Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

Supporting Information

Biomolecular Dynamic Covalent Polymers for DNA complexation and siRNA delivery

Camille Bouillon,[1] Yannick Bessin,[1] Florian Poncet,[1] Magali Gary-Bobo,[1] Pascal Dumy,[1] Mihail Barboiu,[2] Nadir Bettache,[1]* and Sébastien Ulrich[1]*

[1] IBMM, Université de Montpellier, CNRS, ENSCM, UM, Montpellier, France.
E-mail: nadir.bettache@umontpellier.fr; Sebastien.Ulrich@enscm.fr
[2] IEM, Adaptive Supramolecular Nanosystems Group, Université de Montpellier, CNRS, ENSCM, UM, Montpellier, France.

1. General procedures and materials

All solvents and reagents were purchased from Acros Organics, Alfa Aesar, Fluka or Sigma-Aldrich and used without further purifications. For dichloromethane, amylene was the stabilizer. The activated ester **4** was prepared using reported procedures.[1, 2]

TLC. TLC analyses were performed on Merck silica gel $60F_{254}$, with detection under UV light or treatment with ninhydrine solution in ethanol followed by heating. Flash chromatography was performed on silica gel (40-63 µm) purchased from Merck.

NMR. Nuclear Magnetic Resonance (^{1}H NMR, ^{13}C NMR and DOSY-NMR) spectra were recorded at 250 MHz for proton and 63 MHz for carbon (Bruker Avance 250), 400 MHz for proton and 100 MHz for carbon (Bruker Avance 400) and 600 MHz for proton and 150 MHz for carbon (Bruker Avance III) using deuterated chloroform, methanol or dimethyl sulfoxide. Chemical shifts are reported in ppm relative to the residual solvent peak. Data are reported as follows: Chemical shifts (δ), multiplicity (s for singlet, d for doublet, t for triplet, q for quartet, quint for quintuplet and m for multiplet), integration and coupling constant (J in Hertz). DOSY NMR was carried out at the Laboratoire de Mesures Physiques, IBMM-Université de Montpellier.

LCMS. LC/MS analyses were performed on a Shimadzu LCMS2020 (Phenomex Kinetex C_{18}, 2.6 µm * 7.5 cm, 100 Å) equipped with a SPD-M20A detector with the following linear gradient of solvent B (99.9% acetonitrile, 0.1% HCOOH) and solvent A (99.9% water and 0.1% HCOOH): 5 to 95% of solvent B in 5 min; flow 1 ml/min. Retention times (t_R) are given in minutes.

Mass spectrometry. MALDI-TOF and High Resolution mass Spectrometry (HRMS) analyses (positive mode) were carried out in the Laboratoire de Mesures Physiques, IBMM-Université de Montpellier using, respectively, Ultraflex III and Micromass Q-Tof instruments. Fluorescence analyses were carried out on a HITACHI fluorescence spectrophotometer F-2500.

Gel permeation chromatography (GPC). GPC was performed with a Waters equipment fitted with a 30 cm long 5 µm mixed C PLgel column as stationary phase, DMF at 1 $mL.min^{-1}$ flow rate at 60°C as the mobile phase and a Waters 410 refractometric detector. The polymer (10 mg) was dissolved in DMF (2 mL) and the resulting solution was filtered on a 0.45 µm

Millipore filter before injection of 30 µL of sample solution. M_n and Đ were expressed according to calibration using polystyrene standards.

Ethidium bromide displacement assay. Samples were prepared in 3 mL quartz cuvette. Excitation wavelength was set at 546 nm and emission was measured at 590 mm. Samples were prepared in HEPES buffer (100 mM, pH 7.0; 10 µM EDTA; 150 mM NaCl). Ethidium bromide was dissolved in the buffer to provide 42 µM concentration and after standing 1 min the fluorescence was measured. Calf-thymus DNA (ctDNA) (Sigma-Aldrich) was added from a solution in MilliQ H_2O to provide a final concentration of 50 µM. Polymers solutions (100 mM stock solutions) were added in microliter portions. The fluorescence measurement was recorded after shaking the solution for a few seconds. The relative fluorescence emission was calculated as follows:

$$\text{Relative fluorescence emission (\%)} = (I_{EthBr\text{-}DNA} - I) / (I_{EthBr\text{-}DNA} - I_{EthBr})$$

where $I_{EthBr\text{-}DNA}$ represents the fluorescence emission of the EthBr-ctDNA intercalation complex, I represents the measured fluorescence emission, and I_{EthBr} represents the fluorescence emission of EthBr in the absence of DNA.

Electrophoretic mobility shift assay (EMSA) with pDNA. 100 ng of 5.7 kilobase pair expression vector, pET-15b (Novagen) were mixed with appropriate amounts of polymer to achieve the desired N/P ratio, and diluted in TAE buffer (20 mM Tris -acetate / 0.5 mM EDTA pH 8.2) to obtain a final volume of 10 µL. 2 µL of Blue 6X loading dye (Fisher Scientific) was added, after which 12 µL was run on a 0.7% wt/vol agarose gel (50V) in the same TAE buffer. DNA was visualized with SYBER Safe (Life Technologies).

Gel electrophoresis with siRNA. Gel retardation assays with siRNA were performed using 10 µL of a 4 µM siRNA Luciferase targeting sequence (sense: 5'-AACUUACGCUGAGUACUUCGA-3' and anti-sense 5'-UCGAAGUACUCAGCGUAAGUU-3'), which were mixed with the appropriate amounts of DCPs (in order to reach the desired N/P ratio) in RNase free water to obtain a final volume of 20 µL. 4 µL of Blue 6X loading dye (Fisher Scientific) was added. Electrophoresis was carried out on a 2 % wt/vol agarose gel in 1× TBE (90 mM Tris-borate/2 mM EDTA, pH 8.2) at 50 V for 1 h. siRNA was visualized with GelRed™ nucleic acid gel stain (Interchim, France). The reference for the gel is a 100 bp DNA ladder from Sigma-Aldrich (Saint-Quentin-Fallavier,

France). The GelRed-stained siRNA was visualized on an ultraviolet transilluminator (Infinity Gel documentation Imaging, Vilber Lourmat, France).

Dynamic light scattering. Particle size measurements were carried out on a Zetasizer Nano ZS (Malvern, United Kingdom) with transparent ZEN0040 disposable microcuvette cells (40 µl) at 25°C. The DCP/siRNA complexes were prepared in phosphate buffer (100 mM, pH = 7.0) at a defined N/P ratio using a siRNA Luciferase targeting sequence (sense: 5'-AACUUACGCUGAGUACUUCGA-3' and anti-sense 5'-UCGAAGUACUCAGCGUAAGUU-3') at a concentration of 1 µM. Measurements were performed immediately after the complexes formation and after 1 h incubation.

Zeta Potential. Zeta Potential analyses were carried out on a MALVERN Zetasizer Nano series NANO-ZS instrument. The polymer solutions at 200 mM were diluted to 5 mM solution at DMSO, and siRNA solution used was at 20 µM. The polymer/siRNA complexes were prepared by mixing appropriate amounts of polymer and siRNA to achieve the desired N/P ratio and diluted in phosphate buffer (100 mM, pH = 7.0) to obtain a final volume of 1000 µL. Measurements were performed at 25°C and were carried out in zeta potential cuvette. 3 measurements were made with 12 runs for each.

Biological evaluation on cell culture. The MCF7-luc cell line derived from MCF7 human breast cancer cells by stable transfection of firefly luciferase gene (PCDNA 3.1 CMV-Luc-SVNeo) were generously provided by Dr P. Balaguer (ICM Montpellier, France). Selection of resistant clones was performed by geneticin addition at 1 mg.mL-1 until experiments. Cells were grown in phenol red-free F12/ Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum (FCS). The cells were incubated at 37°C in a humidified atmosphere with 5% CO2. Cy5-labeled non-coding siRNA and the siRNA targeting sequence (sense: AACUUACGCUGAGUACUUCGA and anti-sense UCGAAGUACUCAGCGUAAGUU) for luciferase were purchased from Eurogentec (Serring, Belgium). The MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) assay kit was purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). The DCP/siRNA complexes were prepared as follows: the DCPs (20 mM in filtered RNase-free water, prepared from a 200 mM stock solution in DMSO) were vortexed for 5 s, ultrasonically mixed for 5 min, mixed with siRNA in order to reach the desired N/P, and incubated at room

temperature for 20 min.

Cell luciferase assay. MCF7-luc cells were seeded at a density of 5000 cells per well in 96-well white opaque tissue culture plates in 150 µl complete culture medium and incubated for 24 h. The cells were then washed with PBS and incubated with various DCP/siRNA complex formulations in OptiMEM at 37°C for 4 h. Thereafter, 50 µL of 40% serum containing medium was added. Two days after transfection, expression of luciferase was assessed by addition into culture medium of luciferin (10^{-3} M, final concentration) purchased from Promega (France). Living cell luminescence was measured 10 min after by a plate reader CLARIOstar® High Performance Monochromator Multimode Microplate Reader (BMG Labtech, Ortenberg, Germany).

2. Synthetic procedures and characterizations

2.1. General procedure I for the synthesis of protected amino acid hydrazide P_1-AA(P_2)-Hyd-Boc **1a-1c**

Tert-butyl carbazate (1 equiv.) and 1-hydroxybenzotriazole hydrate (HOBt, 1.5 equiv.) were added to a solution of P_1-AA(P_2)-OH (1 equiv.) and triethylamine (1.1 equiv.) in dry dichloromethane (190 mM). Then, the solution was cooled to 0°C and *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC, 1.5 equiv.) was added. The reaction mixture was then warmed to room temperature and stirred overnight. After removal of the solvent, the residue was diluted with EtOAc (100 mL), washed with a saturated $NaHCO_3$ solution (30 mL) and brine (30 mL), dried over anhydrous Na_2SO_4, and concentrated in vacuo. The resulting crude material was purified by flash chromatography to provide P_1-AA(P_2)-Hyd-Boc **1a**-**1c** as a white solid.

- ***Tert*-butyl 2-{2-[((benzyloxy)carbonyl)amino]acetyl}hydrazinecarboxylate 1a:**

Cbz-Gly-Hyd-Boc was synthesized according to the general procedure I using Cbz-Gly-OH (5 g, 23.90 mmol), *tert*-butyl carbazate (3.16 g, 23.90 mmol), HOBt (4.84 g, 35.85 mmol), triethylamine (3.65 mL, 26.29 mmol) and EDC (6.87 g, 35.85 mmol). The compound **1a** was obtained after purification by flash chromatography (cyclohexane/EtOAc gradient 5:5 to 2:8, v/v) with 72 % yield. R_f = 0.47

(cyclohexane/EtOAc 5:5, v/v); t_R = 3.10 min (Method A); ^{1}H NMR (400 MHz, DMSO-d_6): δ = 9.61 (s, 1H, *NH*-NH-Boc), 8.75 (s, 1H, NH-*NH*-Boc), 7.50-7.47 (m, 1H, *NH*-Cbz), 7.36-7.35 (m, 5H, *CH*=*CH*), 5.03 (s, 2H, O*CH*$_2$), 3.71-3.62 (m, 2H, NH*CH*$_2$), 1.42-1.39 (s, 9H, OC(*CH*$_3$)$_3$); ^{13}C NMR (100 MHz, DMSO-d_6) : δ = 168.8, 156.4, 155.1, 137.0, 128.3, 127.7, 127.6, 79.0, 65.4, 41.8, 28.0; HRMS (ESI): *m/z* calcd for $C_{15}H_{22}N_3O_5^+$ [M+H$^+$] 324.1559, found 324.1561.

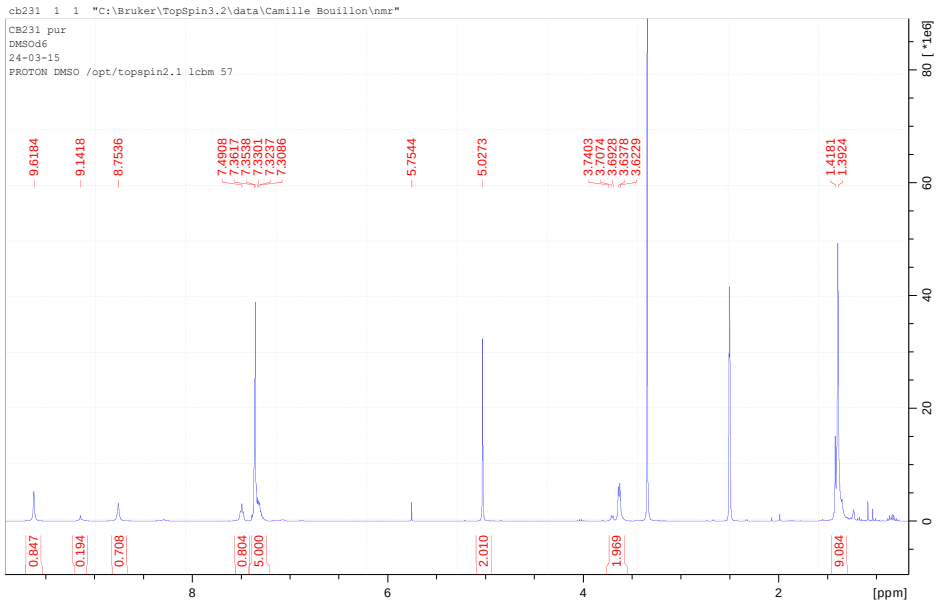


Figure S1: ^{1}H NMR spectrum of Cbz-Gly-Hyd-Boc **1a**

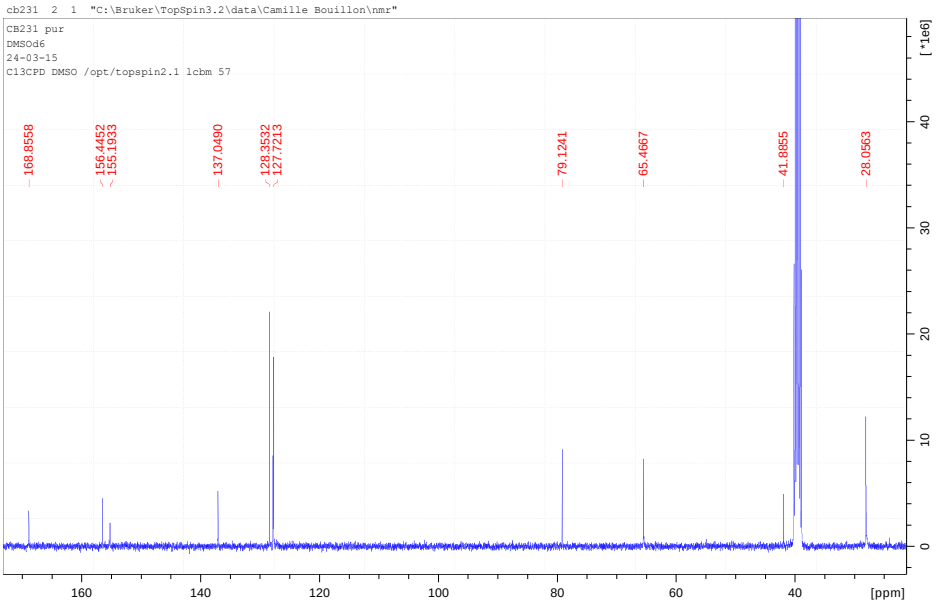


Figure S2: ^{13}C NMR spectrum of Cbz-Gly-Hyd-Boc **1a**

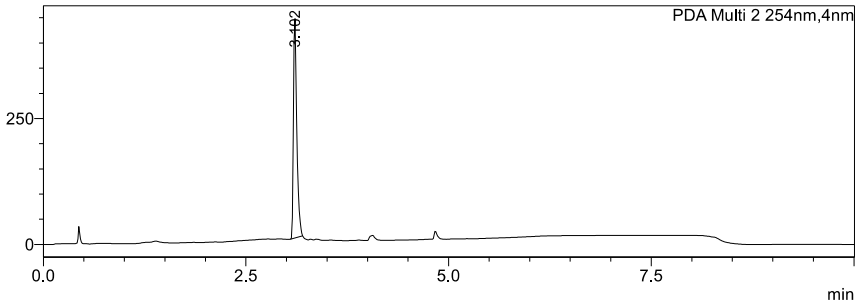


Figure S3: HPLC chromatogram of Cbz-Gly-Hyd-Boc **1a**

- ***Tert*-butyl-2-{2-[(((9H-fluoren-9-yl)methoxy)carbonyl)amino]-5-[3-((2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl)guanidino]pentanoyl}hydrazinecarboxylate 1b :**

Fmoc-Arg(Pbf)-Hyd-Boc was synthesized according to the general procedure I using Fmoc-Arg(Pbf)-OH (10 g, 15.41 mmol), *tert*-butyl carbazate (2.04 g, 15.41 mmol), HOBt (3.12 g, 23.12 mmol), triethylamine (2.96 mL, 16.96 mmol) and EDC (4.43 g, 23.12 mmol). The compound **1b** was obtained after purification by flash chromatography (cyclohexane/EtOAc gradient 3:7 to 2:8, v/v) with 88 % yield. R_f = 0.49 (cyclohexane/EtOAc 3:7, v/v); t_R = 4.77 min (Method A); ^{1}H NMR (400 MHz, DMSO-d_6): δ = 9.68 (s, 1H, *NH*-NH-Boc), 8.76 (s, 1H, NH-*NH*-Boc), 7.88 (d, 2H, ^3J = 7.6 Hz, C*CH*=CH), 7.74-7.71 (m, 2H, C*CH*=CH), 7.54 (d, 1H, ^3J = 8.4 Hz, *NH*-Fmoc), 7.43-7.39 (m, 2H, *CH*=CH), 7.34-7.31 (br, 2H, CH=*CH*), 4.86-4.21 (m, 3H, O*CH$_2$CH*), 4.05-4.00 (br, 1H, *CH*CH$_2$CH$_2$), 3.08-3.00 (m, 2H, *CH$_2$*NHC), 2.94 (s, 2H, *CH$_2$*C(CH$_3$)$_2$), 2.5 (s, 3H, *CH$_3$*), 2.43 (s, 3H, *CH$_3$*), 1.99 (s, 3H, *CH$_3$*), 1.69-1.27 (m, 4H, CH*CH$_2$CH$_2$*), 1.39 (s, 15H, OC(*CH$_3$*)$_3$ + CH$_2$C(*CH$_3$*)$_2$); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 171.7 (2C), 157.3, 155.9, 155.7, 143.8, 140.6, 137.1, 134.3, 131.3, 127.5, 126.9, 125.2, 124.2, 120.0, 116.1, 86.1, 79.0, 65.6, 52.6, 46.5, 42.3, 40.0, 28.9, 28.2 (2C), 25.4, 18.8, 17.5, 12.2; HRMS (ESI): *m/z* calcd for $C_{39}H_{51}N_6O_8S^+$ [M+H$^+$] 763.3489, found 763.3494.

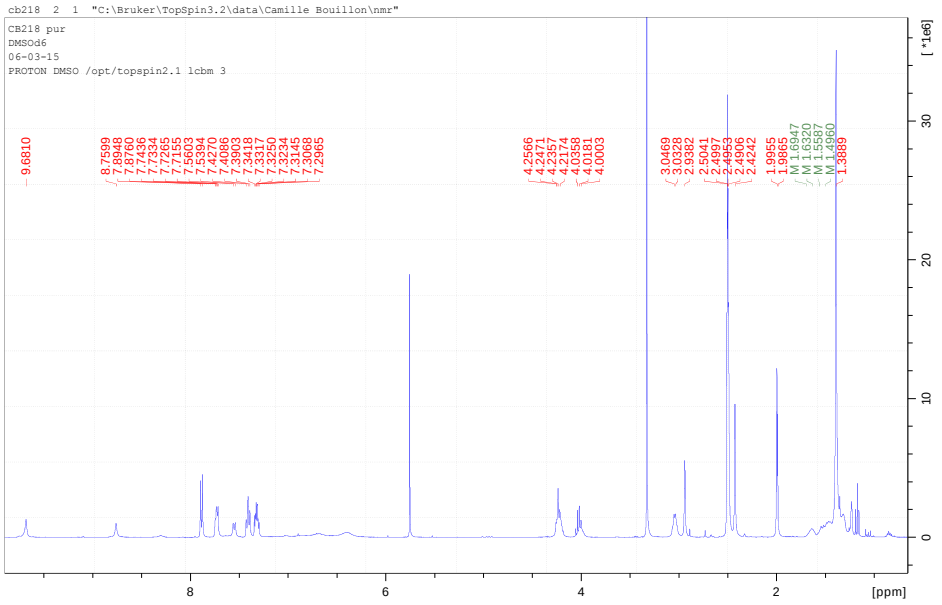


Figure S4: ^{1}H NMR spectrum of Fmoc-Arg(Pbf)-Hyd-Boc **1b**

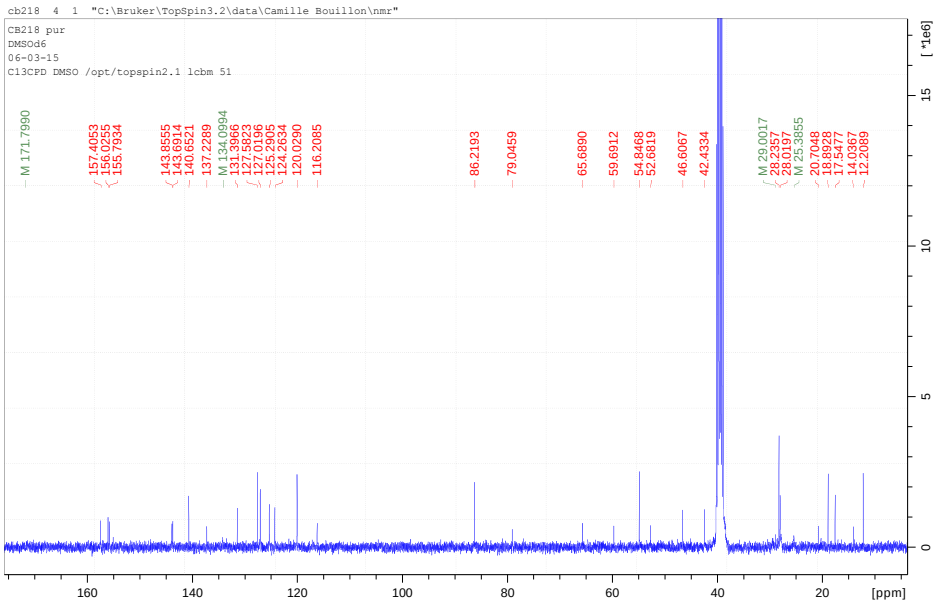


Figure S5: ^{13}C NMR spectrum of Fmoc-Arg(Pbf)-Hyd-Boc **1b**

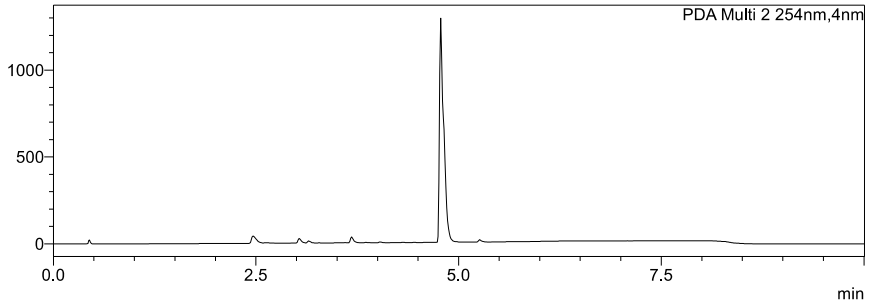


Figure S6: HPLC chromatogram of Fmoc-Arg(Pbf)-Hyd-Boc **1b**

- ***Tert*-butyl-2-{2-[((9H-fluoren-9-yl)methoxy)carbonyl)amino]-3-[1-trityl-1*H*-imidaz ol-5 -yl]propanoyl}hydrazinecarboxylate 1c :**

Fmoc-His(Trt)-Hyd-Boc was synthesized according to the general procedure I using Fmoc-His(Trt)-OH (1 g, 1.61 mmol), *tert*-butyl carbazate (213 mg, 1.61 mmol), HOBt (327 mg, 2.42 mmol), triethylamine (242 μL, 1.77 mmol) and EDC (464 mg, 2.42 mmol). The compound **1c** was obtained after purification by flash chromatography (cyclohexane/EtOAc gradient 3:7 to 2:8, v/v) with 70 % yield. R_f = 0.47 (cyclohexane/EtOAc 3:7, v/v); t_R = 4.23 min (Method A); ^{1}H NMR (400 MHz, DMSO-d_6): δ = 9.74 (s, 1H, *NH*-NH-Boc), 8.76 (s, 1H, NH-*NH*-Boc), 7.88 (d, 2H, ^3J = 7.6 Hz, C*CH*=CH), 7.70-7.66 (m, 2H, C*CH*=CH), 7.55-7.49 (m, 1H, *NH*-Fmoc), 7.44-7.39 (m, 2H, *CH*=CH), 7.36-7.29 (m, 9H, *CH*=*CH*), 7.28 -7.23 (m, 2H, CH=*CH*), 7.23 (s, 1H, N*CH*=N), 7.05-7.03 (m, 6H, C*CH*=CH), 6.80 (s, 1H, N*CH*=C), 4.34-4.26 (m, 1H, NH*CH*CH_2), 4.17-4.12 (m, 3H, OCH_2*CH*), 2.90-2.76 (m, 2H, NCHCH_2), 1.36 (s, 9H, OC(CH_3)$_3$); ^{13}C NMR (100 MHz, DMSO-d_6) : δ = 170.3, 155.6 (2C), 143.7, 142.2, 140.6, 137.4, 137.2, 129.2, 127.9, 127.8, 127.6, 127.0, 125.3, 120.0, 119.0, 79.0, 74.3, 65.7, 60.0, 46.5, 31.3, 28.0; HRMS (ESI): *m/z* calcd for $C_{45}H_{44}N_5O_5^+$ [M+H$^+$] 734.3342, found 734.3342.

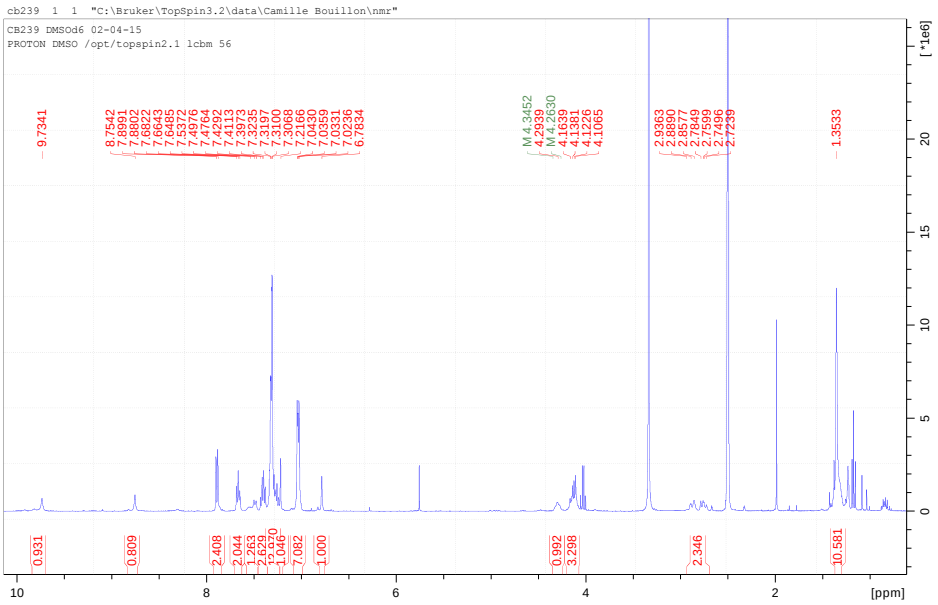


Figure S7: ^{1}H NMR spectrum of Fmoc-His(Trt)-Hyd-Boc **1c**

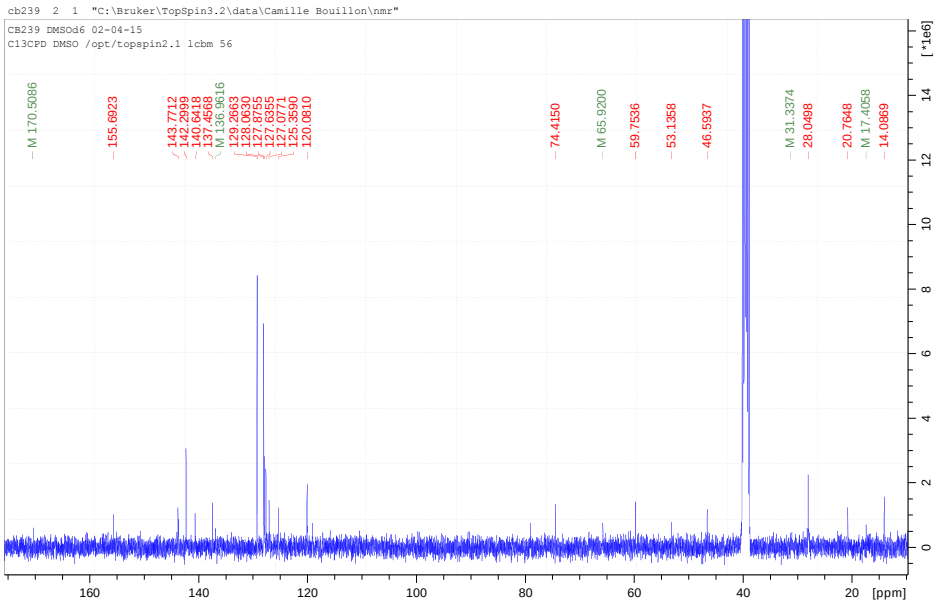


Figure S8: ^{13}C NMR spectrum of Fmoc-His(Trt)-Hyd-Boc **1c**

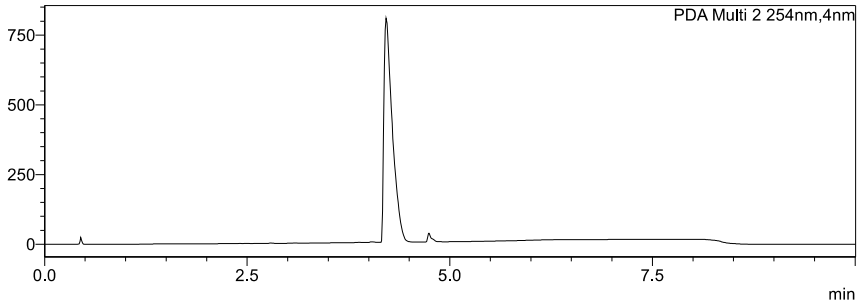


Figure S9: HPLC chromatogram of Fmoc-His(Trt)-Hyd-Boc **1c**

2.2. **Synthesis of *Tert*-butyl 2-*S*-glycylhydrazine-1-carboxylate 2a.**

Cbz-Gly-Hyd-Boc **1a** (2.8 g, 8.61 mmol) was dissolved in MeOH (150 mM) and reduced under H_2 atmosphere for overnight in presence of 10% Pd/C. The reaction mixture was then filtered on Celite and concentrated under reduced pressure to provide H-Gly-Hyd-Boc **2a** with 95% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.11 (s, 2H, CH_2NH_2), 1.40 (s, 9H, OC(CH_3)$_3$); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 172.4, 155.2, 79.0, 43.3, 28.1; HRMS (ESI): *m/z* calcd for $C_7H_{16}N_3O_3^+$ [M+H$^+$] 190.1192, found 190.1192.

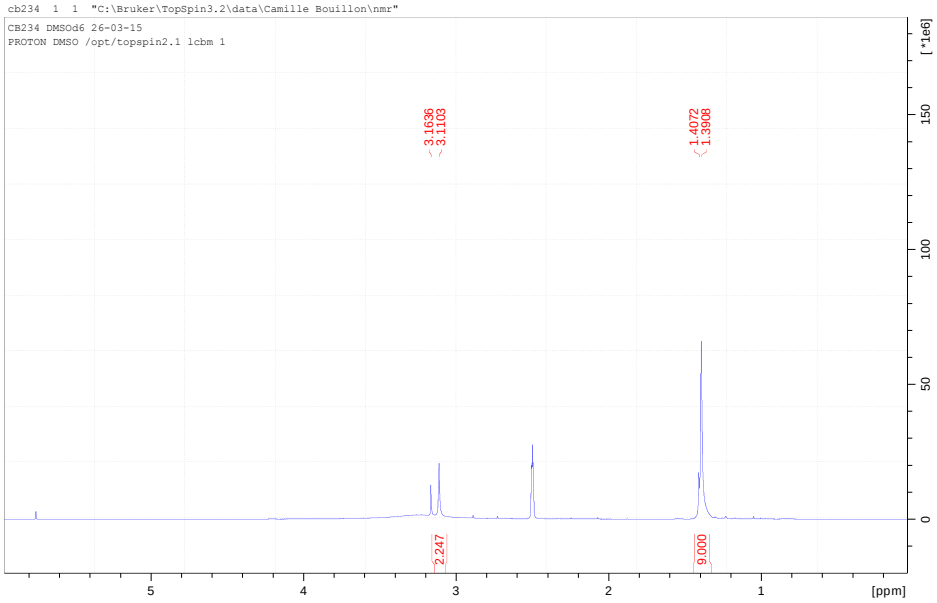


Figure S10: ^{1}H NMR spectrum of H-Gly-Hyd-Boc **2a**

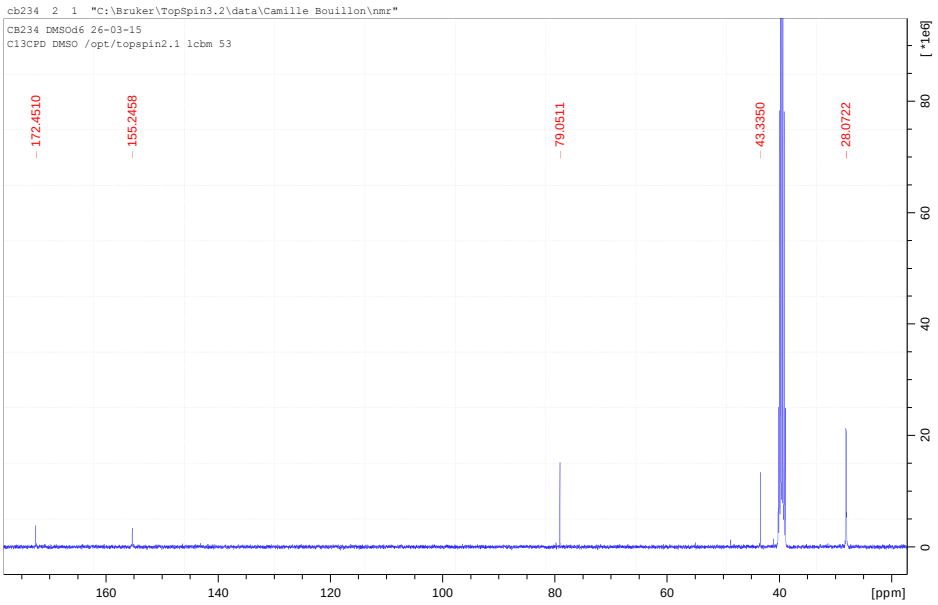


Figure S11: ^{13}C NMR spectrum of H-Gly-Hyd-Boc **2a**

2.3. General procedure II for the deprotection of Fmoc group to provide deprotected amino acid hydrazide H-AA(P_2)-Hyd-Boc **2b-2c**

P_1-AA(P_2)-Hyd-Boc **1b** and **1c** was dissolved in a solution of DMF/piperidine (8:2, v/v; 70 mM). The reaction solution was stirred overnight at room temperature and then concentrated in vacuo. The resulting crude material was purified by flash chromatography to provide H-AA(P_2)-Hyd-Boc **2b** and **2c** as a white solid.

- ***Tert*-butyl-2-{2-amino-5-[3-((2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl)guanidino]pentanoyl}hydrazinecarboxylate 2b :**

H-Arg(Pbf)-Hyd-Boc was synthesized according to the procedure II using Fmoc-Arg(Pbf)-Hyd-Boc **1b** (675 mg, 0.88 mmol). The compound **2b** was obtained after purification by flash chromatography (CH_2Cl_2/MeOH gradient 9:1 to 88:12, v/v) with 84 % yield. R_f = 0.41 (CH_2Cl_2/MeOH 88:12, v/v); t_R = 2.91 min (Method A); ^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.74 (s, 1H, *NH*-NH-Boc), 8.29 (s, 1H, NH-*NH*-Boc), 3.22-3.12 (m, 1H, *CH*CH_2CH_2), 3.03-3.02 (m, 2H, *CH_2*NH(C)NH), 2.96 (s, 2H, *CH_2*C(CH_3)$_2$), 2.48 (s, 3H, *CH_3*), 2.43 (s, 3H, *CH_3*), 2.00 (s, 3H, *CH_3*), 1.55-1.49 (m, 4H, CH*CH_2CH_2*), 1.40 (s, 15H, OC(*CH_3*)$_3$ + CH_2C(*CH_3*)$_2$); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 176.0 (2C), 157.4, 156.0, 155.1, 137.3, 134.2, 131.4, 124.3, 116.2, 86.3, 79.1, 52.7, 42.5, 40.0, 32.1, 28.2, 28.1, 25.1, 20.0, 17.6, 12.3; HRMS (ESI): *m/z* calcd for $C_{24}H_{41}N_6O_6S^+$ [M+H$^+$] 541.2808, found 541.2806.

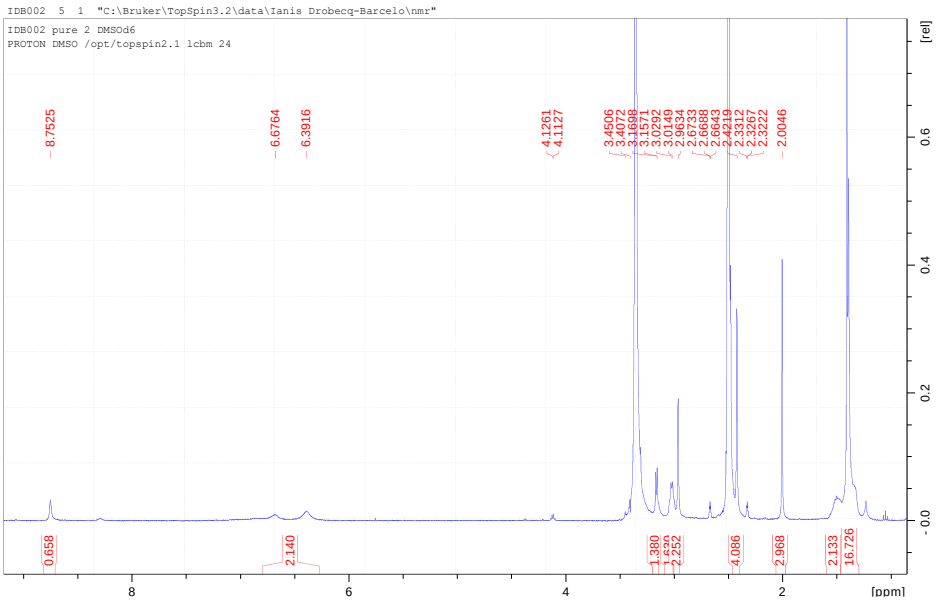


Figure S12: ^{1}H NMR spectrum of H-Arg(Pbf)-Hyd-Boc **2b**

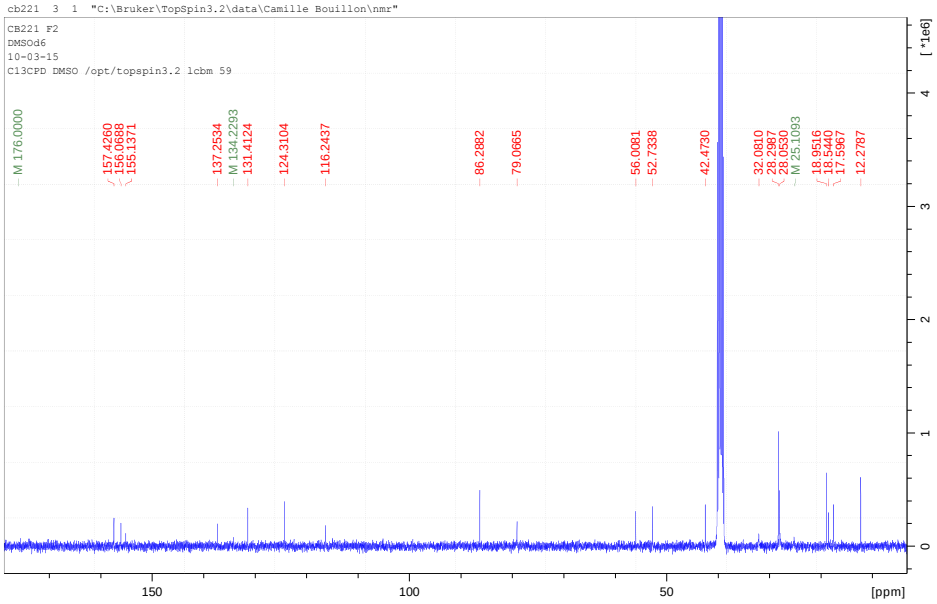


Figure S13: ^{13}C NMR spectrum of H-Arg(Pbf)-Hyd-Boc **2b**

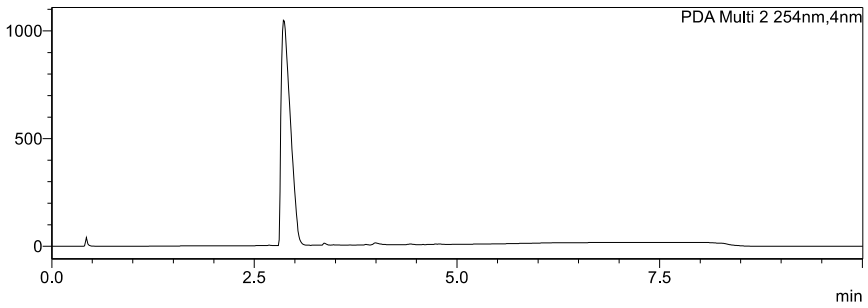


Figure S14: HPLC chromatogram of H-Arg(Pbf)-Hyd-Boc **2b**

- ***Tert*-butyl-2-(2-amino-3-[1-trityl-1*H*-imidazol-5-yl]propanoyl)hydrazine carboxylate 2c :**

H-His(Trt)-Hyd-Boc was synthesized according to the procedure II using Fmoc-His(Trt)-Hyd-Boc **1c** (822 mg, 1.20 mmol). The compound **2c** was obtained after purification by flash chromatography (CH_2Cl_2/MeOH gradient 9:1 to 88:12, v/v) with 69 % yield. R_f = 0.50 (CH_2Cl_2/MeOH 9:1, v/v); t_R = 4.45 min (Method A); ^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.68 (s, 1H, *NH*-NH-Boc), 8.24 (s, 1H, NH-*NH*-Boc), 7.42-7.34 (br, 9H, *CH*=*CH*), 7.26 (s, 1H, N*CH*N), 7.11-7.10 (m, 6H, C*CH*=CH), 6.78 (s, 1H, C*CH*N), 3.44-3.41 (m, 1H, *CH*CH_2), 2.81-2.76 (m, 1H, CHCH_{2a}), 2.53-2.44 (m, 1H, CHCH_{2b}, under DMSOd_6 confirmation COSY), 1.35 (s, 9H, OC(CH_3)$_3$); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 173.8, 155.0, 142.4, 137.7, 137.6, 129.3, 128.1, 127.9, 119.2, 78.9, 74.4, 53.7, 34.0, 28.0; HRMS (ESI): *m/z* calcd for $C_{30}H_{34}N_5O_3^+$ [M+H$^+$] 512.2662, found 512.2663.

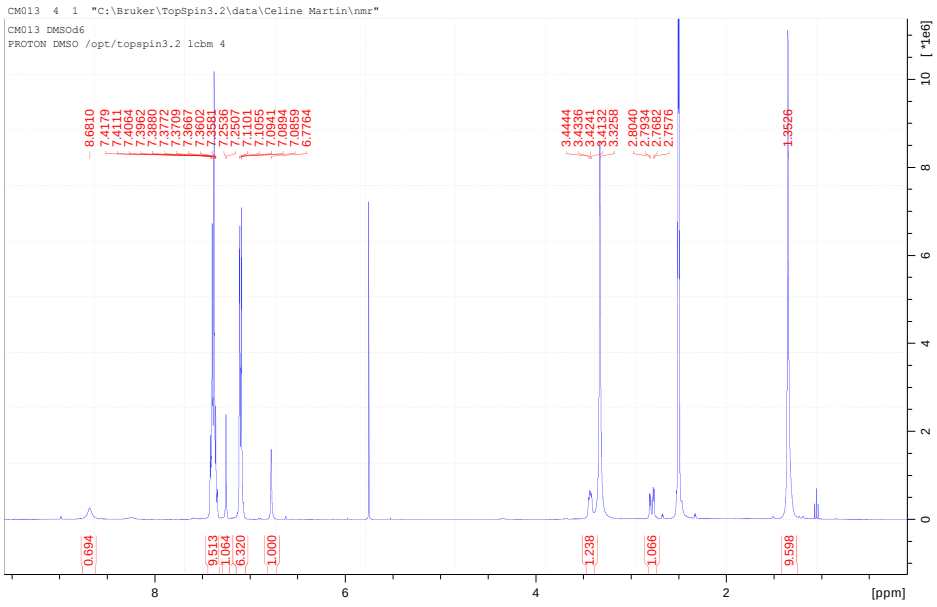


Figure S15: ^{1}H NMR spectrum of H-His(Trt)-Hyd-Boc **2c**

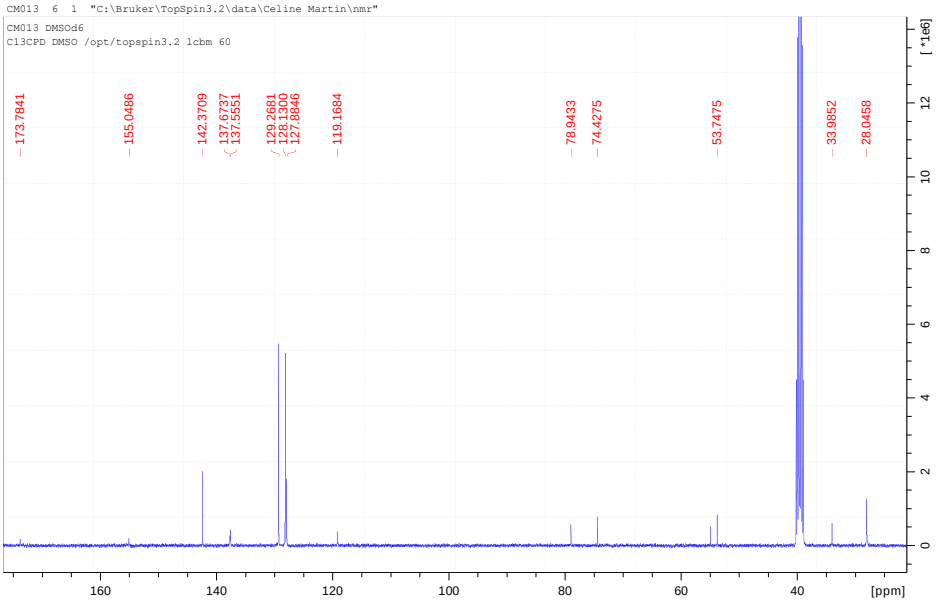


Figure S16: ^{13}C NMR spectrum of H-His(Trt)-Hyd-Boc **2c**

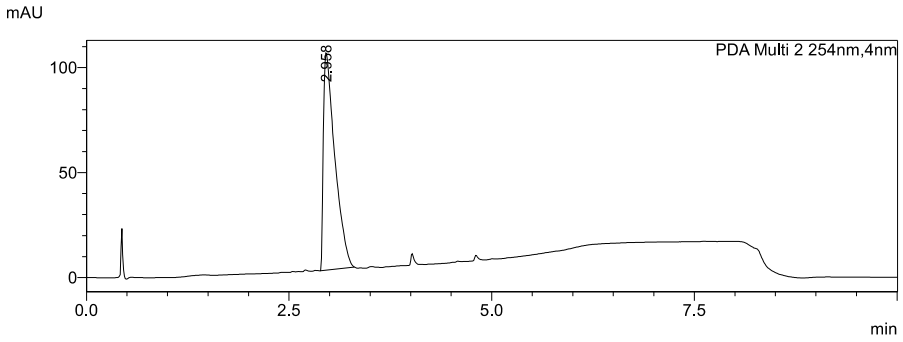


Figure S17: HPLC chromatogram of H-His(Trt)-Hyd-Boc **2c**

2.4. General procedure III for the synthesis of protected amino acids oxyamine hydrazide Boc-Ox-AA(P_2)-Hyd-Boc **3a**-**3c**

Activated ester **4** (1.5 equiv.) and DIEA (4 equiv.) were added to a solution of compound H-AA(P_2)-Hyd-Boc **2a**-**2c** (1 equiv.) in dichloromethane (150 mM). The mixture was stirred overnight. After removal of the solvent, the residue was diluted with EtOAc (50 mL), washed with a saturated $NaHCO_3$ solution (30 mL) and brine (30 mL), dried over anhydrous Na_2SO_4, and concentrated in vacuo. The resulting crude material was purified by flash chromatography to provide Boc-Ox-AA(P_2)-Hyd-Boc **3a**-**3c** as a white solid.

- ***Tert*-butyl-2-{[2-(((*tert*-butoxycarbonyl)amino)oxy)acetyl]-glycyl}hydrazine-1-carboxylate 3a :**

Boc-Ox-Gly-Hyd-Boc **3a** was synthesized according to the general procedure III using H-Gly-Hyd-Boc **2a** (3.1 g, 16.27 mmol), DIEA (11.3 mL, 65.10 mmol) and activated ester (7 g, 24.41 mmol). The compound **3a** was obtained after purification by flash chromatography (petroleum ether/AcOEt gradient 2/8 to 0/100, v/v) with 59 % yield. R_f = 0.35 (petroleum ether/AcOEt 2/8, v/v); t_R = 2.79 min (Method A); ^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.21 (s, 1H, *NH*Boc), 9.63 (s, 1H, *NH*-NH-Boc), 9.24-8.76 (m, 1H, NH-*NH*-Boc), 8.16-7.96 (m, 1H, CH*NH*), 4.20 (s, 2H, CH_2O), 3.86-3.78 (m, 2H, CH_2), 1.42-1.39 (m, 18H, OC(CH_3)$_3$); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 168.2, 168.1, 156.6, 155.1, 80.5, 79.1, 74.6, 39.5 (under DMSO-d_6), 27.9 (2C); HRMS (ESI): *m/z* calcd for $C_{14}H_{27}N_4O_7^+$ [M+H$^+$] 363.1880, found 363.1882.

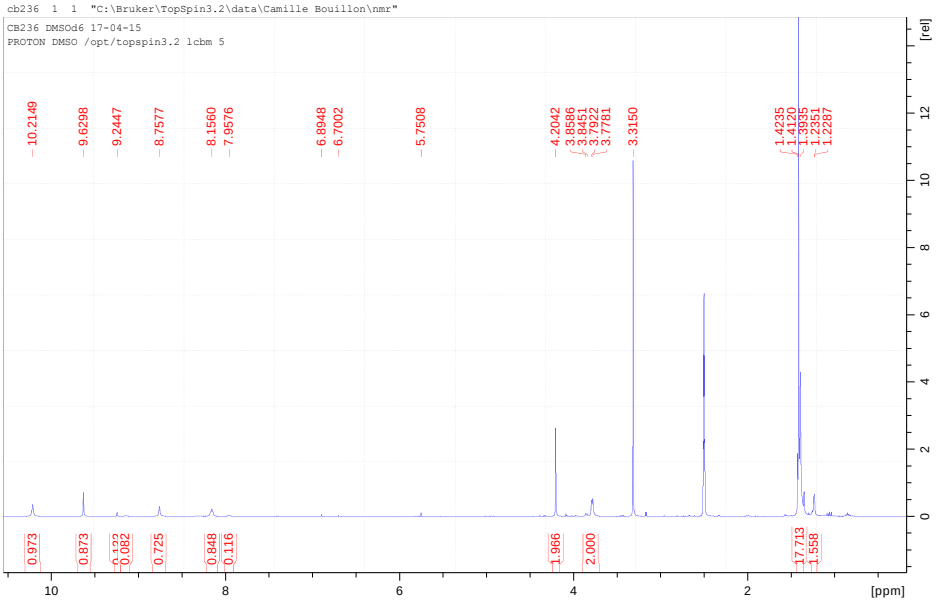


Figure S18: ^{1}H NMR spectrum of Boc-Ox-Gly-Hyd-Boc **3a**

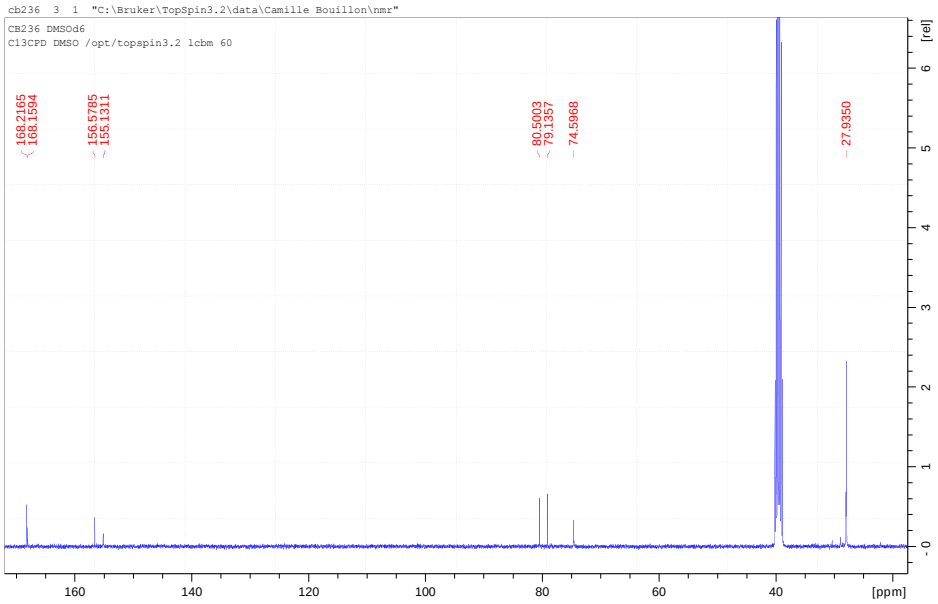


Figure S19: ^{13}C NMR spectrum of Boc-Ox-Gly-Hyd-Boc **3a**

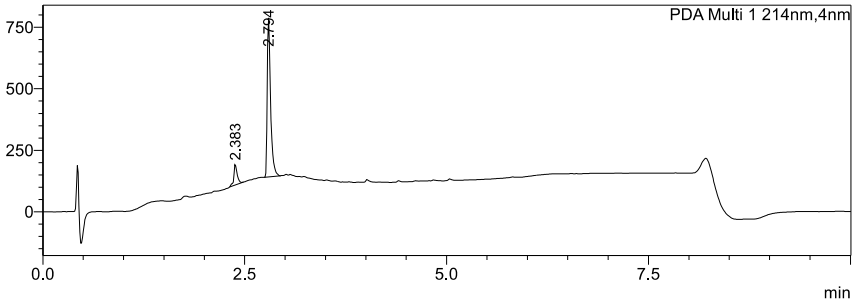


Figure S20: HPLC chromatogram of Boc-Ox-Gly-Hyd-Boc **3a**

- ***Tert*-butyl-2-{N^2-[2(((*tert*-butoxycarbonyl)amino)oxy)acetyl]-N^{ω}-[(2,2,4,6,7-penta methyl-2,3-dihydrobenzofuran-5-yl)sylfonyl]arginyl}hydrazine-1-carboxylate 3b :**

Boc-Ox-Arg(Pbf)-Hyd-Boc **3b** was synthesized according to the general procedure III using H-Arg(Pbf)-Hyd-Boc **2b** (660 mg, 1.31 mmol), DIEA (911 μL, 5.23 mmol) and activated ester (565 mg, 1.96 mmol). The compound **3b** was obtained after purification by flash chromatography (CH_2Cl_2/MeOH gradient 97:3 to 95/5, v/v) with 84 % yield. R_f = 0.31 (CH_2Cl_2/MeOH 95/5, v/v); t_R = 4.01 min (Method A); ^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.31 (s, 1H, , *NH*Boc), 9.77 (s, 1H, *NH*-NH-Boc), 9.20 (s, 1H, NH-*NH*-Boc), 8.14 (d, 1H, ^3J = 8.3 Hz, CH*NH*CO), 4.38-4.37 (m, 1H, *CH*CH_2), 4.27-4.23 (m, 1H, CH_{2a}O), 4.14-4.10 (m, 1H, CH_{2b}O), 3.06-2.99 (m, 2H, CH_2NH(C)NH), 2.96 (s, 2H, $CH_2C(CH_3)_2$), 2.48 (s, 3H, CH_3), 2.42 (s, 3H, CH_3), 2.00 (s, 3H, CH_3), 1.72-1.39 (m, 28H, $CHCH_2CH_2$ + $OC(CH_3)_3$ + $CH_2C(CH_3)_2$); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 170.5, 167.8, 157.5, 157.0, 156.0, 155.1, 137.3, 131.5, 124.3 (2C), 116.3, 86.3, 80.7, 79.2, 74.6, 50.0, 42.5, 39.6, 29.9, 28.3, 28.1, 27.9, 25.1, 19.0, 17.6, 12.3; HRMS (ESI): *m/z* calcd for $C_{31}H_{52}N_7O_{10}S^+$ [M+H$^+$] 714.3496, found 714.3494.

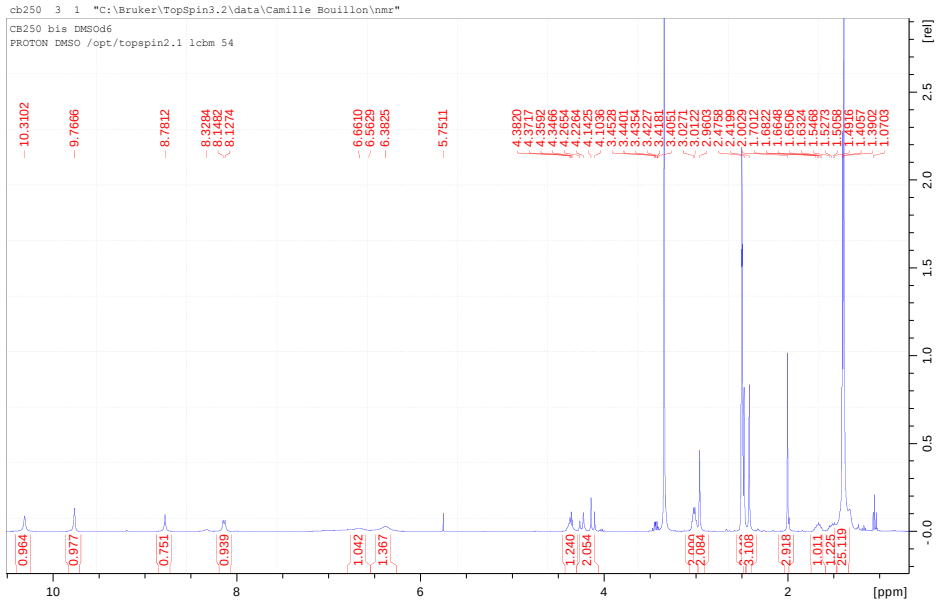


Figure S21: ^{1}H NMR spectrum of Boc-Ox-Arg(Pbf)-Hyd-Boc **3b**

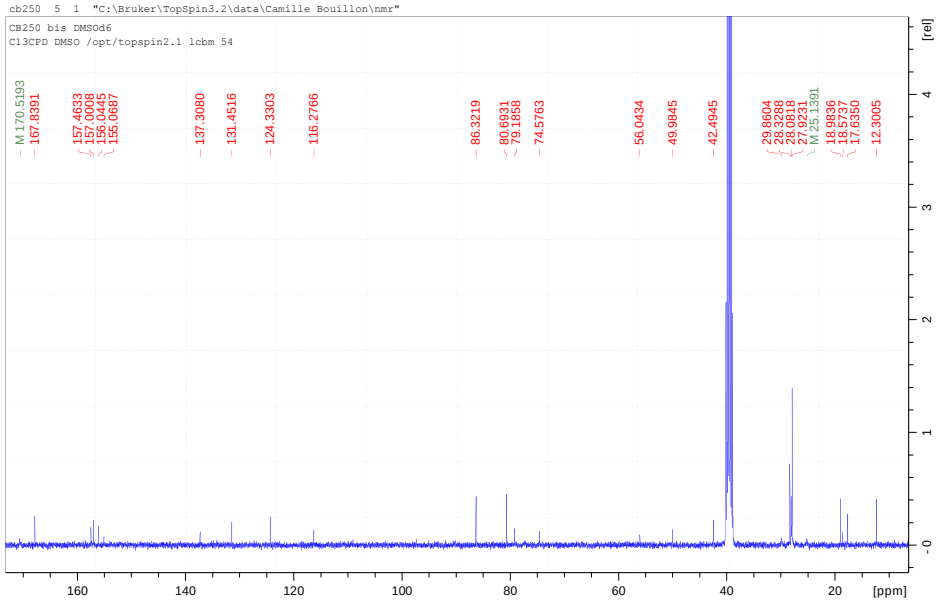


Figure S22: ^{13}C NMR spectrum of Boc-Ox-Arg(Pbf)-Hyd-Boc **3b**

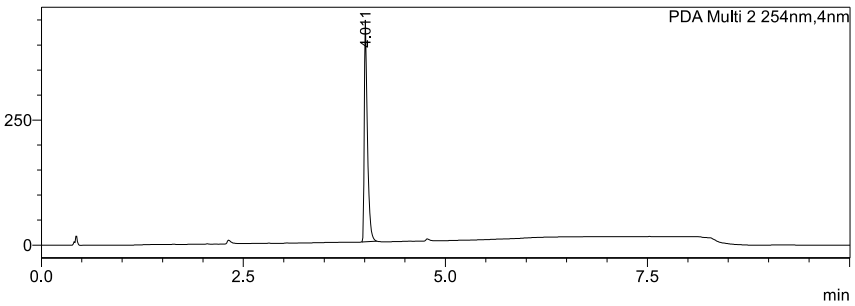


Figure S23: HPLC chromatogram of Boc-Ox-Arg(Pbf)-Hyd-Boc **3b**

- ***Tert*-butyl-2-{*N*$^{\alpha}$-[2(((*tert*-butoxycarbonyl)amino)oxy)acetyl]-*N*$^{\tau}$tritylhistidyl}hydr azine-1-carboxylate 3c :**

Boc-Ox-His(Trt)-Hyd-Boc **3c** was synthesized according to the general procedure III using H-His(Trt)-Hyd-Boc **2c** (1 g, 1.96 mmol), DIEA (1.4 mL, 7.82 mmol) and activated ester (669 mg, 2.93 mmol). The compound **3c** was obtained after purification by flash chromatography (CH_2Cl_2/MeOH 96/4, v/v) with 87 % yield. R_f = 0.45 (CH_2Cl_2/MeOH 95/5, v/v); t_R = 3.62 min (Method A); ^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.60 (s, 1H, *NH*Boc), 9.79 (s, 1H, *NH*-NH-Boc), 8.77 (s, 1H, NH-*NH*-Boc), 8.15 (d, 1H, ^3J = 8.0 Hz, CH*NH*CO), 7.41-7.36 (m, 9H*, CH*=*CH*), 7.22 (s, 1H, N=*CH*-N), 7.07-7.05 (m, 6H, C*CH*=CH), 6.72 (s, 1H, N-*CH*=C), 4.63-4.53 (m, 1H, *CH*CH_2), 4.23-4.19 (m, 1H, CH_{2a}O), 3.99-3.96 (m, 1H, CH_{2b}O), 2.93-2.88 (m, 1H, CHCH_{2a}), 2.77-2.71 (m, 1H, CHCH_{2b}), 1.35-1.29 (m, 18H, OC(CH_3)$_3$); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 170.8, 168.1, 157.1, 155.5, 142.7, 138.0, 137.1, 129.8, 128.6, 128.3, 119.6, 80.9, 79.6, 75.3, 75.0, 51.2, 31.6, 28.3 (2C); HRMS (ESI): *m/z* calcd for $C_{37}H_{45}N_6O_7^+$ [M+H$^+$] 685.3350, found 685.3348.

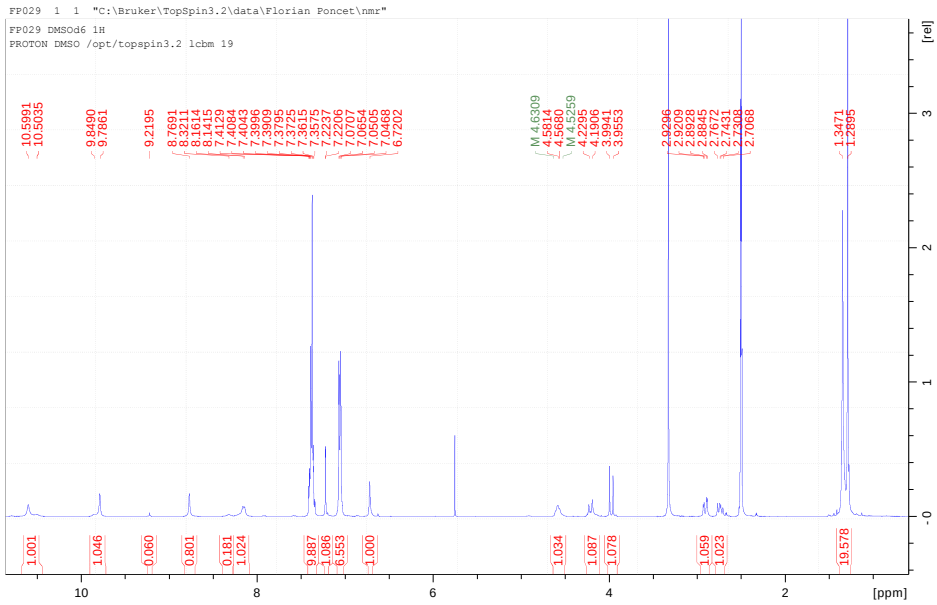


Figure S24: ^{1}H NMR spectrum of Boc-Ox-His(Trt)-Hyd-Boc **3c**

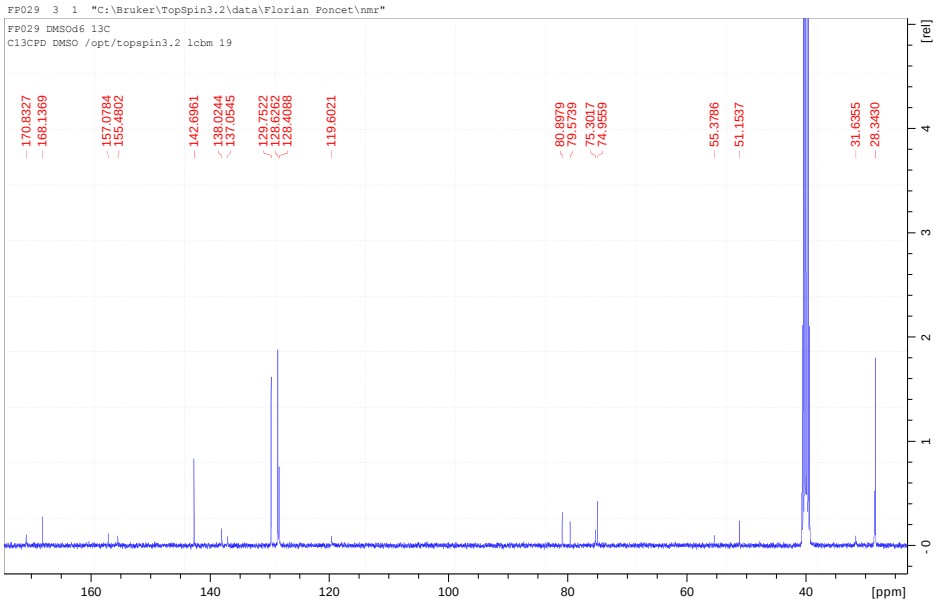


Figure S25: ^{13}C NMR spectrum of Boc-Ox-His(Trt)-Hyd-Boc **3c**

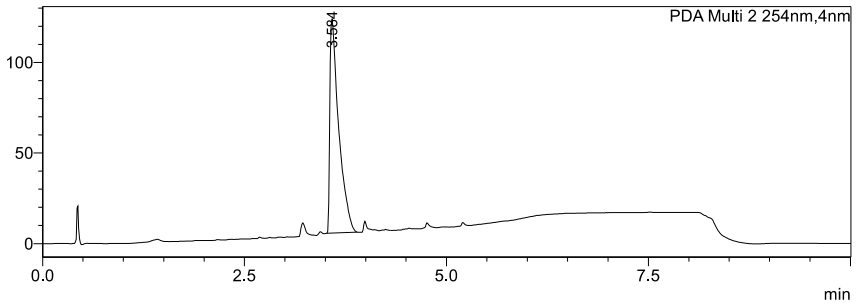


Figure S26: HPLC chromatogram of Boc-Ox-His(Trt)-Hyd-Boc **3c**

2.5. General procedure IV for the synthesis of deprotected amino acids oxyamine hydrazide **Ox-AA-Hyd**

Boc-Ox-AA(P_2)-Hyd-Boc **3a**-**3c** were dissolved in TFA/TIS/H_2O (95/2.5/2.5) (50 mM) and stirred for overnight at room temperature. After removal of 90% of the solvent, diethyl ether was added to the residue. The precipitate was triturated with Et_2O and filtered. The crude material was then lyophilized twice to afford the product **Ox-AA-Hyd** as a white solid. The number of mole of monomer **Ox-AA-Hyd** was calculated by ^{1}H NMR titration method using *tert*-butyl alcohol as an internal reference.

- **2-(aminooxy)-*N*-(2-hydrazinyl-2-oxoethyl)acetamide Ox-Gly-Hyd :**

Ox-Gly-Hyd was synthesized according to the general procedure IV using Boc-Ox-Gly-Hyd-Boc **3a** (433.6 mg, 1.19 mmol). ^{1}H NMR (400 MHz, $DMSOd_6$): δ = 8.51-8.48 (m, 1H, CH_2*NH*), 4.37-4.34 (m, 2H, *CH_2*O), 390-3.86 (m, 2H, *CH_2*NH); ^{13}C NMR (100 MHz, $DMSOd_6$): δ = 168.6, 168.3, 158.5 (TFA), 72.5, 40.5; HRMS (ESI): *m/z* calcd for $C_4H_{11}N_4O_3^+$ [M+H$^+$] 163.0831, found 163.0832.

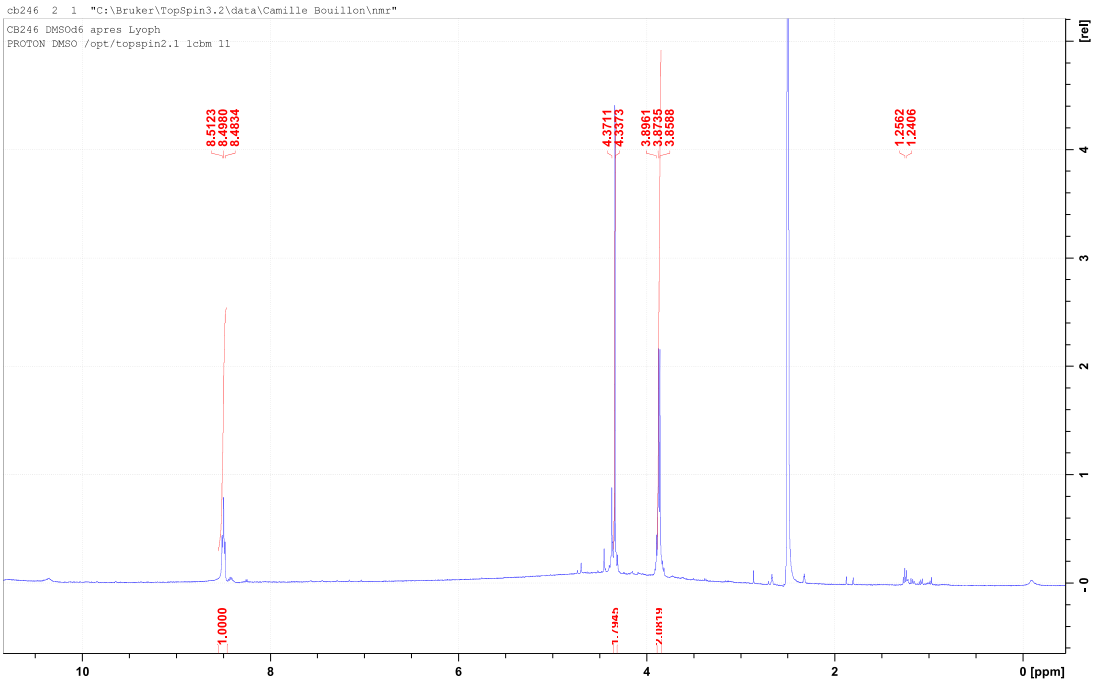


Figure S27: ^{1}H NMR spectrum of **Ox-Gly-Hyd**

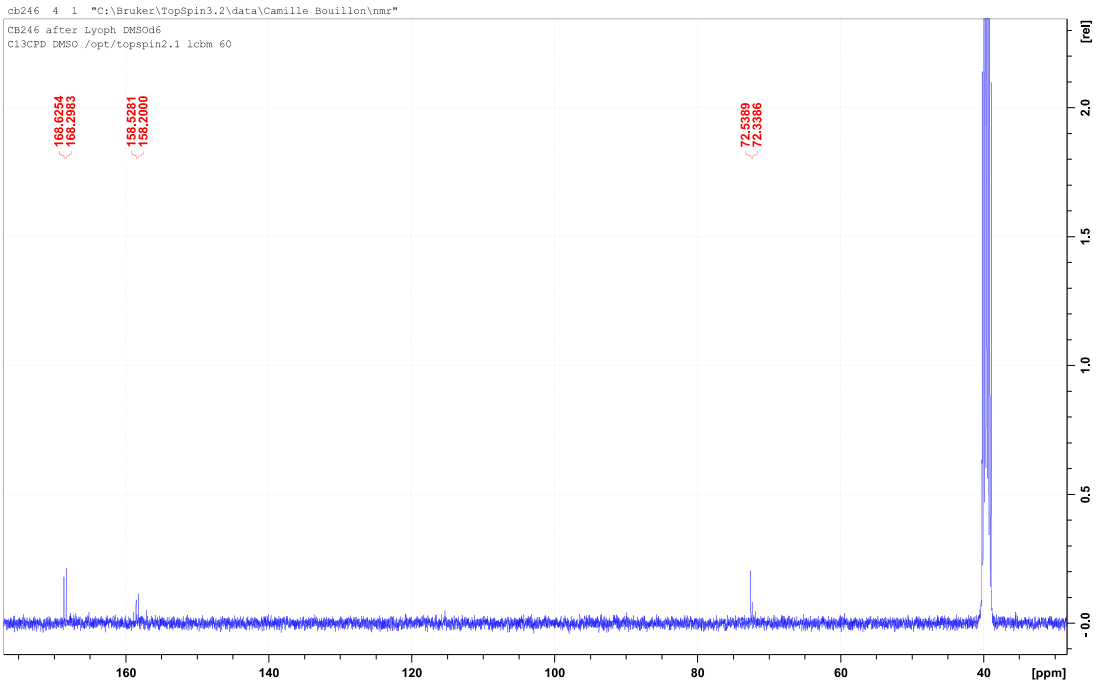


Figure S28: ^{13}C NMR spectrum of **Ox-Gly-Hyd**

- **2-(aminooxy)-*N*-(5-guanidino-1-hydrazinyl-1-oxopentan-2-yl)acetamide**

  Ox-Arg-Hyd :

Ox-Arg-Hyd was synthesized according to the general procedure IV using Boc-Ox-Arg(Pbf)-Hyd-Boc **3b** (782 mg, 1.10 mmol). ^{1}H NMR (400 MHz, D_2O): δ = 4.65 (s, 2H, CH_2O), 4.42-4.39 (m, 1H, $CHCH_2$), 3.14 (t, 2H, ^{3}J = 6.7, $CH_2NH(C)NH_2$), 1.90-1.71 (m, 2H, $CHCH_2$), 1.62-1.54 (m, 2H, $CHCH_2CH_2$); ^{13}C NMR (100 MHz, D_2O): δ = 171.5, 169.5, 162.8 (TFA), 156.7, 117.7 (TFA), 114.8 (TFA), 71.6, 51.8, 40.3, 27.7, 24.2; HRMS (ESI): *m/z* calcd for $C_8H_{20}N_7O_3^+$ [M+H$^+$] 262.1628, found 262.1629.

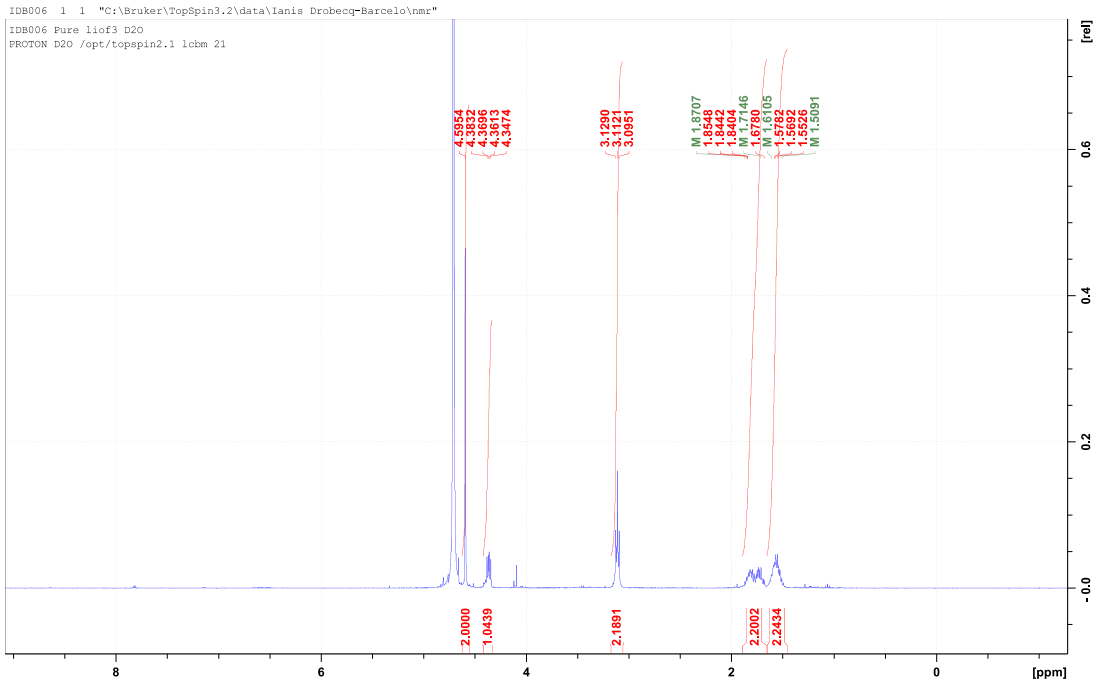


Figure S29: ^{1}H NMR spectrum of **Ox-Arg-Hyd**

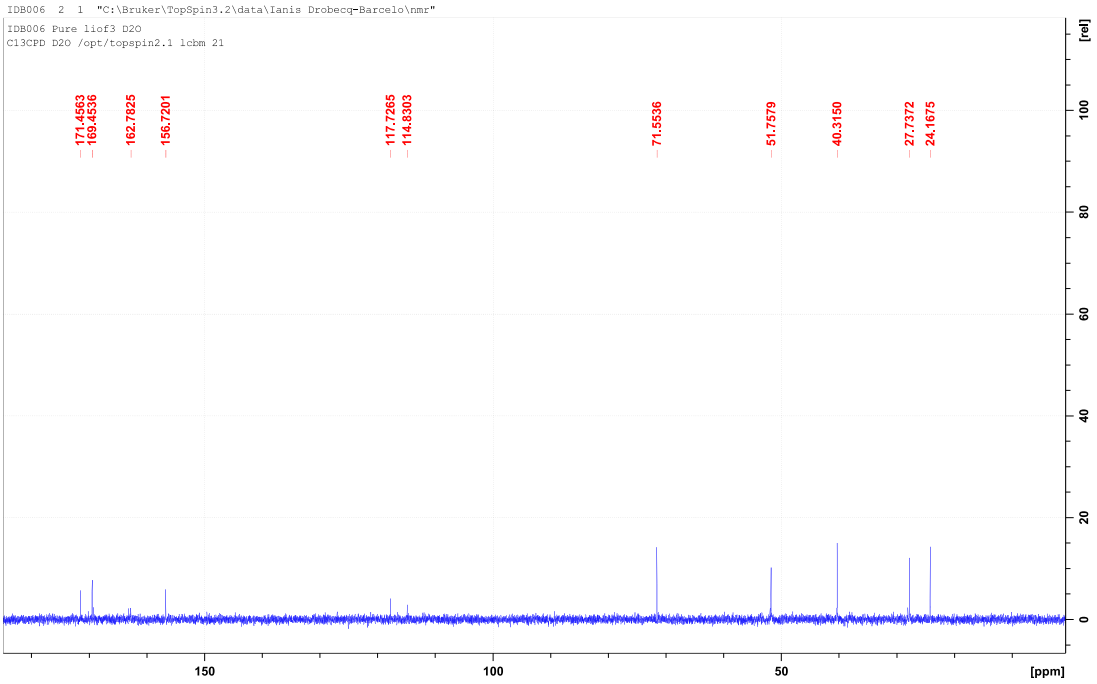


Figure S30: ^{13}C NMR spectrum of **Ox-Arg-Hyd**

- **2-(aminooxy)-*N*-(1-hydrazinyl-3-(1*H*-imidazol-4-yl)-1-oxopropan-2-yl)acetamide Ox-His-Hyd :**

Ox-His-Hyd was synthesized according to the general procedure IV using Boc-Ox-His(Trt)-Hyd-Boc **3c** (1.1 g, 1.69 mmol). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.97 (s, 1H, N=*CH*NH), 8.50-8.44 (m, 1H, CH*NH*C(O)), 7.38-7.36 (m, 1H, NH*CH*=C), 4.77-4.65 (m, 1H, *CH*CH$_2$), 4.23-4.20 (m, 2H, *CH*$_2$O), 3.27-3.14 (M, 1H, CH*CH*$_{2a}$), 3.09-3.00 (m, 1H, CH*CH*$_{2b}$); ^{13}C NMR (100 MHz, D$_2$O): δ = 169.1, 168.9, 158.4 (TFA), 133.9, 129.0, 117.1, 72.8, 50.1, 26.7.

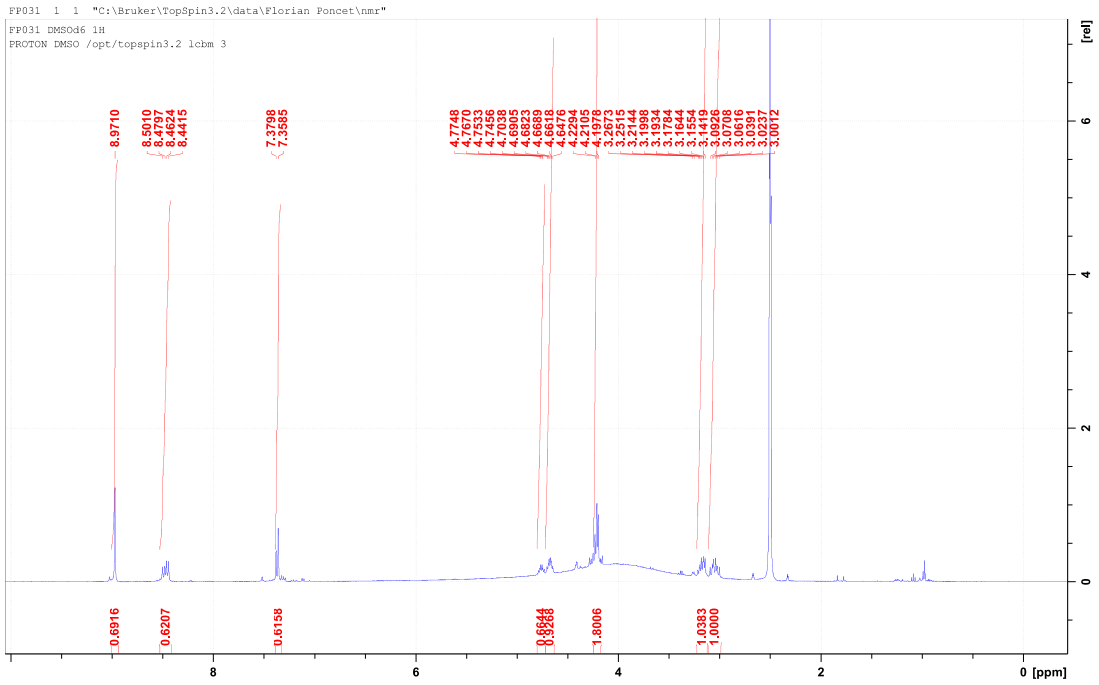


Figure S31: ^{1}H NMR spectrum of **Ox-His-Hyd**

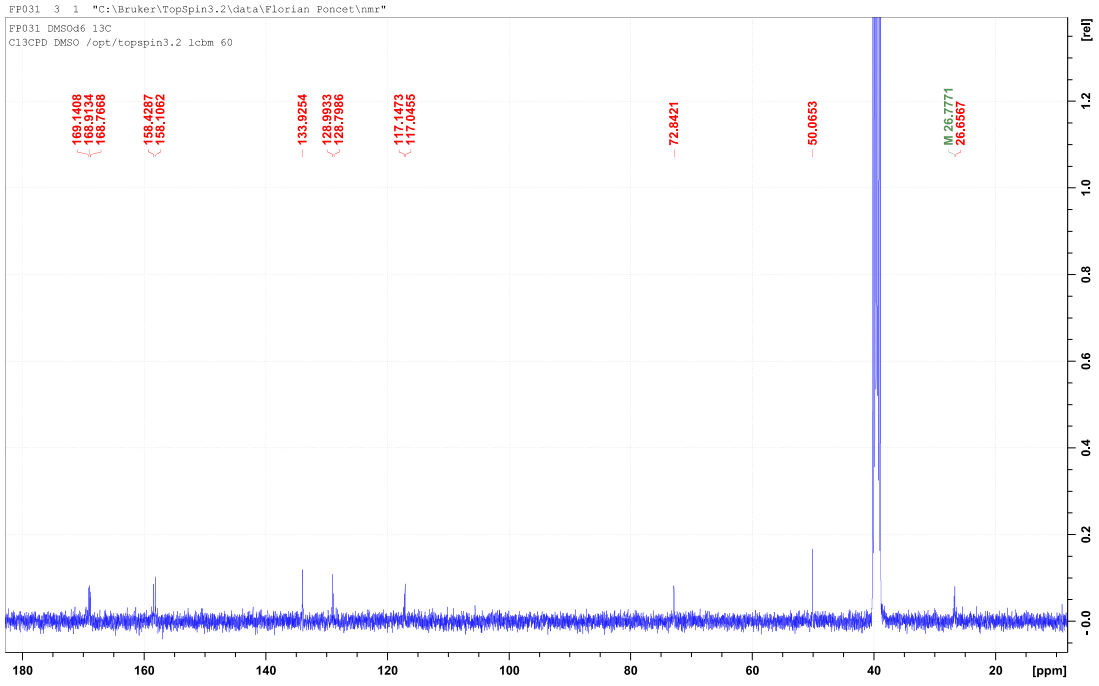


Figure S32: ^{13}C NMR spectrum of **Ox-His-Hyd**

2.6. **Synthesis of *N*-*N*-(disulfanediylbis(ethane-2,1-diyl))formylbenzamide S_2-ALD**

4-Formylbenzoic acid (1.33 g, 8.88 mmol) and 1-hydroxybenzotriazole hydrate (1.8 g, 13.22 mmol) were added to a solution of cystamine dihydrochloride (1 g, 4.44 mmol) and triethylamine (1.36 mL, 19.54 mmol) in DMF (111 mM). Then the solution was cooled to 0°C and *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (2.5 g, 13.32 mmol) was added. The reaction mixture was warmed to room temperature and stirred for overnight. Then water (40 mL) and brine solution (150 mL) have been added to precipitate the product. The resulting precipitate has been filtrate, solubilized in a mixture CH_2Cl_2/MeOH solution and purified by flash chromatography (CH_2Cl_2/AcOEt gradient 9/1 to 7/3) to provide **S_2-ALD** as a white solid with 30 % yield. R_f = 0.22 (CH_2Cl_2/AcOEt 8/2, v/v); t_R = 3.16 min (Method A); ^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.07 (s, 1H, C*H*O), 8.89 (t, 2H, ^3J = 5.6 Hz, *NH*), 8.03-7.98 (m, 8H, *CH=CH*), 3.61-3.56 (m, 4H, NH*CH*$_2$), 2.95 (t, 4H, ^3J = 6.8 Hz, S*CH*$_2$); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 192.9, 165.5, 139.2, 137.8, 129.4, 127.9, 39.5 (under DMSO-d_6), 37.0; HRMS (ESI): *m/z* calcd for $C_{20}H_{21}N_2O_4S_2^+$ [M+H$^+$] 417.0943, found 417.0947.

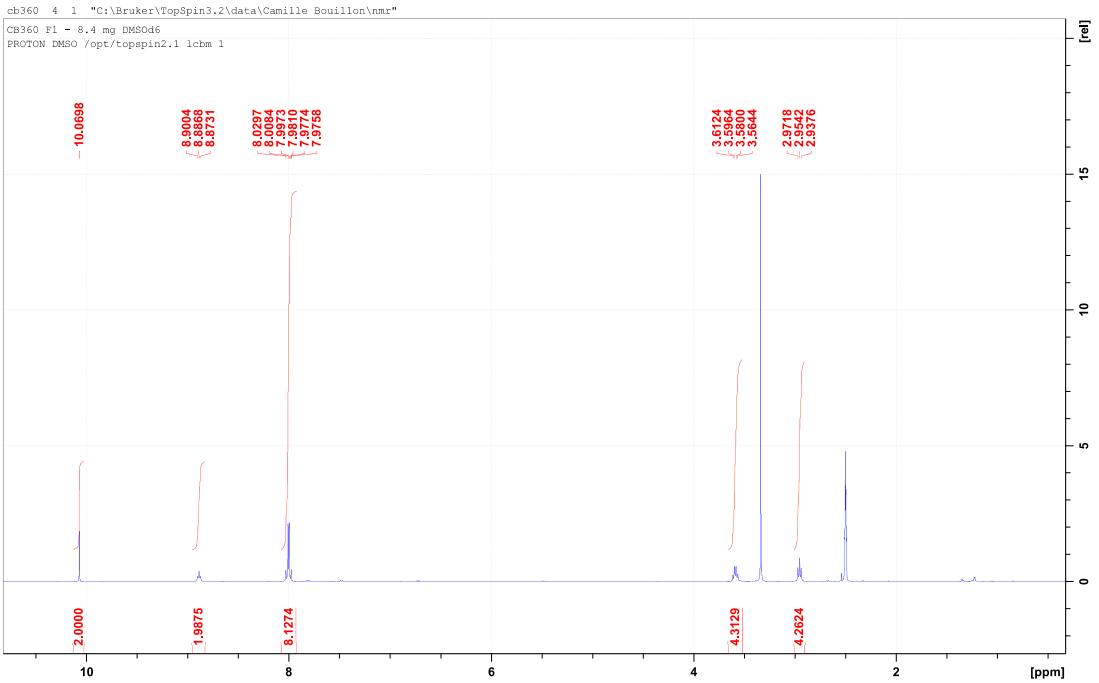


Figure S33: ^{1}H NMR spectrum of **S_2-ALD**

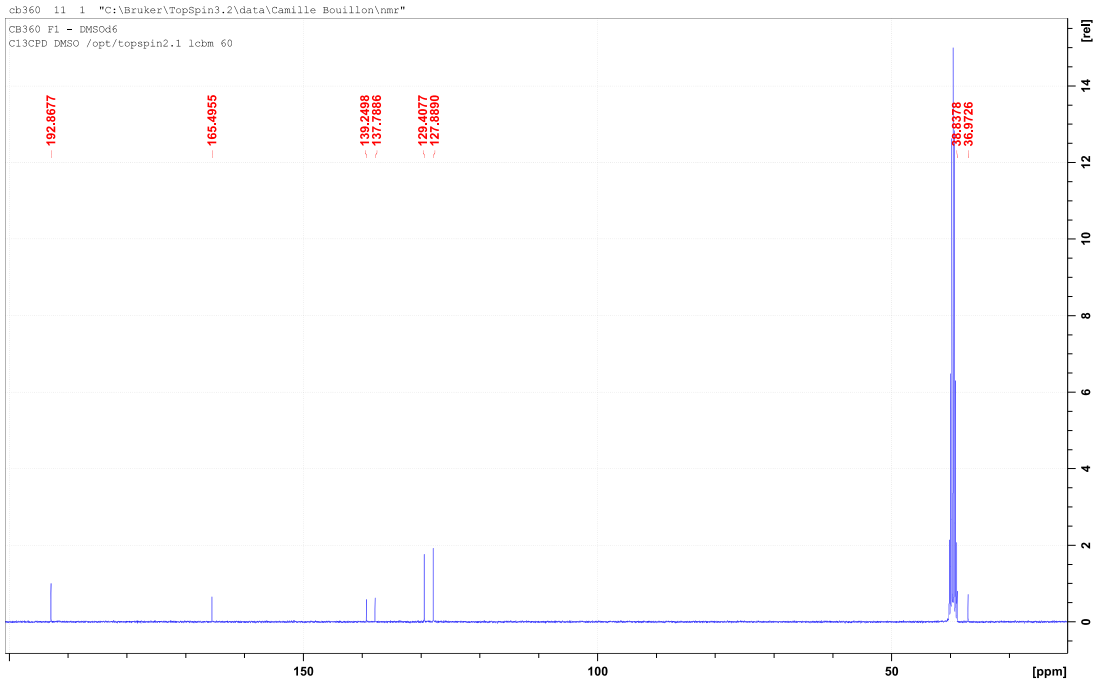


Figure S34: ^{13}C NMR spectrum of **S_2-ALD**

3. Double condensation reaction

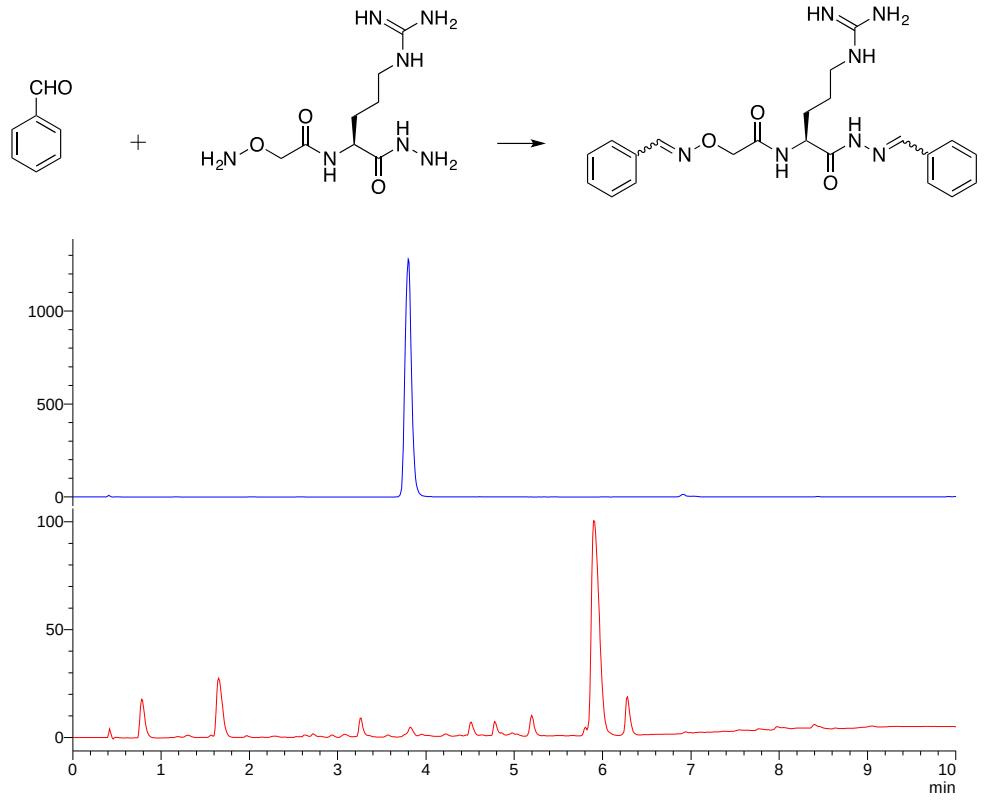


Figure S35: Model bisconjugation reaction involving acylhydrazone and oxime ligations, and HPLC monitoring. **Ox-Arg-Hyd** (10 mM in DMSO) with 2.0 equiv. of benzaldehyde. Top: HPLC chromatogram of pure benzaldehyde; bottom: HPLC chromatogram of the reaction after 48 hours.

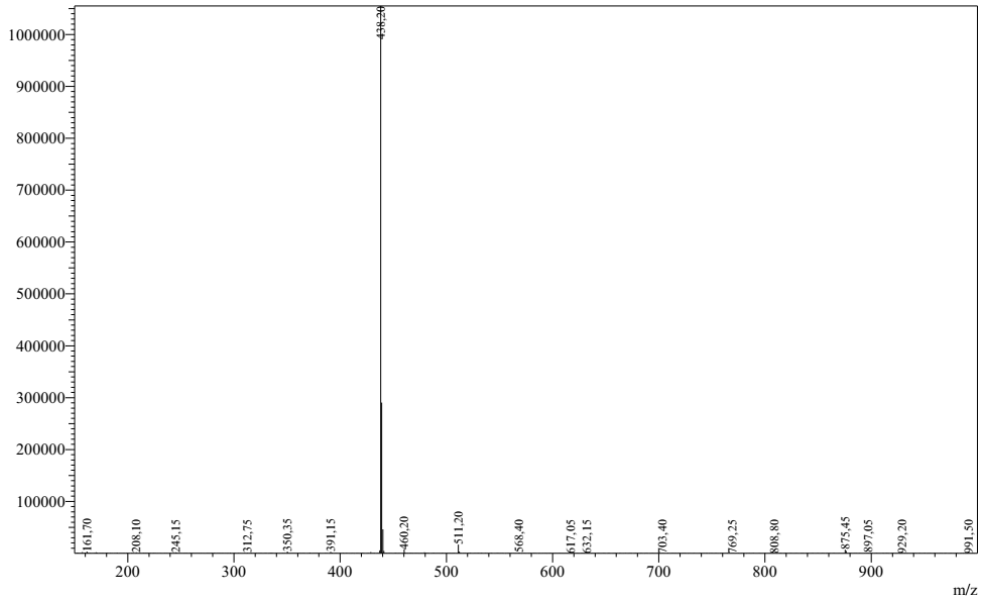


Figure S36: Extracted ESI mass spectrum of the bisconjugated product of the model bisconjugation reaction. M/z calcd for $C_{22}H_{28}N_7O_3^+$ [M+H$^+$] 438.23, found 438.20.

4. MALDI-ToF mass spectra

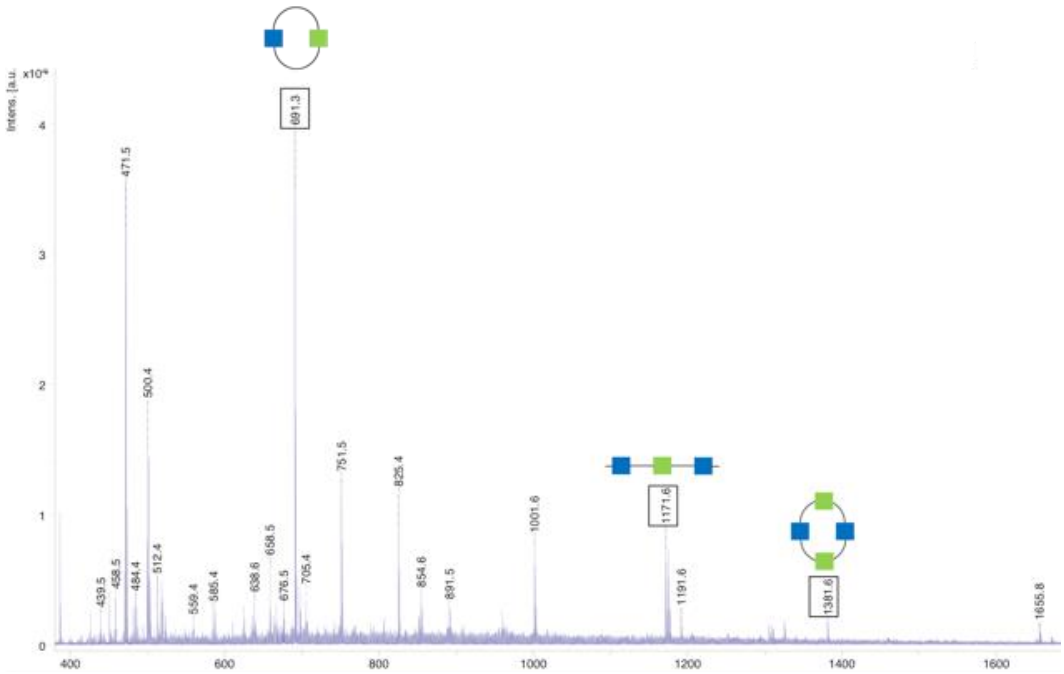


Figure S37: MALDI-TOF (HCCA matrix) mass spectrometry analysis of **Poly(EG-His)**, prepared by the self-assembly of **EG-ALD** and **Ox-His-Hyd** carried out at 100 mM in DMSO. Macrocycles and oligomers are schematically represented using alternate green and blue squares.

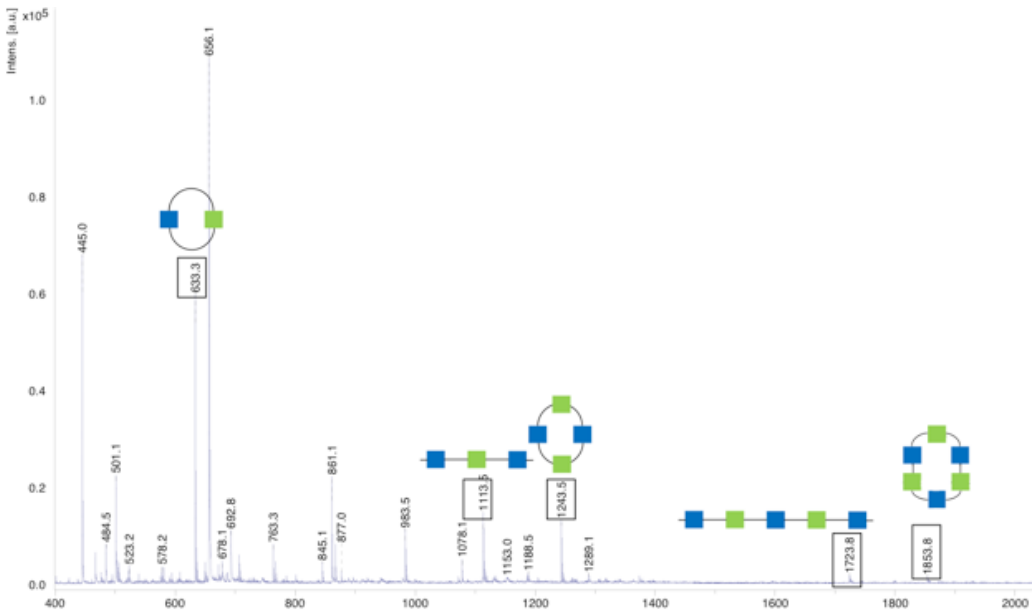


Figure S38: MALDI-TOF (HCCA matrix) mass spectrometry analysis of **Poly(EG-Gly)**, prepared by the self-assembly of **EG-ALD** and **Ox-Gly-Hyd** carried out at 100 mM in DMSO. Macrocycles and oligomers are schematically represented using alternate green and blue squares.

5. DOSY-NMR Spectra

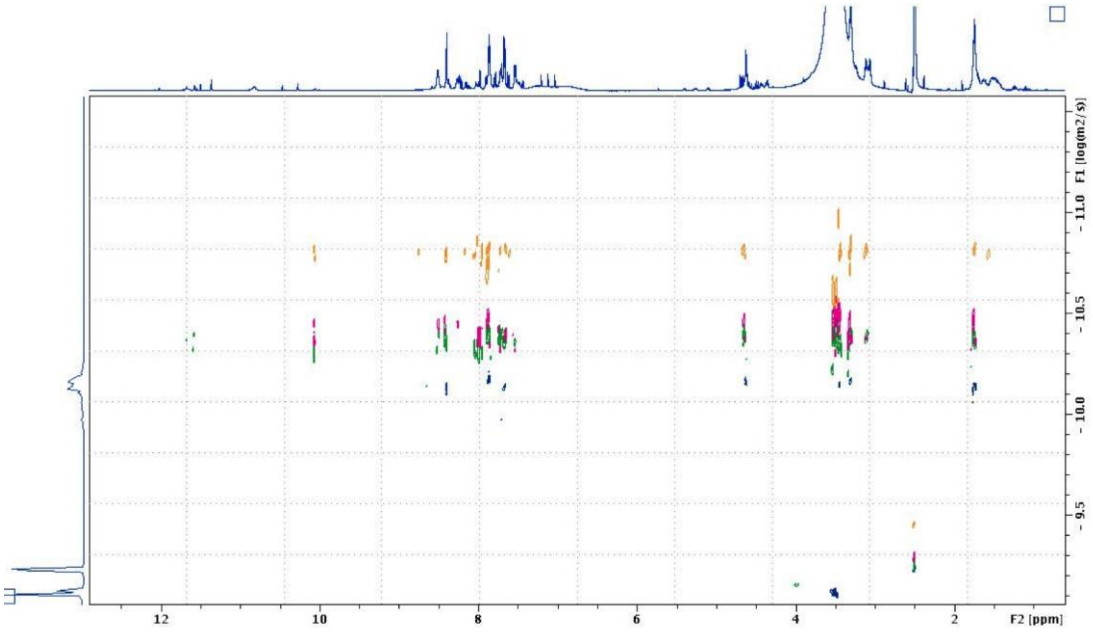


Figure S359: DOSY-NMR spectrum (600 MHz) of **Poly(EG-Arg)** at 200 mM (yellow), 100 mM (pink), 50 mM (green) and 10 mM (blue) in d_6-DMSO

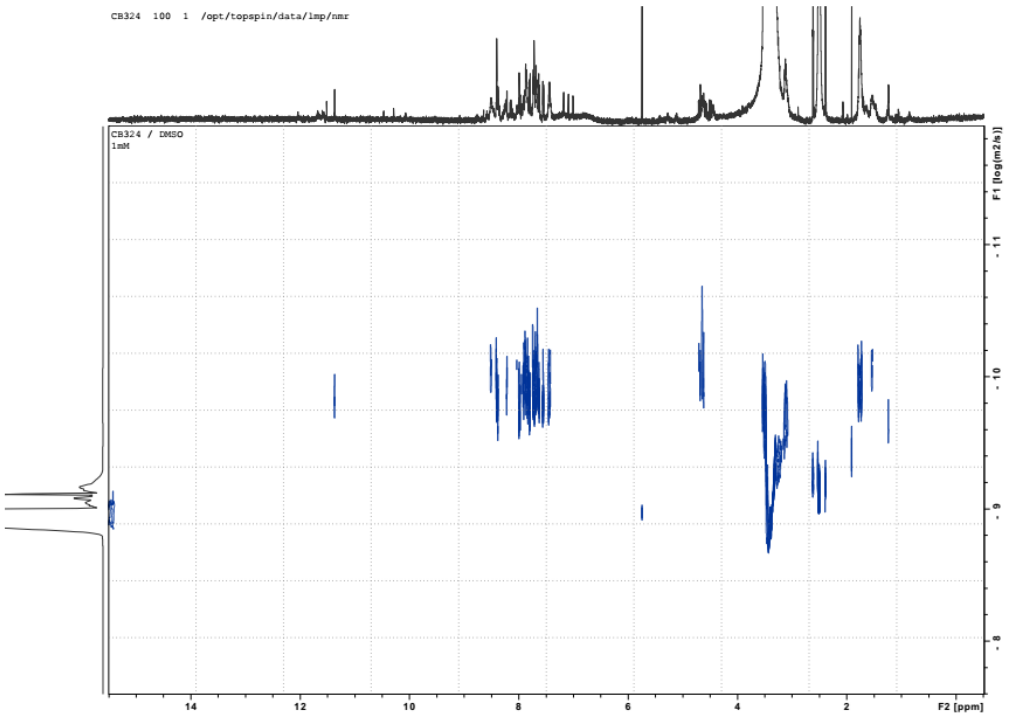


Figure S40: DOSY-NMR spectrum (600 MHz) of **Poly(EG-Arg)** at 1 mM in d_6-DMSO

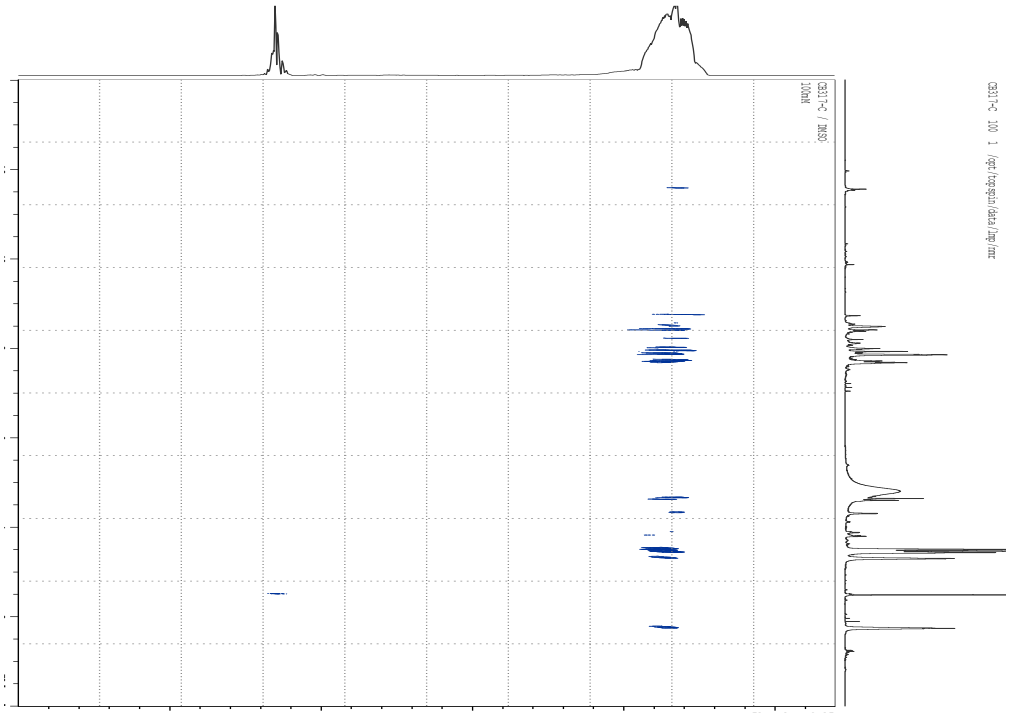


Figure S41: DOSY-NMR spectrum (600 MHz) of **Poly(EG-Gly)** at 100 mM in d_6-DMSO

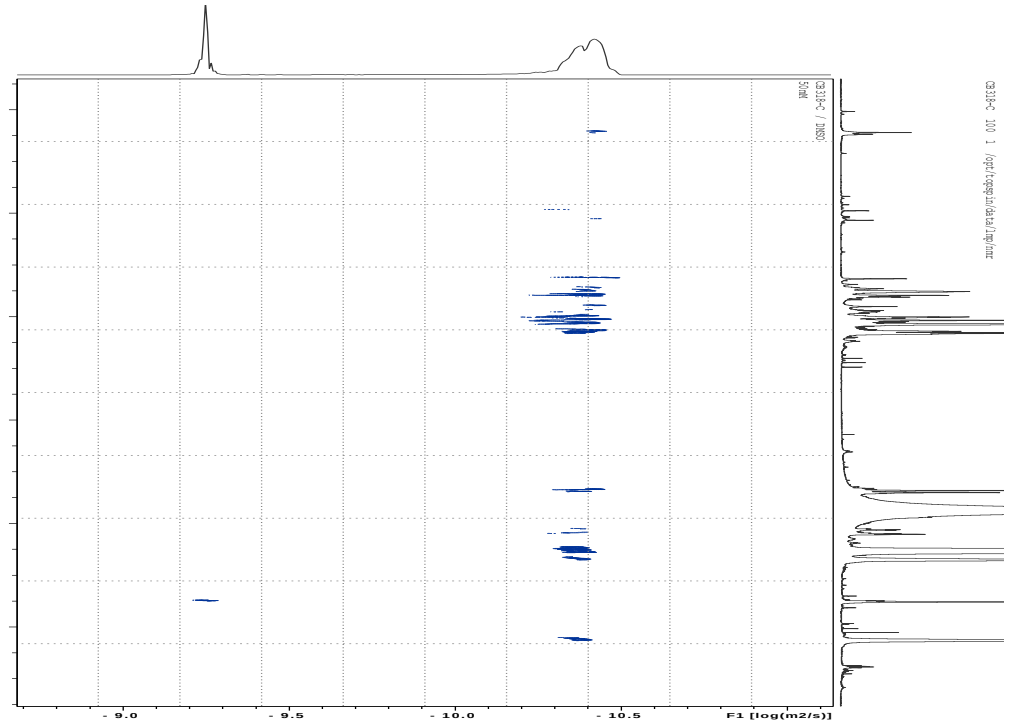


Figure S42: DOSY-NMR spectrum (600 MHz) of **Poly(EG-Gly)** at 50 mM in d_6-DMSO

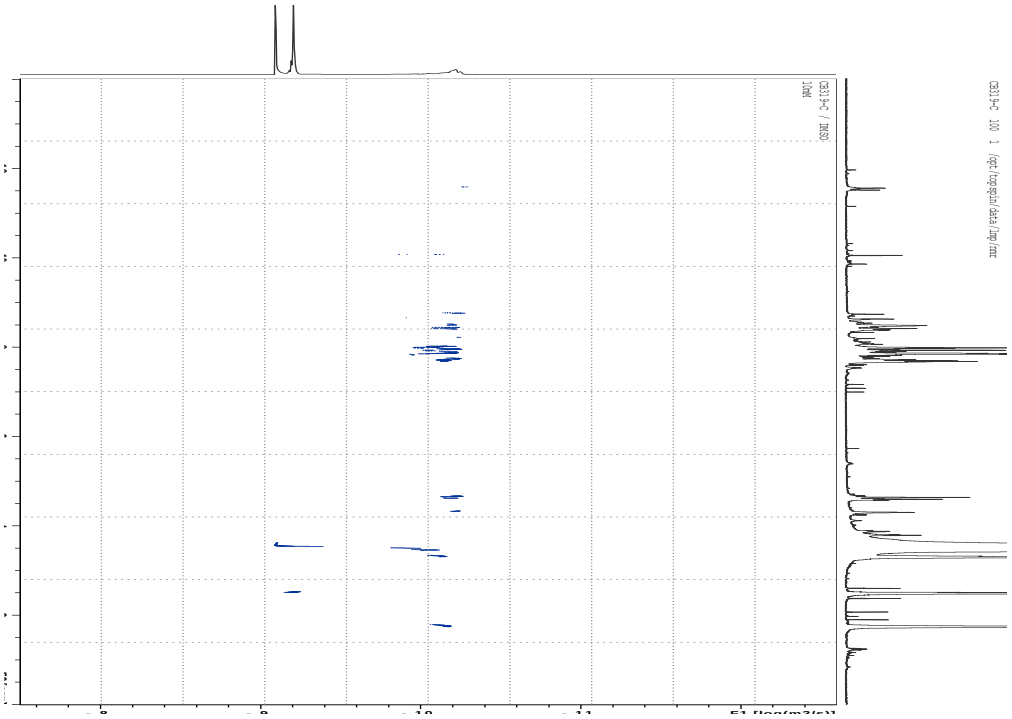


Figure S43: DOSY-NMR spectrum (600 MHz) of **Poly(EG-Gly)** at 10 mM in d_6-DMSO

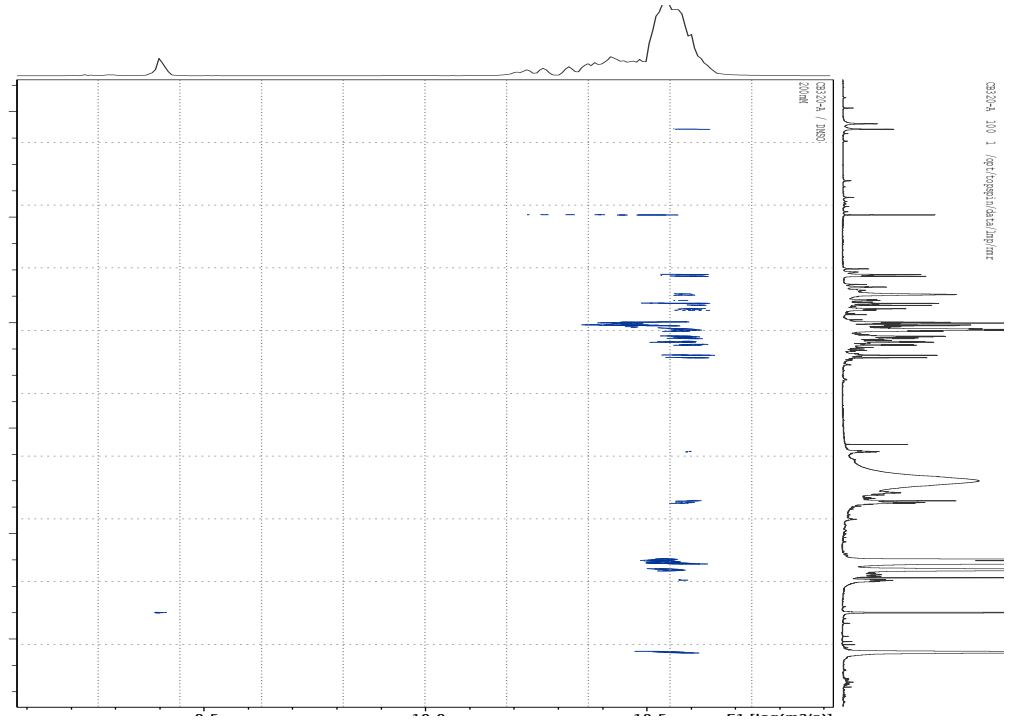


Figure S364: DOSY-NMR spectrum (600 MHz) of **Poly(EG-His)** at 200 mM in d_6-DMSO

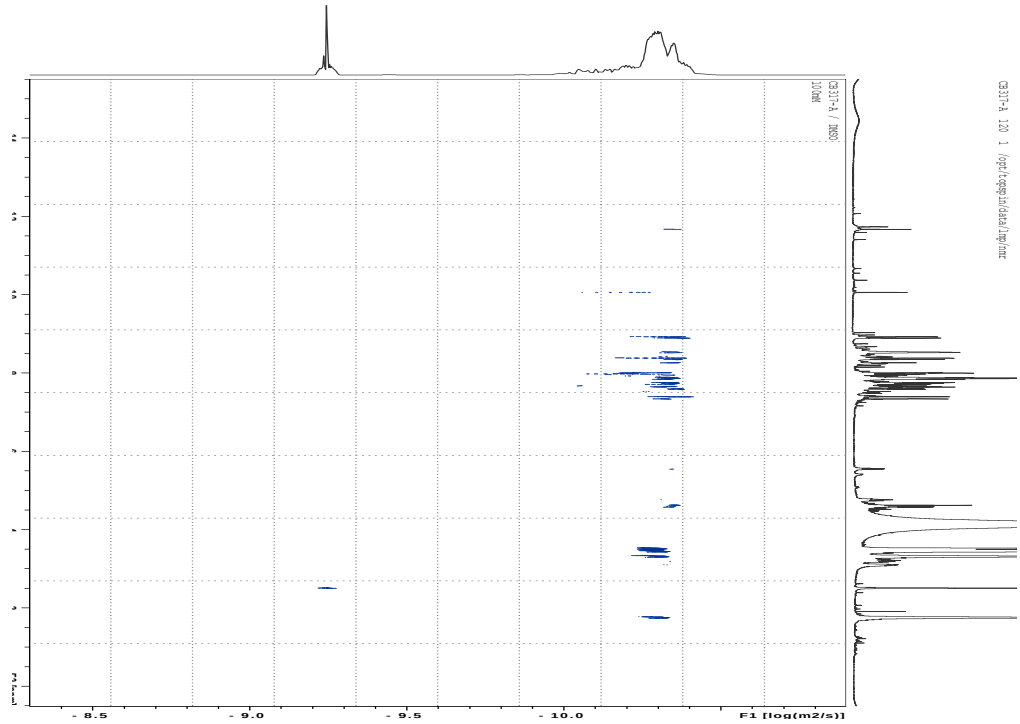


Figure S375: DOSY-NMR spectrum (600 MHz) of **Poly(EG-His)** at 100 mM in d_6-DMSO

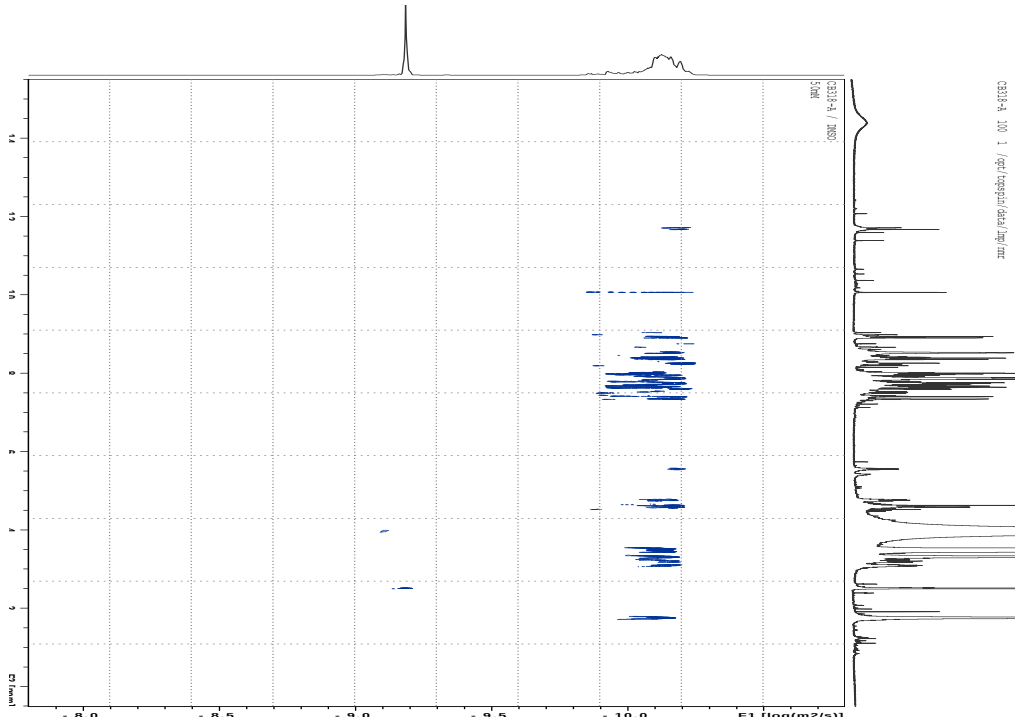


Figure S386: DOSY-NMR spectrum (600 MHz) of **Poly(EG-His)** at 50 mM in d_6-DMSO

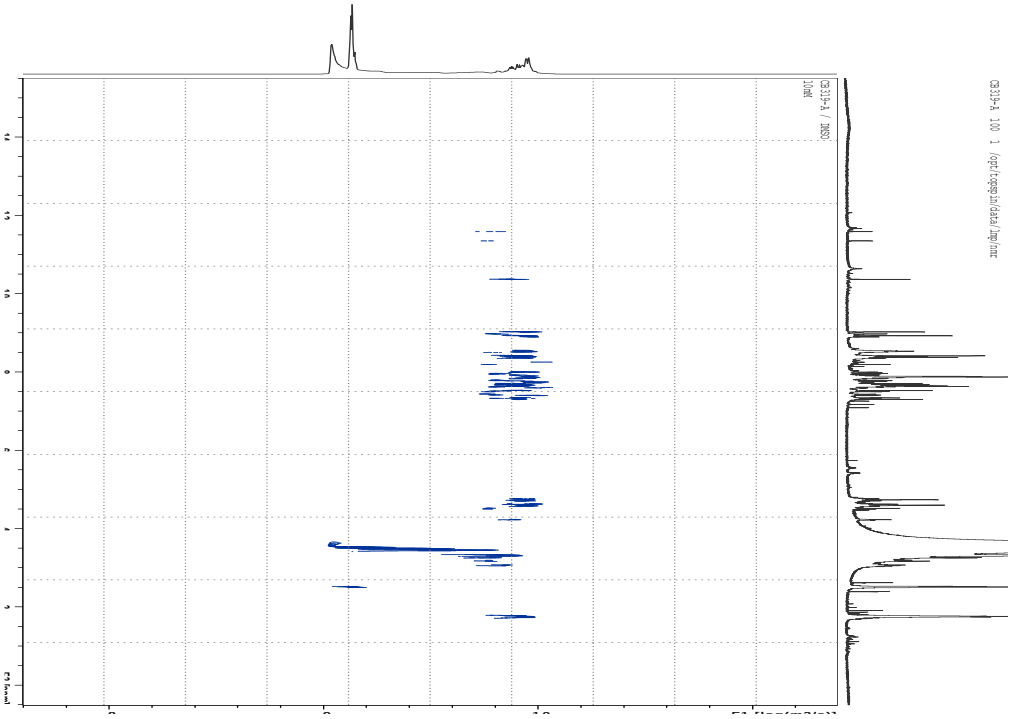
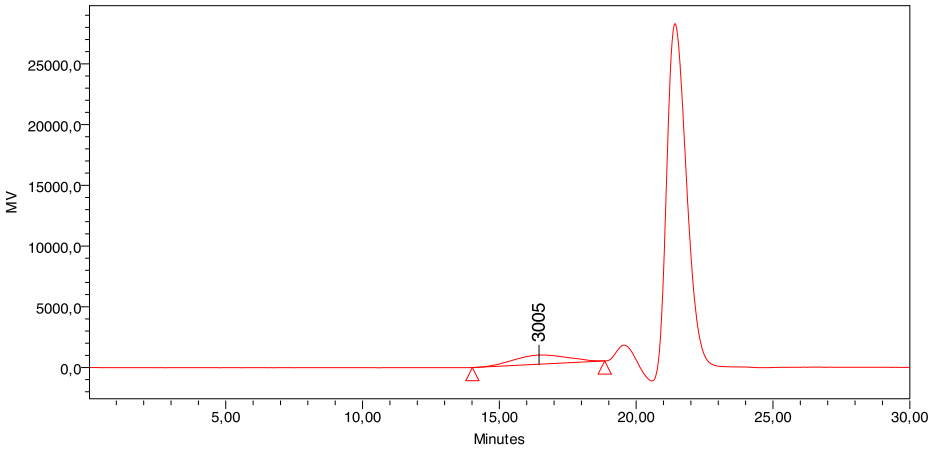


Figure S397: DOSY-NMR spectrum (600 MHz) of **Poly(EG-His)** at 10 mM in d_6-DMSO

6. Gel permeation chromatography



GPC Results

	Dist Name	Mn	Mw	MP	Mz	Mz+1	Mv	Poly dispersity	MW Marker 1	MW Marker 2
1		2144	3651	3005	5718	7880		1,702940		

Figure S408: GPC chromatogram and results of **Poly(EG-Gly)**

7. Gel retardation assay

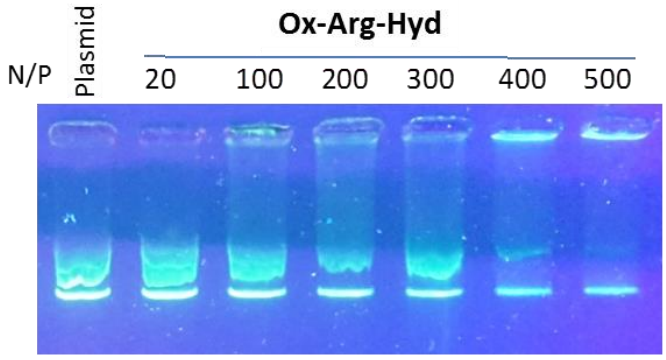


Figure S419: Gel electrophoresis of plasmid DNA with **Ox-Arg-Hyd**.

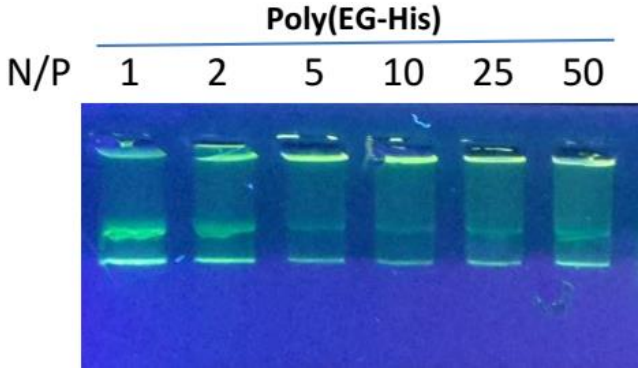


Figure S50: Gel electrophoresis of plasmid DNA with **Poly(EG-His)**. In this case, the polyplexes were prepared in an acidic buffer (100 mM acetate buffer, pH = 5.0).

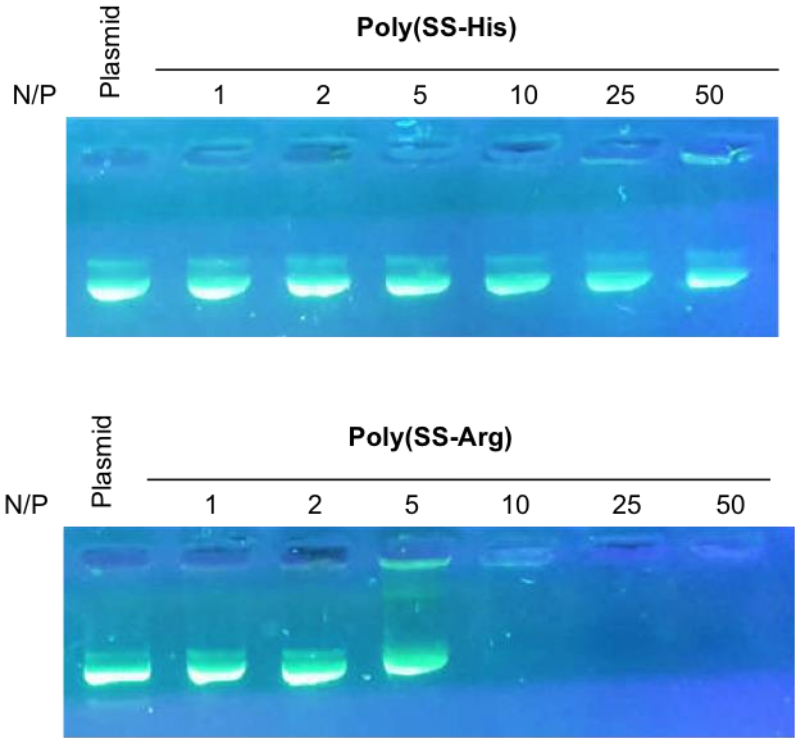


Figure S51: Gel electrophoresis of plasmid DNA with **Poly(S_2-His)** (top) and **Poly(S_2-Arg)** (bottom).

8. References

[1] Ethoxyethylidene protecting group prevents N-overacylation in aminooxy peptide synthesis, V. Dulery, O. Renaudet, P. Dumy, *Tetrahedron*, **2007**, *63*, 11952.

[2] Synthesis of photoaffinity probes of tautomycin, M. Kurono, A. Shimomura, M. Isobe, *Tetrahedron*, **2004**, *60*, 1773.