Expanding the toolbox of chemoselective modifications of protein-like polymers at methionine residues

Marie Rosselin,[a] Ye Xiao,[a] Ludovic Belhomme,[a] Sébastien Lecommandoux[a] and Elisabeth Garanger*[a]

[a] Univ. Bordeaux, CNRS, Bordeaux INP, LCPO, UMR 5629, F-33600, Pessac, France.

I. Materials and methods

I.1. Materials

All of the reagents and solvents are commercially available from standard suppliers, except for oxaziridine derivatives that have been prepared following the published procedure.[1] Methyl iodine, propylene oxide, allyl glycidyl ether, glycidyl propargyl ether, formic acid, glacial acetic acid, Trizma®, hexafluoroisopropanol (HFIP), N,N,N′,N″,N″-pentamethyldiethylenetriamine (PMDETA), sodium azide (99.5%), 3-chloroperoxybenzoic acid (≤77%) and sodium cyanate (96%) were obtained from Sigma-Aldrich (FR). Deionized water (18 MΩ-cm) was obtained by using a Millipore Milli-Q Biocel A10 purification unit. Cuprisorb was purchased from Seachem. Ethanol (96.0%, EtOH), methanol (98.5%, MeOH), tetrahydrofuran (99%), DMF (99%), DCM (99.5%), acetonitrile (99.9%, ACN) and $CuSO_4.5H_2O$ were obtained from VWR international. 1-ethyl urea (98%), benzaldehyde (99%), potassium carbonate (99%), propargylamine (98%), and 3-chloropropylamine hydrochloride (98%) and NaCl (99%) was purchased from Alfa Aesar (FR). β-D-galactopyranosyl azide (Gal-N_3) was obtained from Carbosynth (UK). Ammonium Acetate and Ammonium pyrrolidinedithiocarbamate, APDC and sodium ascorbate were obtained from Fisher Scientific (FR). Titanium isopropoxide (98%) was purchased from Acros Organics. Silica gel 60 M was purchased from Macherey-Nagel. Amicon® ultra-15 centrifugal filter tube 3 000 MWCO was obtained from Merck millipore. Tris buffer was prepared with 0.05 M of Trisma-HCland 0.15 M of NaCl in Milli-Q water, the pH of the solution was then adjusted to 7.6 with NaOH 0.1M.

I.2. NMR spectrometry analysis

^{1}H NMR analyses were performed in D_2O at 298 K on a Bruker AVANCE III HD 400 apparatus equipped with a 5 mm Bruker multinuclear z-gradient direct probe operating at 400.2 MHz. The solvent signal was used as the reference signal (δ = 4.79 ppm). HSQC analyses were performed on a Bruker AVANCE NEO 400 spectrometer operating at 100.7

MHz, equipped with a 5 mm Bruker multinuclear z-gradient direct cryoprobe-head operating at 298 K. Data processing was performed using Bruker Topspin Software.

I.3. Transition temperature measurements

Turbidity assays were performed on a Cary 100 Bio UV-visible spectrometer equipped with a multi-cell thermoelectric temperature controller from Varian (Palo Alto, CA) operating at 600 nm between 20°C and 80°C at a 1°C.min-1 scan rate for ELP[V_3M_1-*40*], compound 1A, 1B, 1C and 1D in Tris buffer at four different concentrations (25 µM, 50 µM, 125M and 250 µM).

I.4. Size-exclusion chromatography (SEC)

Measurements in water were performed on an Ultimate 3000 system from Thermoscientific equipped with diode array detector DAD. The system also include a multi-angles light scattering detector MALS and differential refractive index detector dRI from Wyatt technology. Polymers were separated on two TOSOH successive columns (one G4000PWXL (7.8*300) column with exclusion limits from 2 000 Da to 300 000 Da and one G3000PWXL (7.8*300) column with exclusion limit bellow 40 000 Da). Measurements were performed at a flowrate of 0.6 mL/min and columns temperature was held at 26°C. Aqueous solvent composed by Acetic Acid (AcOH) 0.3 M, Ammonium Acetate 0.2 M and ACN (6.5/3.5, v/v) was used as the eluent. Ethylene glycol was used as flow marker.

II. Experimental section

II.1. Alkylation

Alkylation of ELP[M_1V_3-40] (procedure A). ELP[M_1V_3-40] was dissolved in 0.2 M aqueous formic acid (20 mg/mL). Alkyl halide (40 equiv. per Met residue) was added as a solution in THF (50 mg/mL). The reaction was stirred for 5 days at room temperature under Argon atmosphere. The excess of alkyl halide was removed by extraction with diethyl ether, the organic layer was pipetted off and discarded. The aqueous phase was then transferred to a 3,000 MWCO ultra-centrifugal filter tube and washed with 40 mL of Milli-Q water at 20°C. The product was lyophilized to dryness to give a white powder.

Alkylation of ELP[M_1V_3-40] (procedure B). ELP[M_1V_3-40] was dissolved an AcOH/HFIP mixture (9/1, v/v) at 20 mg/mL. The solution was degassed with Ar. and the epoxide was added to the mixture (10 equiv. per methionine residue). After 24h of stirring, a second portion of epoxide (10 equiv. per methionine residue) was added and the reaction was stirred

48h in total, at room temperature and under Ar. atmosphere. The obtained mixture was transferred into a 3 000 MWCO Amicon® ultra-15 centrifugal filter tube, washed with 40 mL of Milli-Q water and freeze-dried.

II.1.1. Alkylation of ELP[M_1V_3-40] with alkyl halide: Route (A)

Compound 1A. Alkylation with methyl iodine, procedure A. ^{1}H NMR (400 MHz, D_2O, 25°C): (main peaks) δ 4.67−4.61 (m, 11 H, CHα Met), δ 4.47−4.40 (m, 80 H, CHα Val and Pro, VPGXG), 4.18-4.16 (d, 30 H, CHα Val, VPGVG), 3.41-3.37 (t, 22H, CH_2S Met), 2.95−2.94 (d, 66 H, SCH_3 Met), 1.00−0.93 (br m, 420 H, CH_3 Val). Yield= 61 %.

II.1.2. Alkylation of ELP[M_1V_3-40] with epoxide: Route (B)

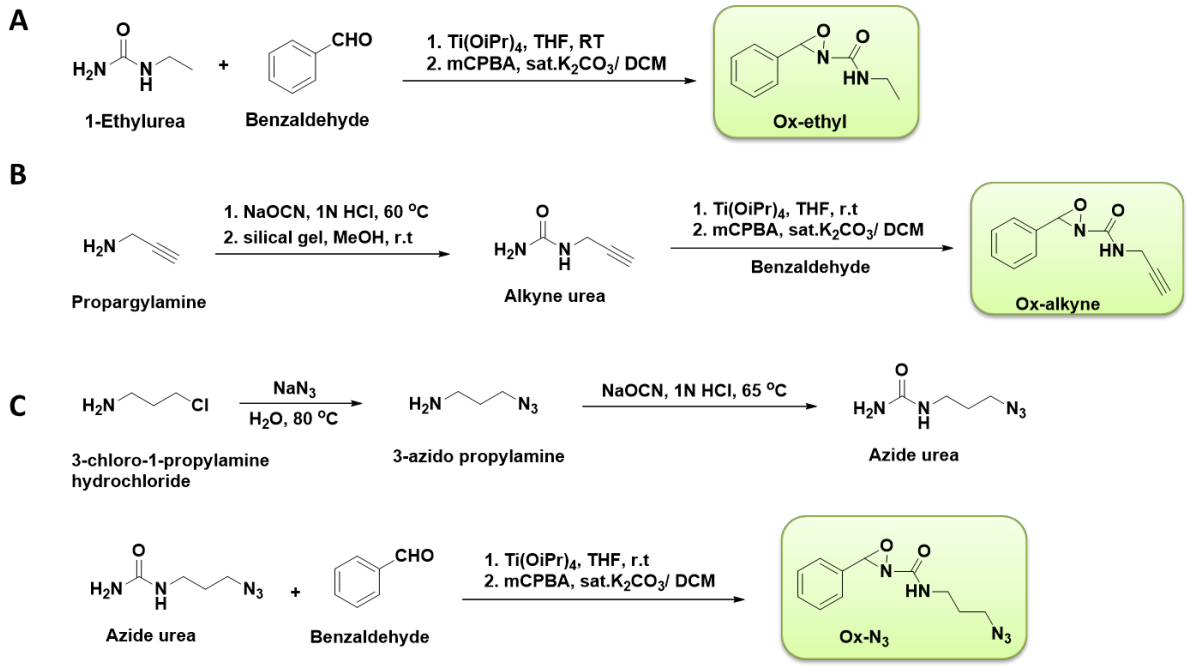
Compound 1B. Alkylation with propylene oxide, procedure B. ^{1}H NMR (400 MHz, D_2O, 25°C): (main peaks) δ 4.66−4.63 (m, 11 H, CHα Met), δ 4.48−4.41 (m, 80 H, CHα Val and Pro, VPGXG), 4.37-4.28 (m, 11H, CHOH Met, signal β), 4.19-4.17 (d, 30 H, CHα Val, VPGVG), 3.59-3.39 (m, 22H, CHCH_2S Met, signal α), 3.01-2.98 (dd, 33 H, SCH_3 Met), 1.38-1.36 (dd, 33 H, CH_3CHOH Met, signal γ), 1.00−0.93 (br m, 420 H, CH_3 Val). Yield= 88 %. MS-ESI: Theoretical MW = 17684.9 Da, Experimental [M11]11+ = 1607.4 Da.

Compound 2B. Alkylation with glycidyl propargyl ether, procedure B. ^{1}H NMR (400 MHz, D_2O, 25°C): (main peaks) δ 4.66−4.62 (m, 11 H, CHα Met), δ 4.47−4.41 (m, 80 H, CHα Val and Pro, VPGXG), 4.38-4.36 (m, 11 H, CHOH Met, signal β), 4.29-4.28 (d, 22 H, CH_2 Met, signal δ), 4.18-4.16 (d, 30 H, CHα Val, VPGVG), 3.63-3.52 (m, 22 H, CHCH_2S Met, signal α), 3.03-3.00 (dd, 33 H, SCH_3 Met), 2.95 (t, 3 H, CH alkyne), 1.38-1.36 (dd, 33 H, CH_3CHOH Met), 1.00−0.94 (br m, 420 H, CH_3 Val). Yield= 86 %. MS-ESI: Theoretical MW = 18279.1 Da, Experimental [M11]11+ = 1661.8 Da.

Compound 3B. Alkylation with allyl glycidyl ether, procedure B. ^{1}H NMR (400 MHz, D_2O, 25°C): (main peaks) δ 6.01-5.91 (m, 11 H, CH alkene, signal ε), δ 5.37-5.27 (m, 22 H, CH_2 alkene, signal #), 4.64−4.62 (m, 11 H, CHα Met), δ 4.47−4.40 (m, 80 H, CHα Val and Pro, VPGXG), 4.37-4.35 (m, 11 H, CHOH Met, signal β), 4.18-4.16 (d, 30 H, CHα Val, VPGVG), 4.11-4.10 (d, 22 H, CH_2 Met, signal δ), 3.02-2.99 (dd, 33 H, SCH_3 Met), 1.00−0.93 (br m, 420 H, CH_3 Val). Yield= 89 %, functionalization 95 %.

II.1.3. Alkylation of ELP[M_1V_3-40] with oxaziridine: Route (C)

Synthesis of oxaziridine derivatives (ReACT method)



Oxaziridine derivatives were synthesized follow the procedure reported by Lin and co-coworkers.[1] Briefly, the synthetic strategy were based on the amination of benzaldehyde by urea to generate imine and the imine was subsequently oxidized by mCPBA to form oxaziridine derivatives. The chemical shift of the only proton on oxaziridine ring was found 4.92 ppm in ^{1}H NMR spectra of ox-ethyl, ox-alkyne, ox-N_3, which are in agreement with the literature data.

General procedure of alkylation with oxaziridine. To a solution of ELP[V_3M_1-*40*] (50 mg, 2.9 μM) in degassed water (50 mL) under Ar. atmosphere was added a solution of oxaziridine derivative synthesized as described by Lin and co-workers[1] (6 equiv. per Met) in DMF (200 μL). After stirring for 30-60 mins, DCM (20 mL) was added and the mixture was extracted with DCM for two times to remove the benzaldehyde. The combined water layers were then dialyzed (MW. cut 3k) against Milli-Q water for 12h (changing water every 4 h). The final product was obtained by lyophilization.

Compound 1C. Alkylation with ethyl-oxaziridine (Ox-ethyl). ^{1}H NMR (400 MHz, D_2O, 25°C): (main peaks) δ 4.47−4.40 (m, 80 H, CHα Val and Pro, VPGXG), 4.18-4.17 (d, 30 H, CHα Val, VPGVG), 3.14-3.09 (q, 22 H, CH_2 Met signal α), 3.07-2.95 (m, 22 H, CH_2S Met,

signal l), 2.70 (s, 33 H, SCH_3 Met), 1.09-1.05 (dt, 33 H, CH_3CH_2 Met, signal β), 1.00−0.93 (br m, 420 H, CH_3 Val). Yield = 63 %.

Compound 2C. Alkylation with oxaziridine-alkyne (Ox-alkyne). ^{1}H NMR (400 MHz, D_2O, 25°C): (main peaks) δ 4.47−4.40 (m, 80 H, CHα Val and Pro, VPGXG), 4.18-4.17 (d, 30 H, CHα Val, VPGVG), 3.13-3.01 (m, 22 H, CH_2 Met signal l), 2.73 (s, 33 H, SCH_3 Met), 2.58 (t, 11 H, CH-Alkyne, signal β), 1.00-0.93 (br m, 420 H, CH_3 Val). Yield = 94 %.

Compound 3C. Alkylation with oxaziridine-azide (Ox-N_3). ^{1}H NMR (400 MHz, D_2O, 25°C): (main peaks) δ 4.47−4.41 (m, 80 H, CHα Val and Pro, VPGXG), 4.19-4.17 (d, 30 H, CHα Val, VPGVG), 6.40-3.36 36 (t, 22 H, CH_2-N_3, signal γ), 3.24-3.20 (t, 22 H, CH_2-NH Met signal α), 3.13-3.03 (m, 22 H, CH_2S Met, signal l), 2.75 (bs, 33 H, SCH_3 Met), 1.80-1.73 (q, 22 H, -CH_2-CH_2-CH_2- Met, signal β), 1.00−0.93 (br m, 420 H, CH_3 Val). Yield = 85 %.

II.2. Demethylation

Demethylation of modified-ELP (compound 1D). To a solution of modified- ELP[M_1V_3-40] (50 mg, 10 mM)) in 75% EtOH(aq) was added ammonium pyrrolidinedithiocarbamate (APDC) (5.0 equiv per Met residue). The solution was stirred under Ar. for 24 h at room temperature. The obtained mixture was transferred to a 1 kDa MWCO dialysis bag and dialyzed against 50% MeOH(aq) during 24 h with 3 solvent changes followed by 8 h dialysis against Milli-Q water with 3 changes. The dialysis bag contents were then lyophilized to provide demethylated ELPs. ^{1}H NMR (400 MHz, D_2O, 25°C): (main peaks) δ 4.58−4.55 (m, 11 H, CHα Met), δ 4.47−4.40 (m, 80 H, CHα Val and Pro, VPGXG), 4.19-4.17 (d, 30 H, CHα Val, VPGVG), 2.70-2.55 (m, 22 H, CH_2S, signal l), 1.25-1.23 (d, 33 H, CH3CHOH), 1.00−0.93 (br m, 420 H, CH_3 Val). Yield= 44 %.

II.3. Click-Reaction

II.3.1. Click reaction in water. To a solution of ELP[M_1V_3-40] derivative in degassed water (5 mg/mL) under Ar. Atmosphere, the desired azido-galactose (1.5 equiv. per alkyne) was added. A solution of Cu(I) was prepared by addition of sodium ascorbate (1.3 equiv. per alkyne) to a degassed solution of Cu(II)SO4 (0.26 equiv. per alkyne) and pentamethyldiethylenetriamine (0.26 equiv. per alkyne) in Milli-Q water. The fresh Cu(I) solution was then transferred to the reaction mixture with a syringe. The reaction was stirred under Ar. at room temperature for 72 hrs. Cuprisorb was added to remove cupper by shaking

overnight. Few drops of an aqueous solution of EDTA (0.15 M) were added and the solution was purified by ultracentrifugation with Amicon® 3000 MWCO ultra-centrifugal filter tube against Milli-Q water (40 mL). The remaining mixture was lyophilized.

Compound 4B. Click-reaction of compound 4B with β-D-galactopyranosyl azide. ^{1}H NMR (400 MHz, D_2O, 25°C): (main peaks) δ 8.34 (s, 11 H, CH triazole, signal *), δ 5.74-5.72 (d, 11 H, $CH_{1'}$), δ 4.65−4.61 (m, 11 H, CHα Met), δ 4.47−4.42 (m, 80 H, CHα Val and Pro, VPGXG), 4.40-4.36 (m, 11 H, CHOH Met, signal β), 4.18-4.16 (d, 30 H, CHα Val, VPGVG), 3.81-3.80 (d, 11 H, $CH_{6'}$), 299-2.97 (m, 33 H, SCH_3 Met), 1.00−0.92 (br m, 420 H, CH_3 Val). Yield= 71 %.

II.3.2. Click reaction in DMSO. To a solution of ELP[M_1V_3-40] derivative in degassed DMSO (10 mg/mL) under Ar. Atmosphere, Cu(II)SO4 (0.05 equiv. per alkyne), sodium ascorbate (0.2 equiv. per alkyne) and tris((1-benzyl-4-triazolyl)methyl)amine TBTA (0.05 equiv. per alkyne) were added followed by the azido-galactose (0.05 equiv. per alkyne) dissolved in DMSO. The reaction was stirred under Ar. at room temperature for 72 hrs., 4 mL of Milli-Q water were added, the mixture was cooled at 4°C during 20 min. and TBTA was precipitate and removed by centrifuge. Cuprisorb was added to remove cupper by shaking overnight. Few drops of an aqueous solution of EDTA (0.15 M) were added and the solution was purified by dialysis using an Amicon® 3000 MWCO ultra-centrifugal filter tube against Milli-Q water (40 mL). The remaining mixture was lyophilized.

Compound 4C. Click-reaction of compound 4C with β-D-galactopyranosyl azide. ^{1}H NMR (400 MHz, D_2O, 25°C): (main peaks) δ 8.06 (s, 11 H, CH triazole, signal *), δ 5.61-5.59 (d, 11 H, $CH^{1'}$), δ 4.39−4.33 (m, 91 H, CHα Val and Pro, VPGXG and CH_2 α), 4.11-4.09 (d, 30 H, CHα Val, VPGVG), 4.03-4.02 (m, 11 H, $CH^{5'}$), 3.73-3.71 (d, 22 H, $CH_2{}^{6'}$), 0.92−0.86 (br m, 420 H, CH_3 Val). Yield= 84 %.

III. Supplementary figures

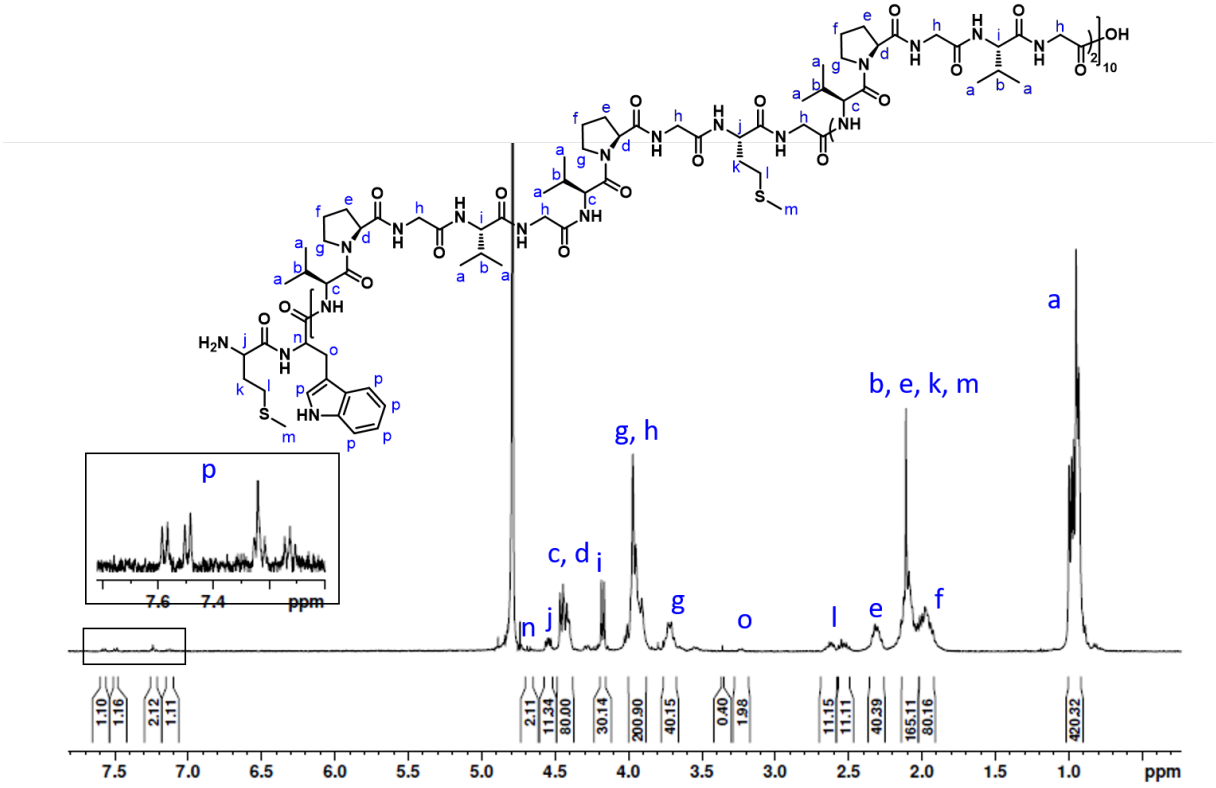


Figure S1. ^{1}H NMR spectrum of ELP[V_3M_1-*40*] in D_2O

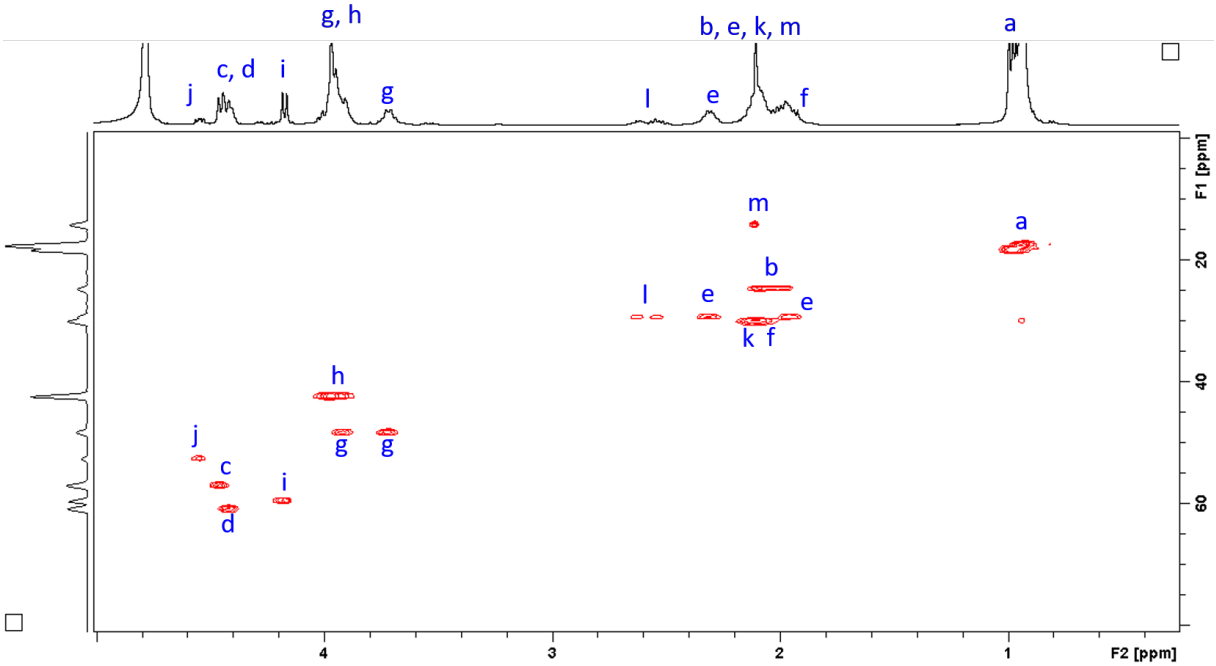


Figure S2. 2D NMR spectrum of ELP[V_3M_1-*40*] in D_2O, HSQC

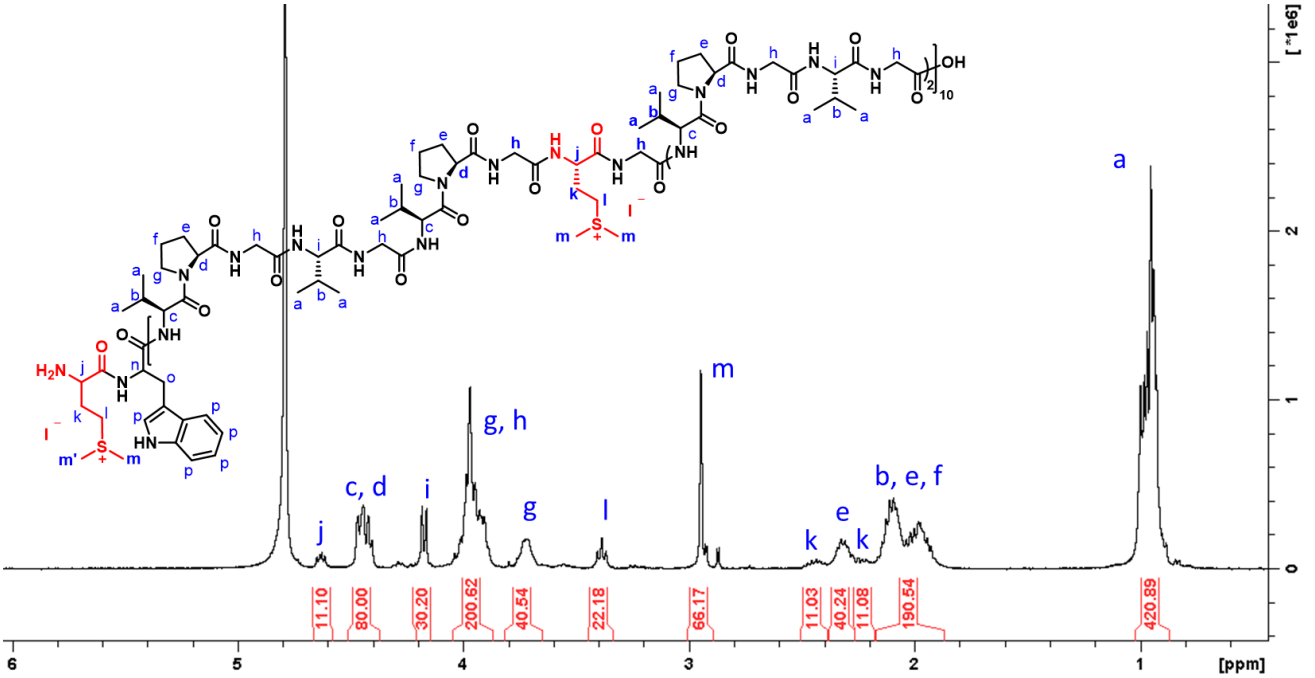


Figure S3. ^{1}H NMR spectrum of compound 1A in D_2O

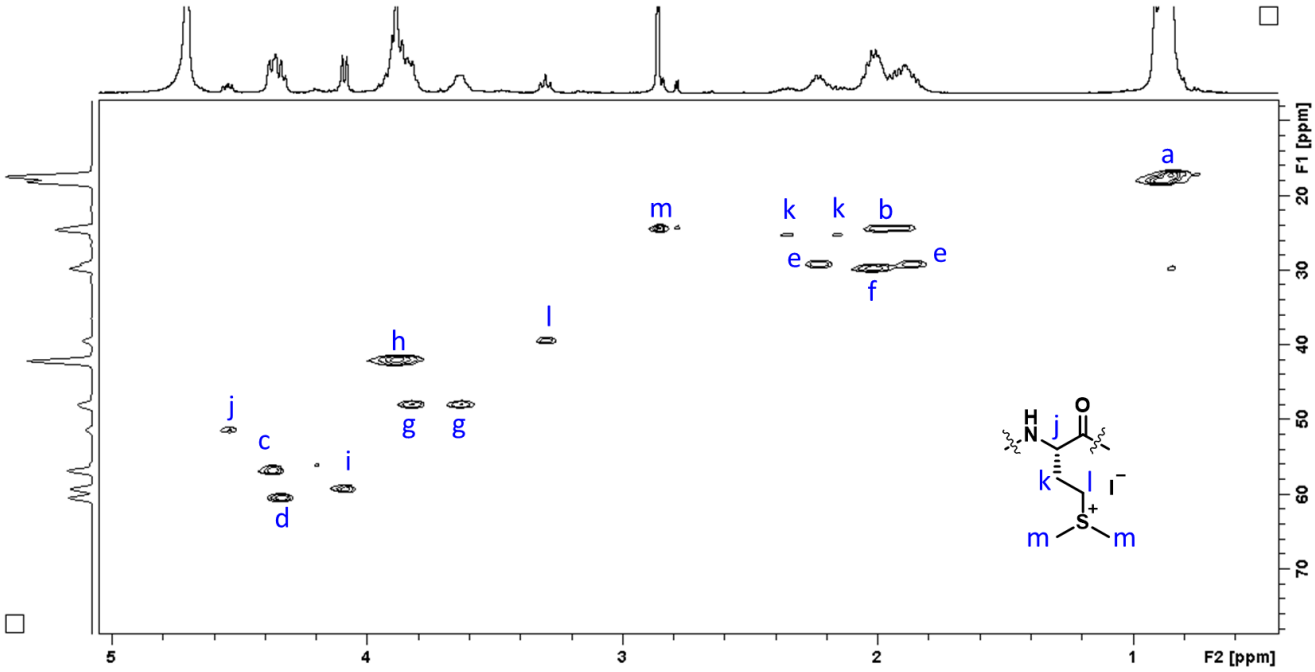


Figure S4. 2D NMR spectrum of compound 1A in D_2O, HSQC

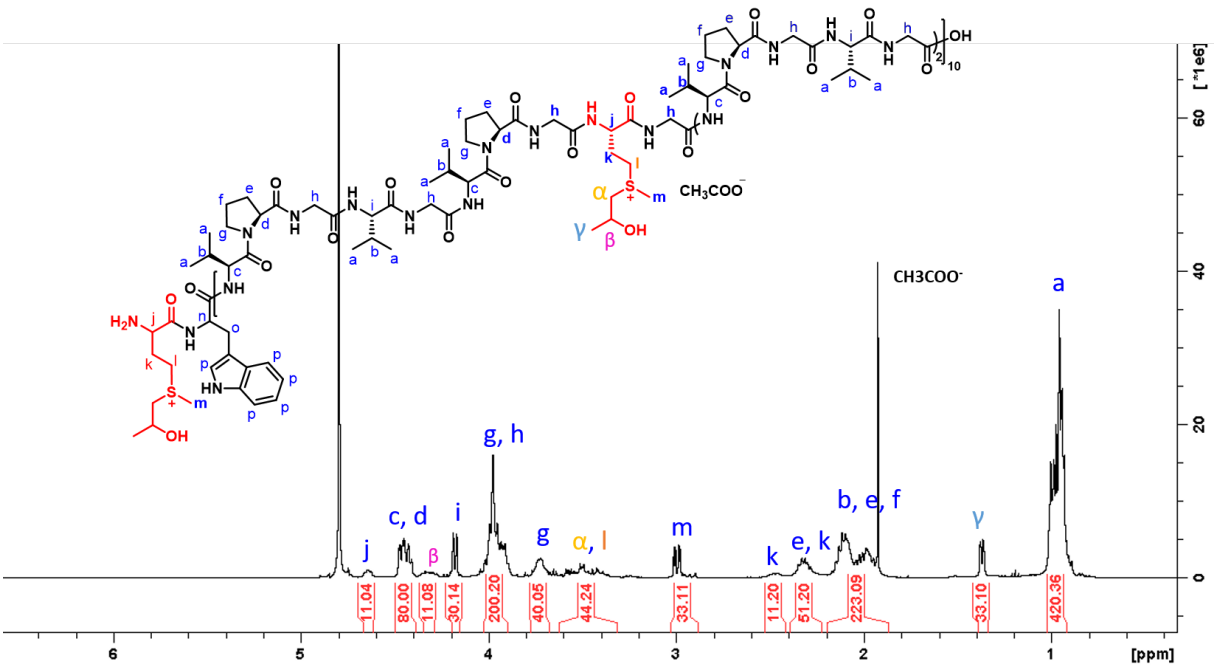


Figure S5. ^{1}H NMR spectrum of compound 1B in D_2O

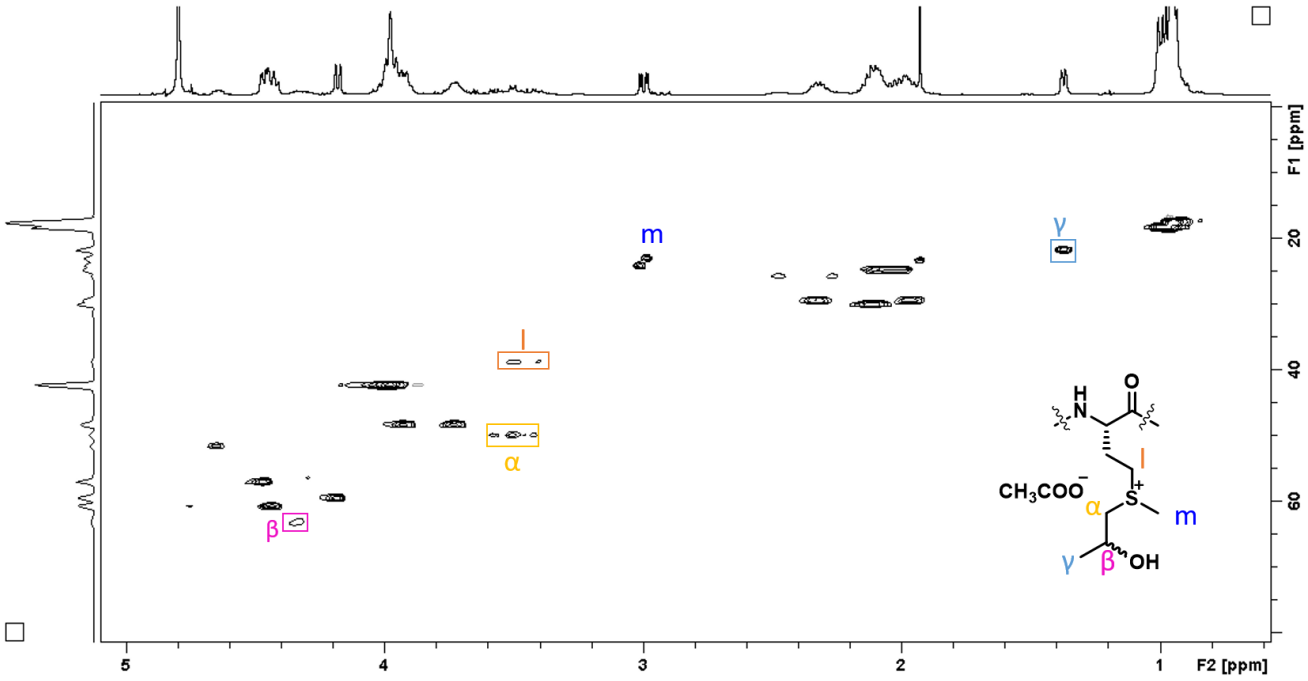


Figure S6. 2D NMR spectrum of compound 1B in D_2O, HSQC

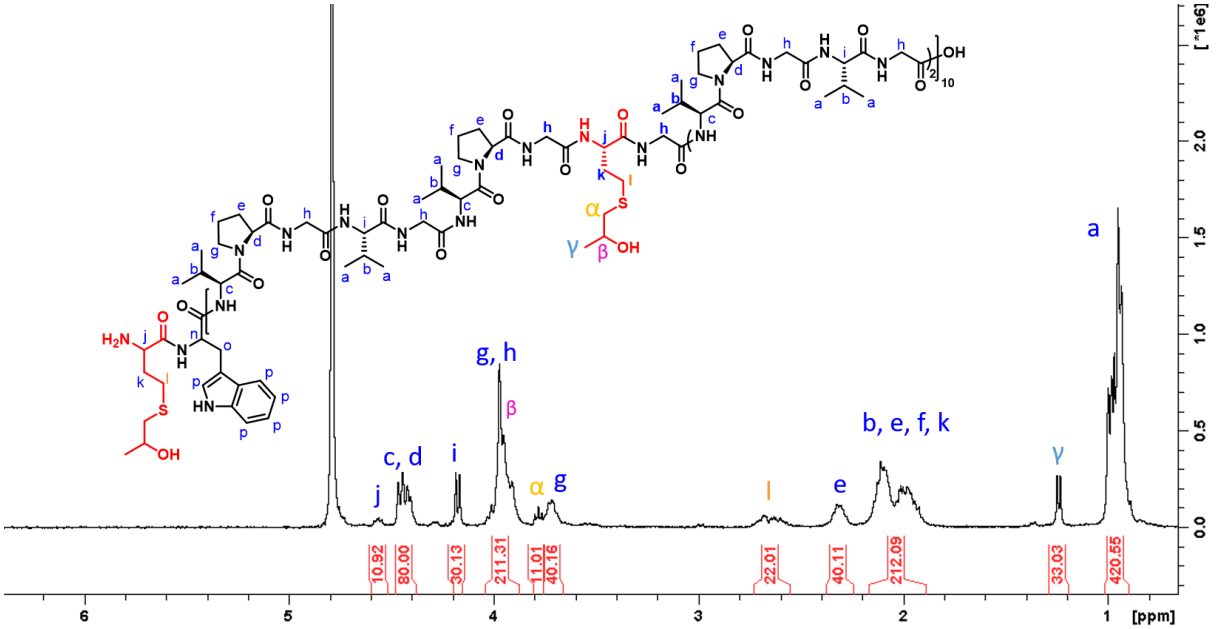


Figure S7. ^{1}H NMR spectrum of compound 1D in D_2O

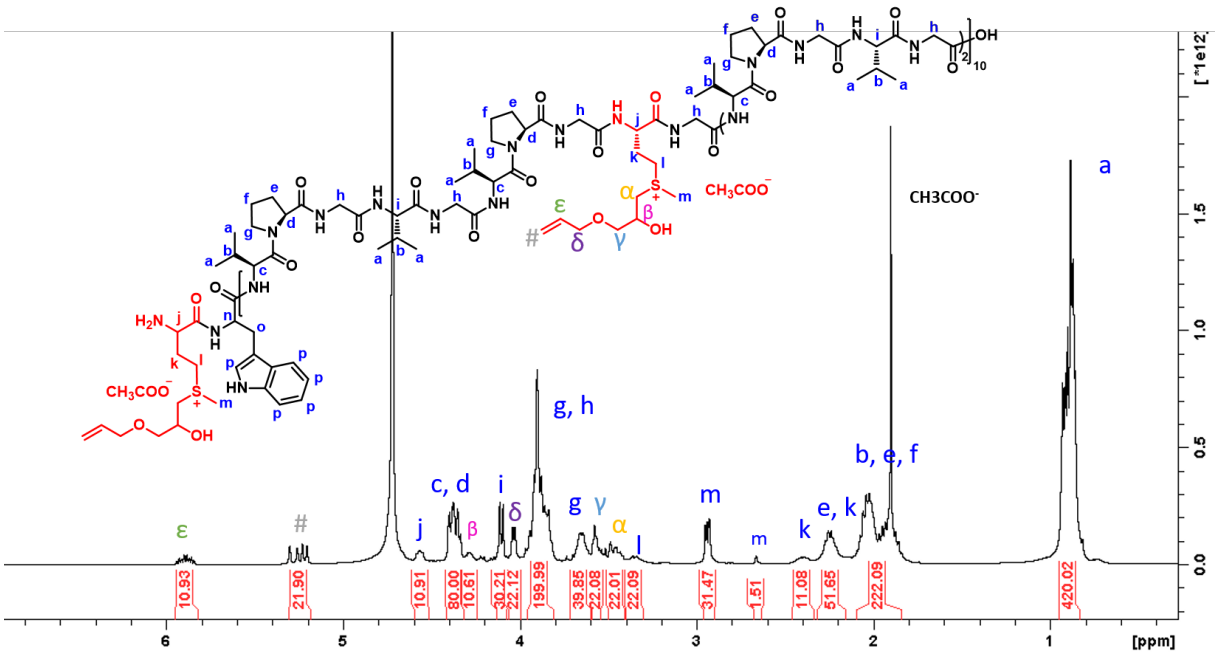


Figure S8. ^{1}H NMR spectrum of compound 3B in D_2O

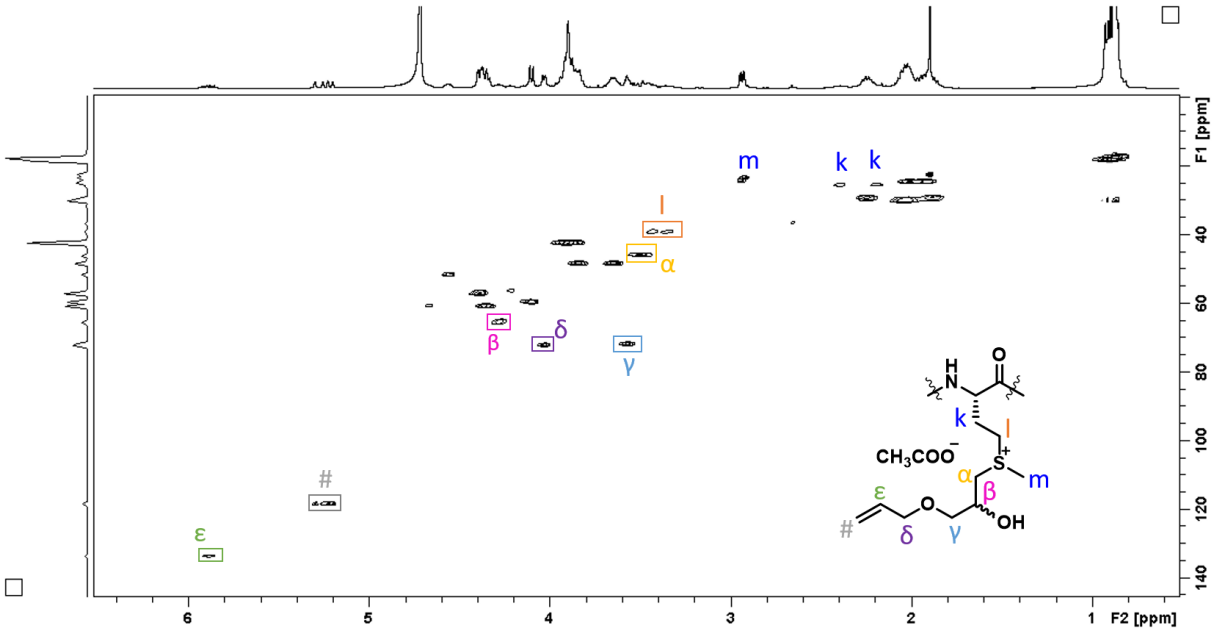


Figure S9. 2D NMR spectrum of compound 3B in D_2O, HSQC

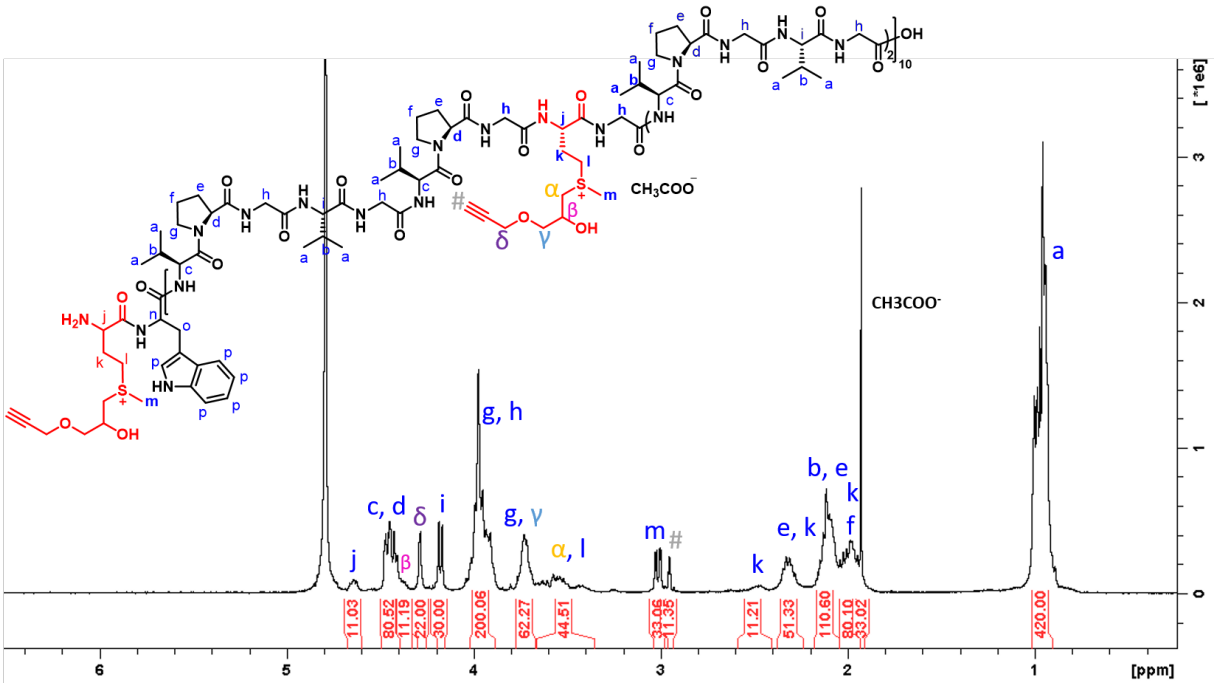


Figure S10. ^1H NMR spectrum of compound 2B in D_2O

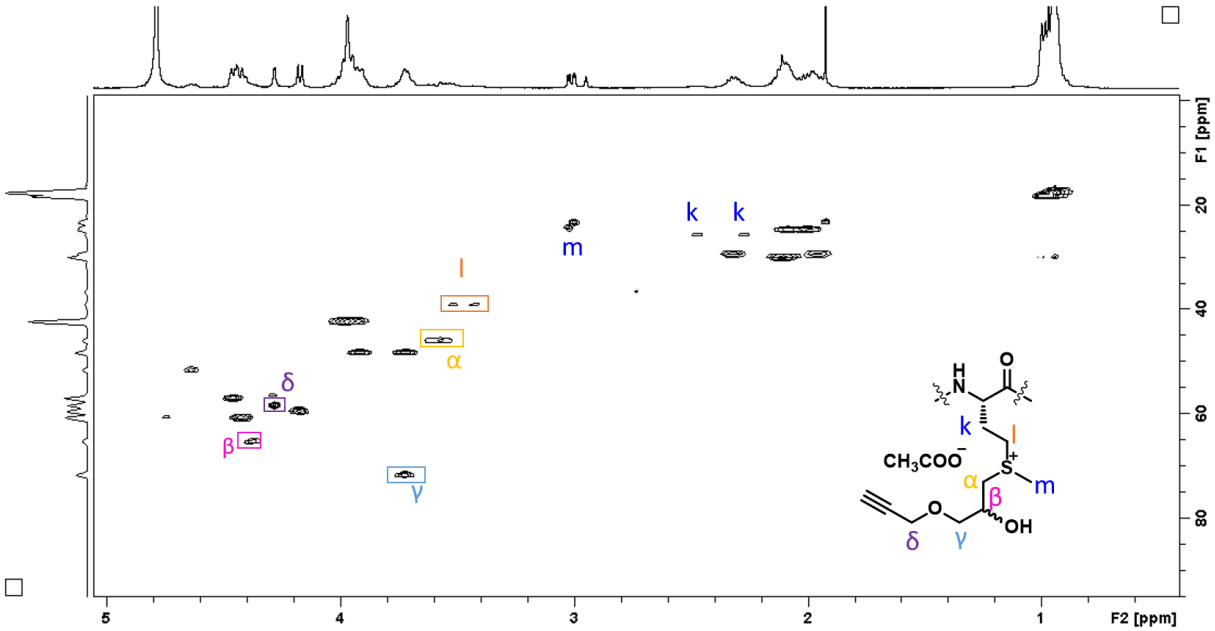


Figure S11. 2D NMR spectrum of compound 2B in D_2O, HSQC

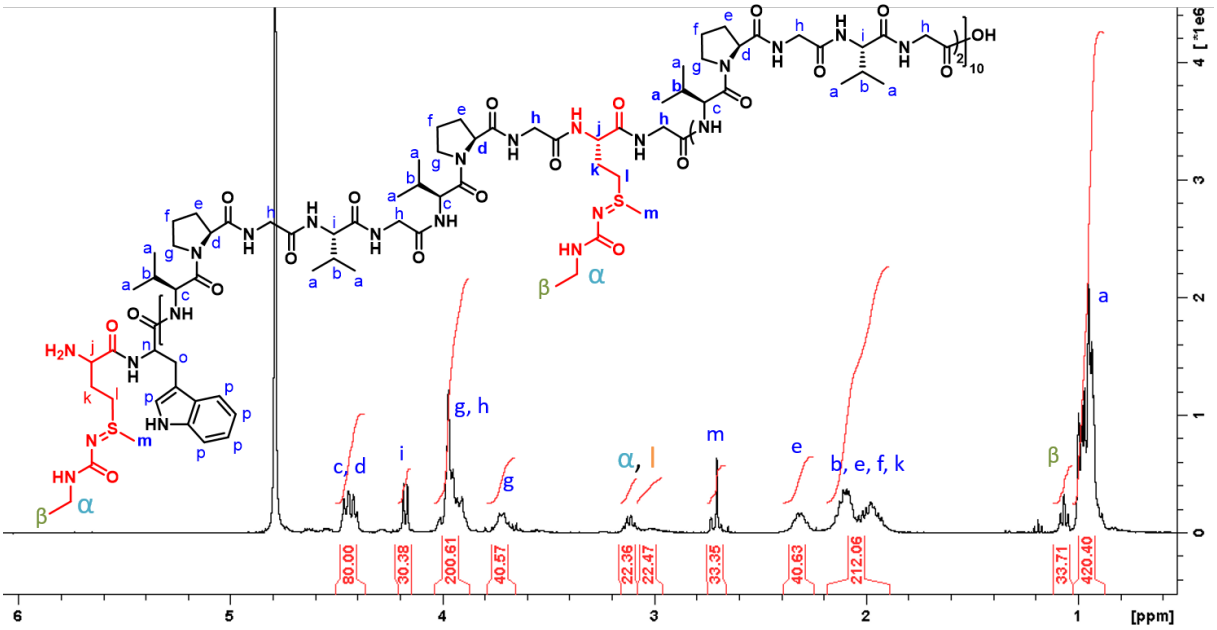


Figure S12. ^1H NMR spectrum of compound 1C in D_2O

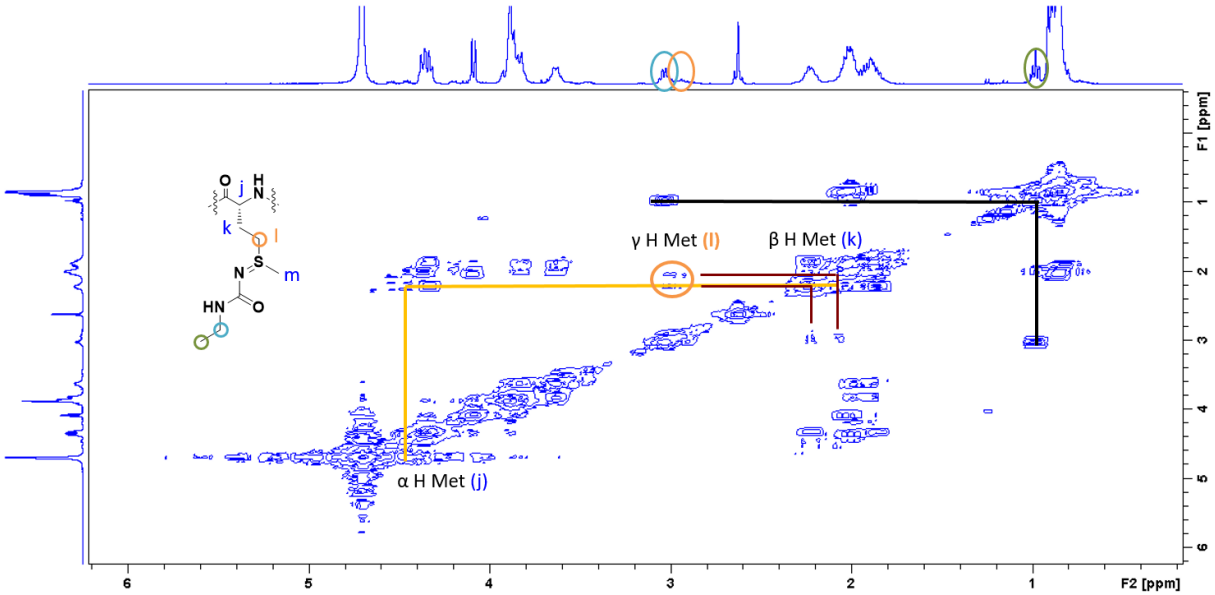


Figure S13. 2D NMR spectrum of compound 1C in D_2O, COSY

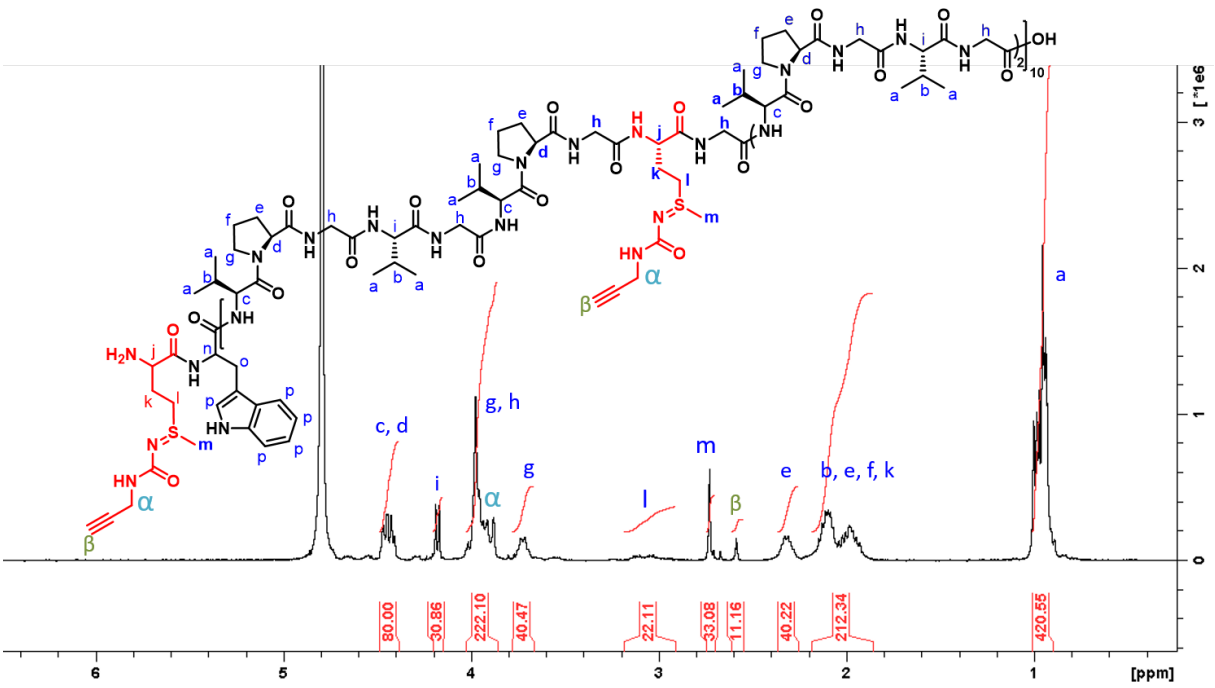


Figure S14. ^{1}H NMR spectrum of compound 2C in D_2O

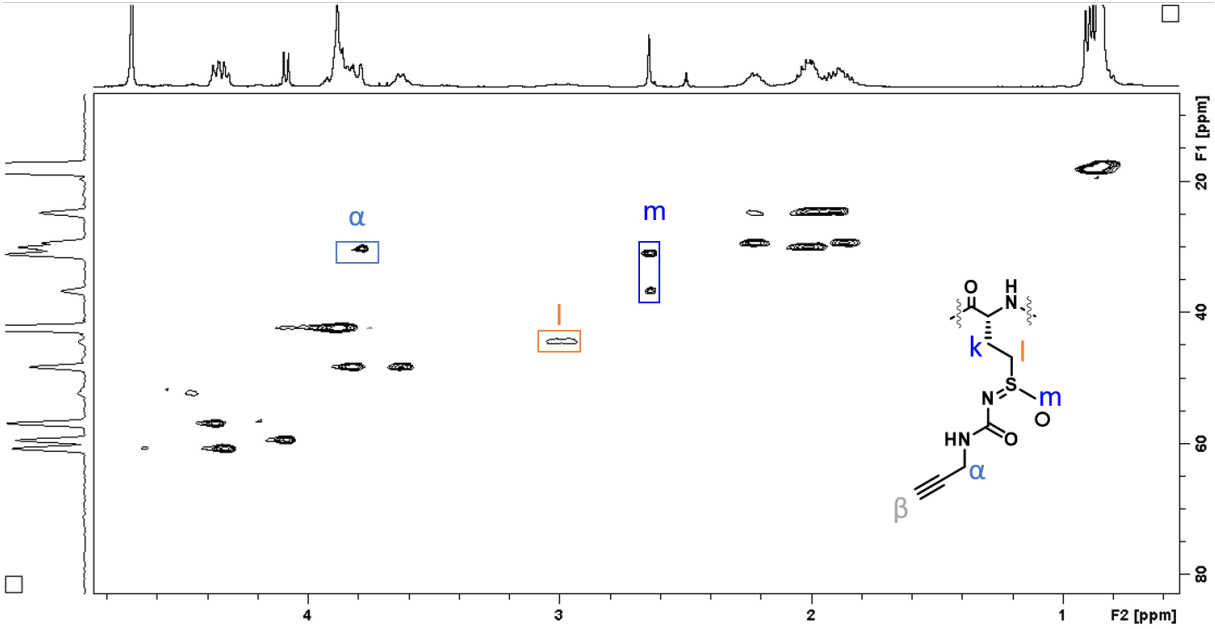


Figure S15. 2D NMR spectrum of compound 2C in D_2O, HSQC

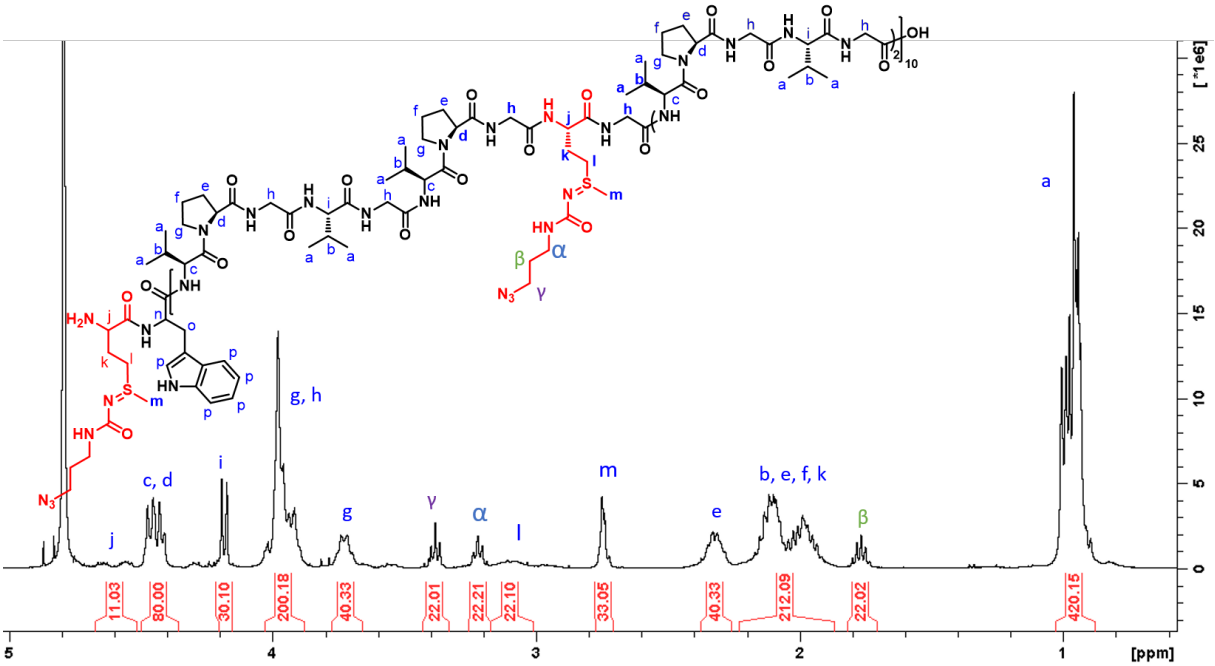


Figure S16. ^1H NMR spectrum of compound 3C in D_2O

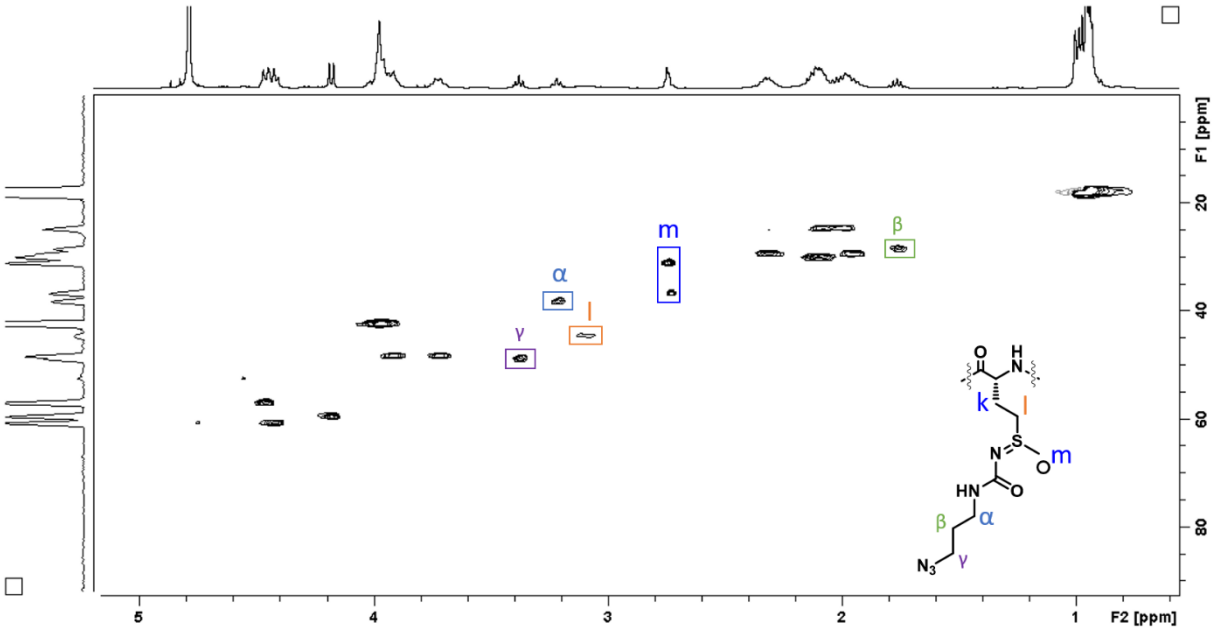


Figure S17. 2D NMR spectrum of compound 3C in D_2O, HSQC

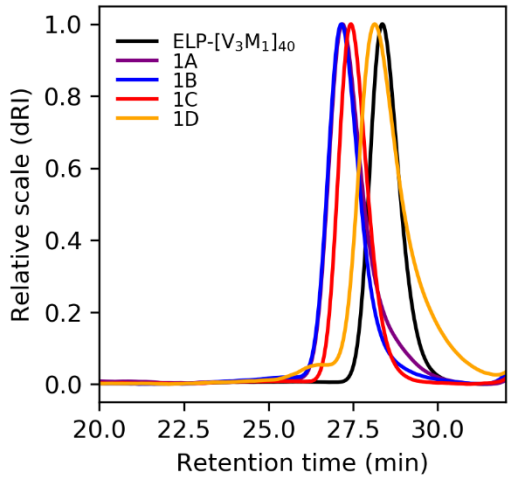


Figure S18. Size exclusion chromatography of ELP[M_1V_3-40], compound 1A, 1B, 1C and 1D in AcOH/Ammonium Acetate/ACN eluent.

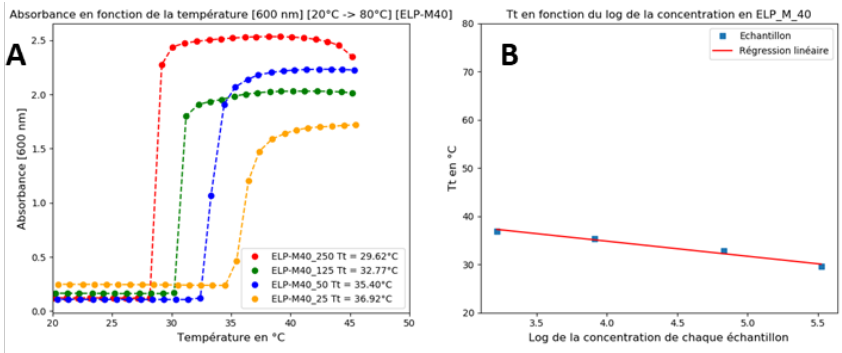


Figure S19. (A) Absorbance of 125 µM solutions of ELP[V_3M_1-*40*] in Tris buffer as function of temperature. (B) Tt values of ELP[V_3M_1-*40*], plotted as functions of sample concentration in Tris buffer; data fitted using Chilokotti equation.

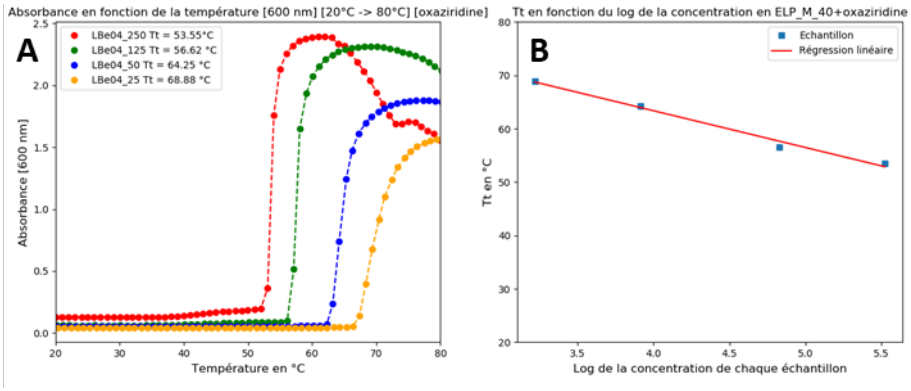


Figure S20. (A) Absorbance of 125 µM solutions of compound 1C in Tris buffer as function of temperature. (B) Tt values of compound 1C, plotted as functions of sample concentration in Tris buffer; data fitted using Chilokotti equation.

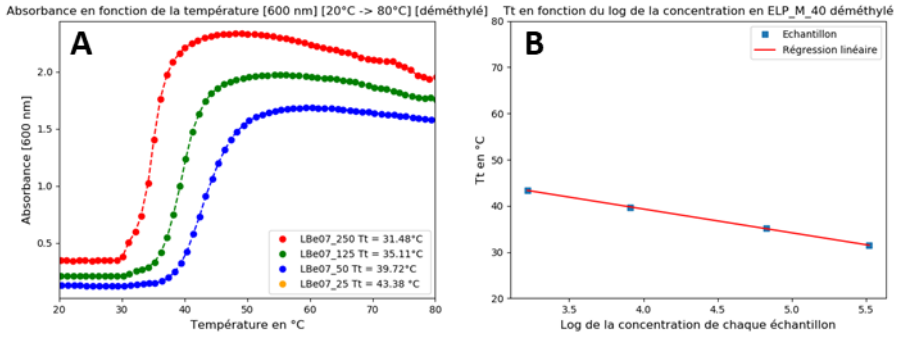


Figure S21. (A) Absorbance of 125 µM solutions of compound 1D in Tris buffer as function of temperature. (B) Tt values of compound 1D, plotted as functions of sample concentration in Tris buffer; data fitted using Chilokotti equation.

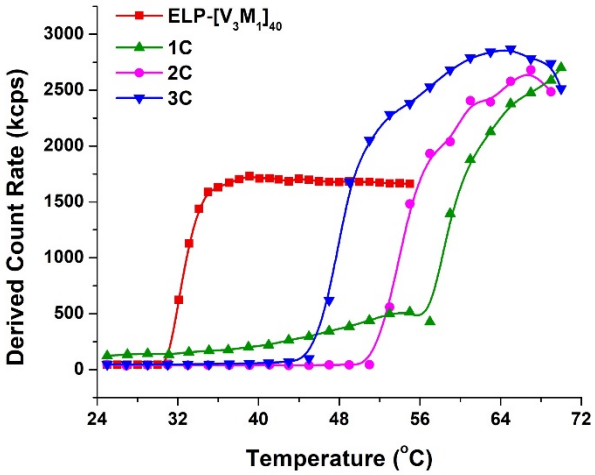
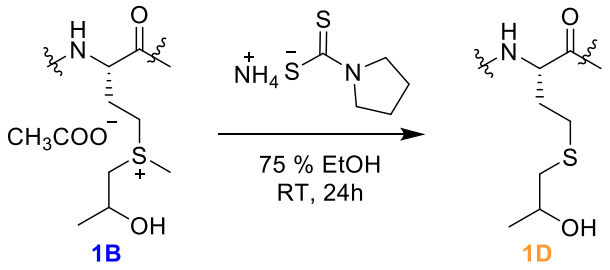


Figure S22. Scattered light intensity of ELP[V_3M_1-*40*], compound 1C, 2C and 3C at 125 µM in Mili-Q water as function of temperature.

Concentrations	ELP-$[V_3M_1]_{40}$	1A	1B	1C	1D
25 µM	37°C	> 80°C	> 70°C	69°C	43°C
50 µM	35°C	> 80°C	> 70°C	64°C	40°C
125 µM	33°C	> 80°C	> 70°C	57°C	35°
250 µM	30°C	> 80°C	> 70°C	54°C	31°C

Table S1. Table summarizing the onset temperature of aggregation (Tt) of the derivatives at 125 µM in Tris buffer.



Scheme S1. Demethylation reaction conditions of compound 1B to obtain compound 1D.

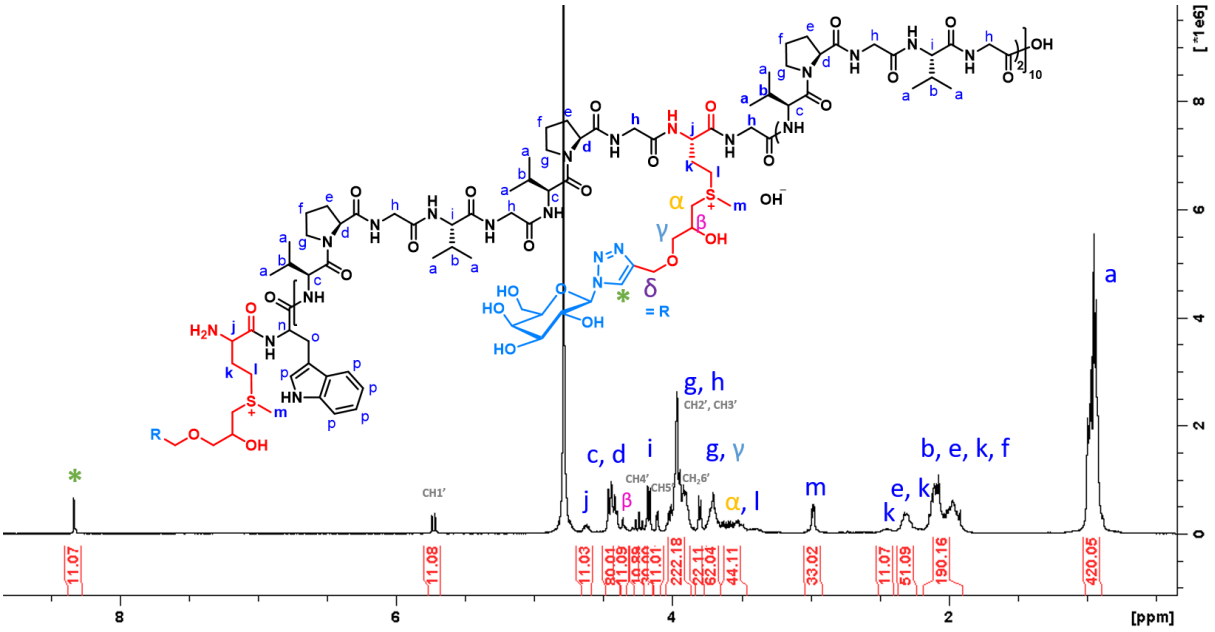


Figure S23. ^{1}H NMR spectrum of compound 4B in D_2O

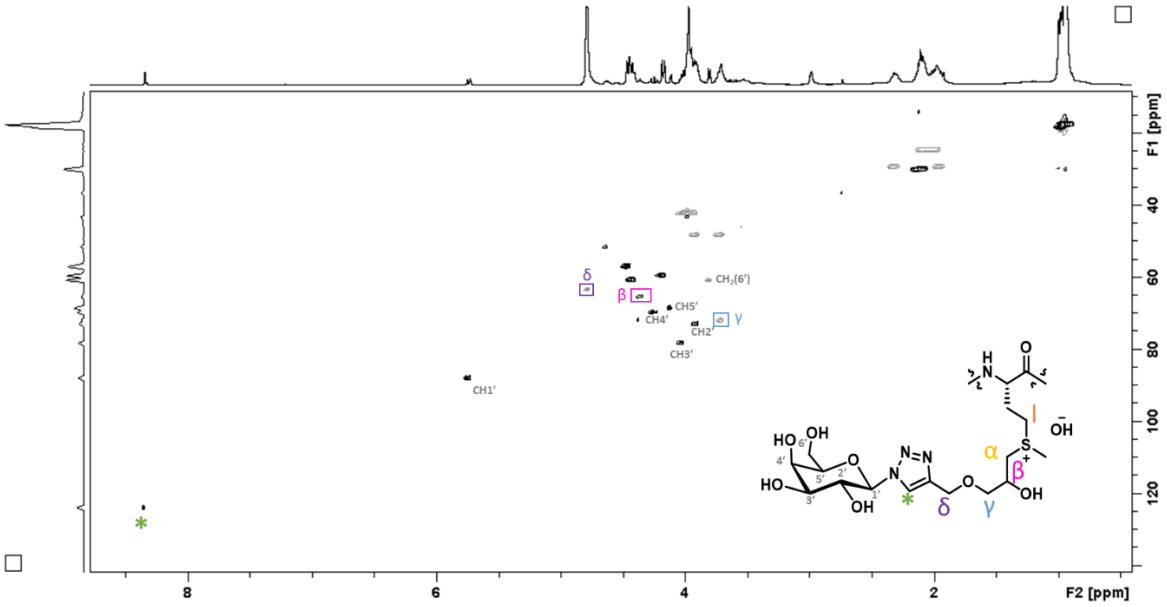


Figure S24. 2D NMR spectrum of compound 4B in D_2O, HSQC

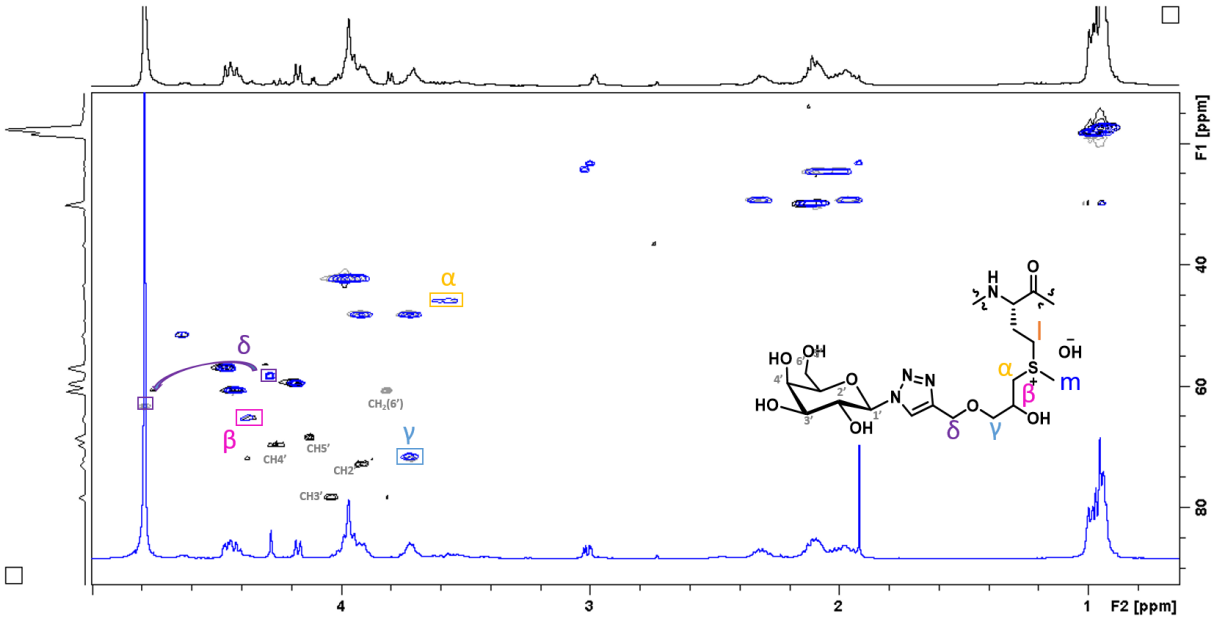


Figure S25. 2D NMR spectrum of compound 4B (in black) and compound 2B (in blue) in D_2O, HSQC

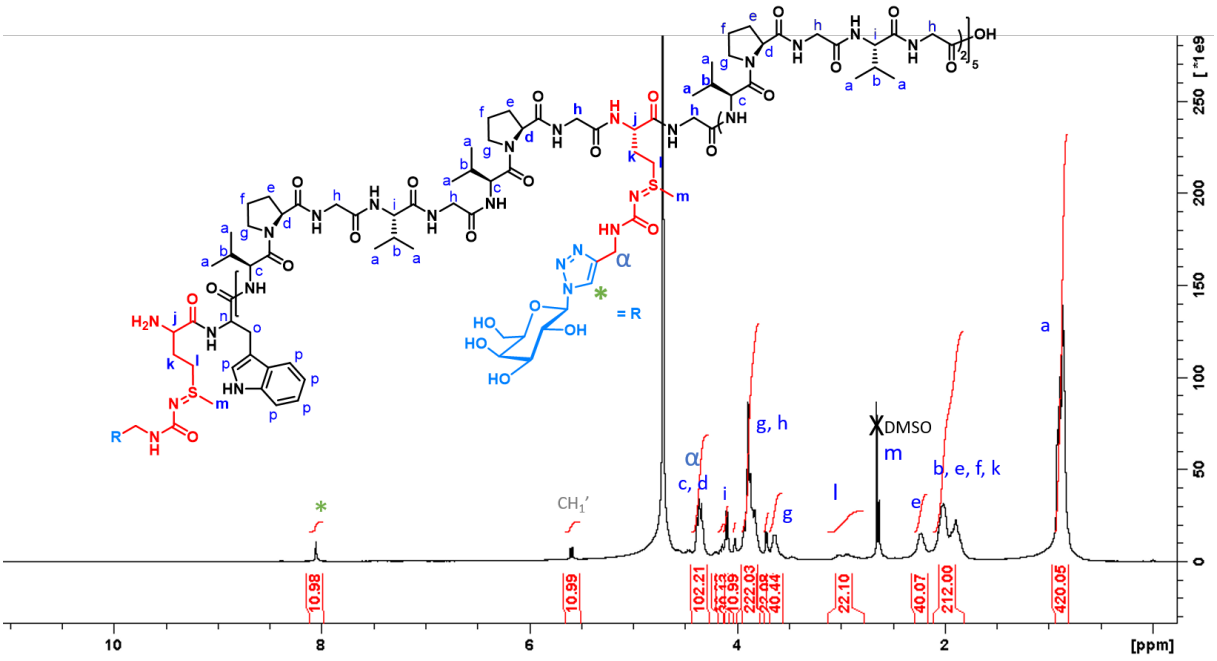


Figure S26. ^{1}H NMR spectrum of compound 4C in D_2O

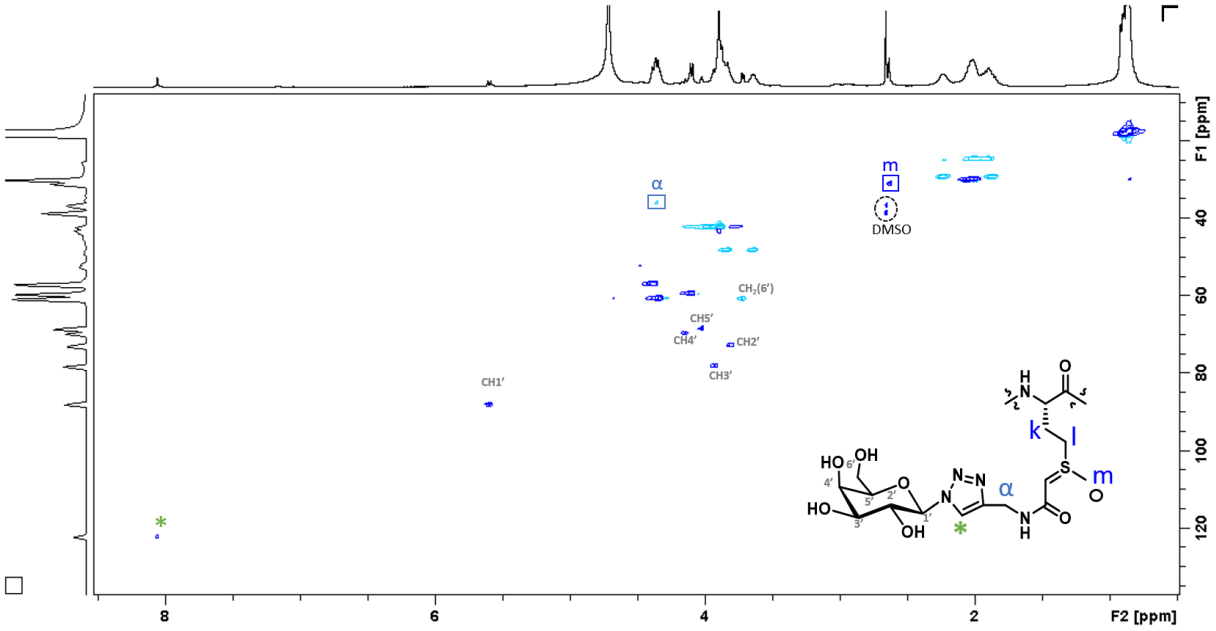


Figure S27. 2D NMR spectrum of compound 4C in D_2O

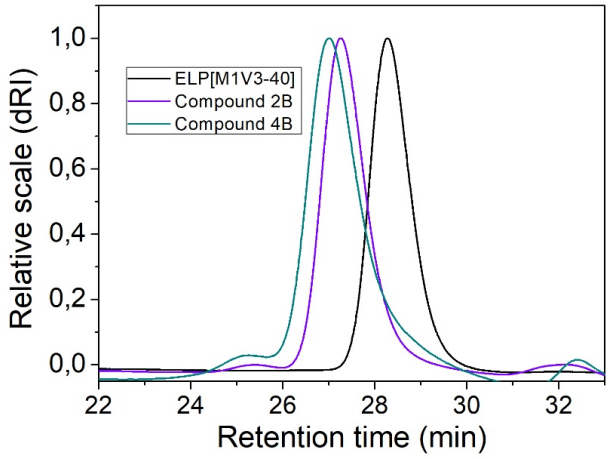


Figure S28. Size exclusion chromatography of ELP[M_1V_3-40], compounds 2B and 4B in AcOH/Ammonium Acetate/ACN eluent.

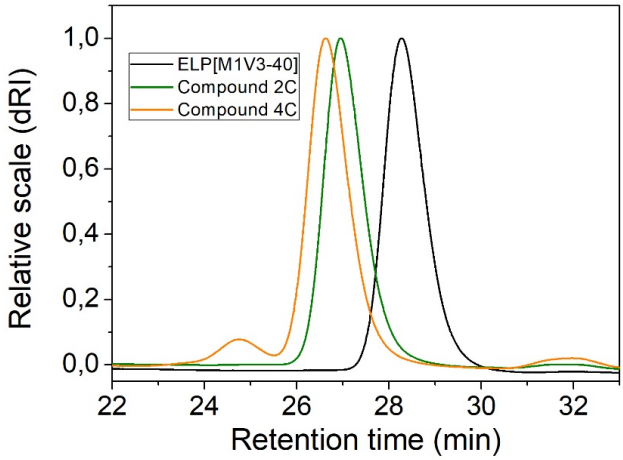


Figure S29. Size exclusion chromatography of ELP[M_1V_3-40], compounds 2C and 4C in AcOH/Ammonium Acetate/ACN eluent.

IV. Reference

(1) Lin, S.; Yang, X.; Jia, S.; Weeks, A. M.; Hornsby, M.; Lee, P. S.; Nichiporuk, R. V.; Iavarone, A. T.; Wells, J. A.; Toste, F. D.; Chang C. J. Redox-Based Reagents for Chemoselective Methionine Bioconjugation. *Science* 2017, *355* (6325), 597–602. https://doi.org/10.1126/science.aal3316.