-Vis spectra were taken with the RP-HPLC diode array detectors directly in the RP-HPLC flow cells at t_r = 12.81 min.

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