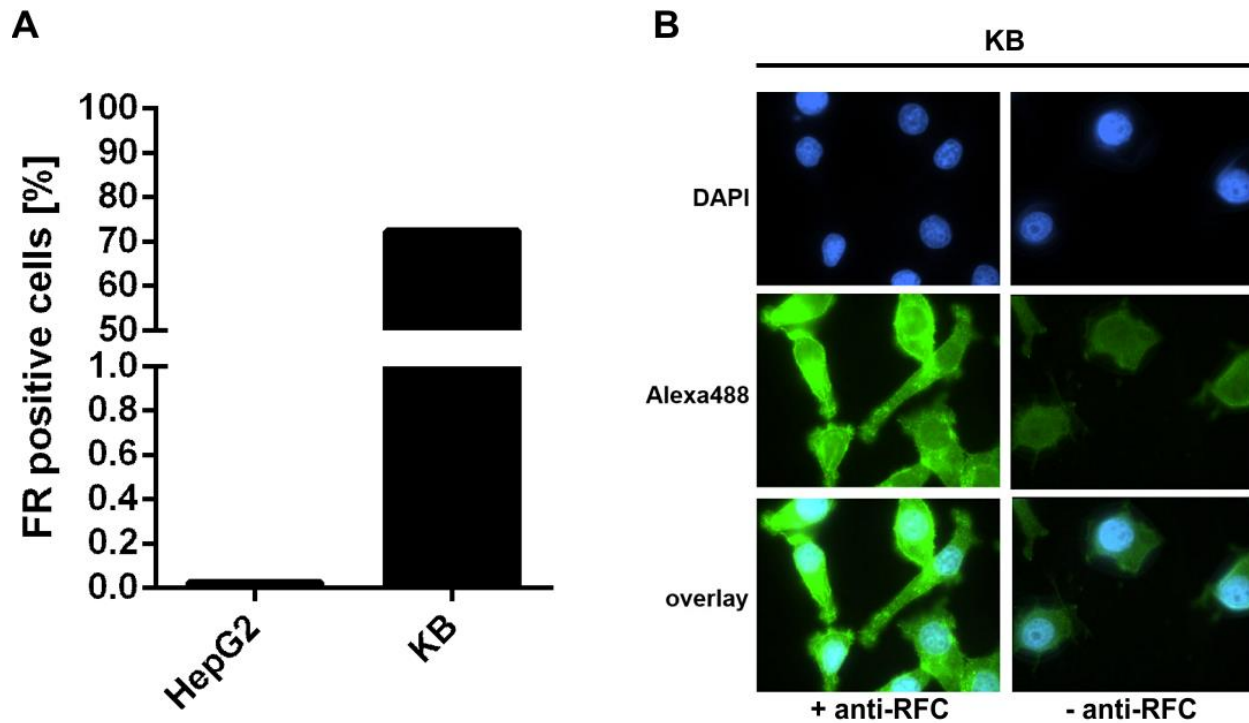


Supporting Information

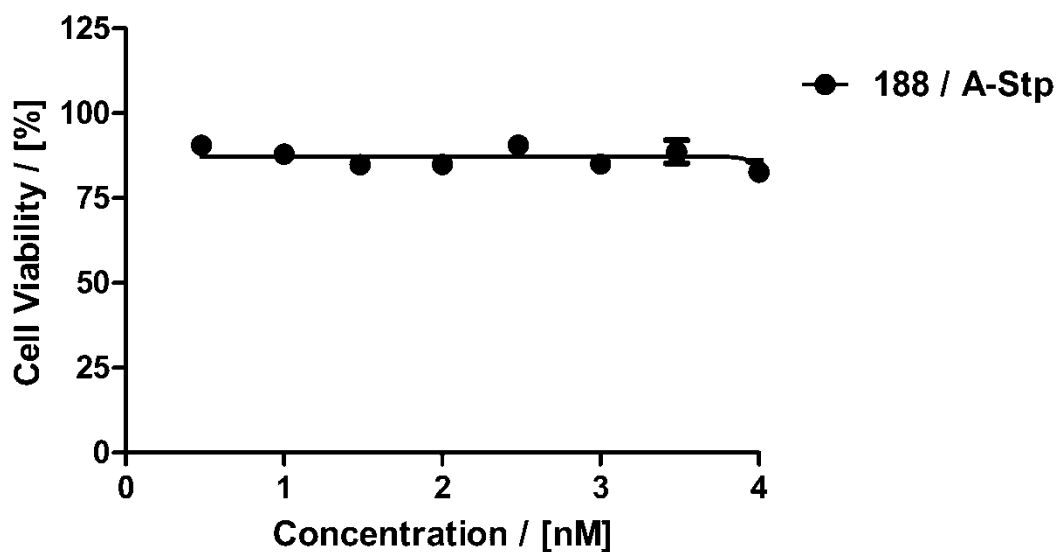
Synthetic polyglutamylation of dual-functional MTX-ligands for enhanced combined cytotoxicity of poly(I:C) nanoplexes.

Ulrich Lächelt, Valentin Wittmann, Katharina Müller, Daniel Edinger, Petra Kos, Miriam Höhn,
and Ernst Wagner.

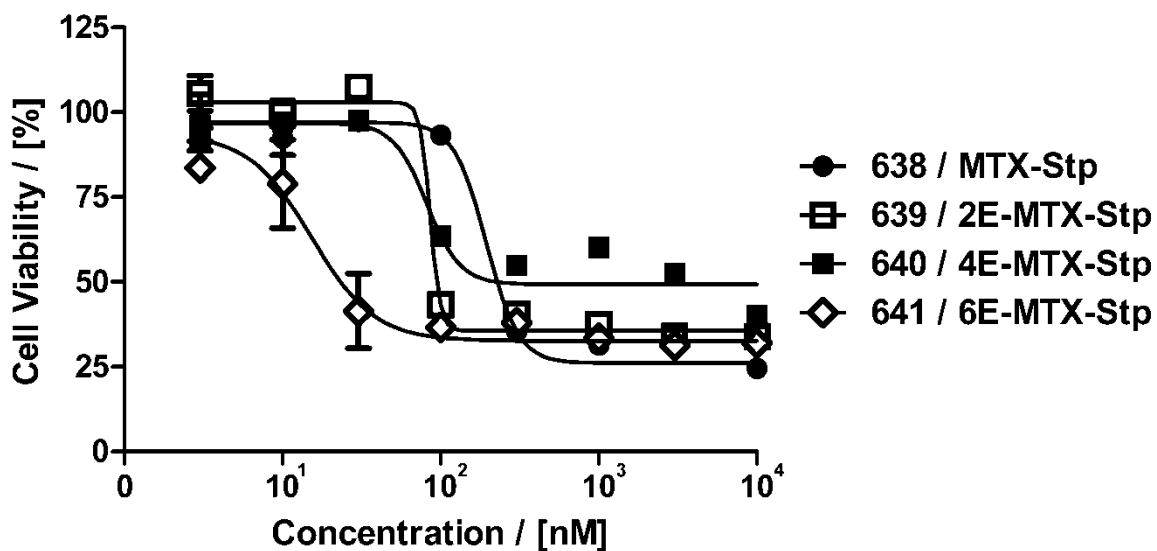
Supplementary Figures	S2
Particle size and zeta potential	S7
¹ H-NMR and RP-HPLC	S8



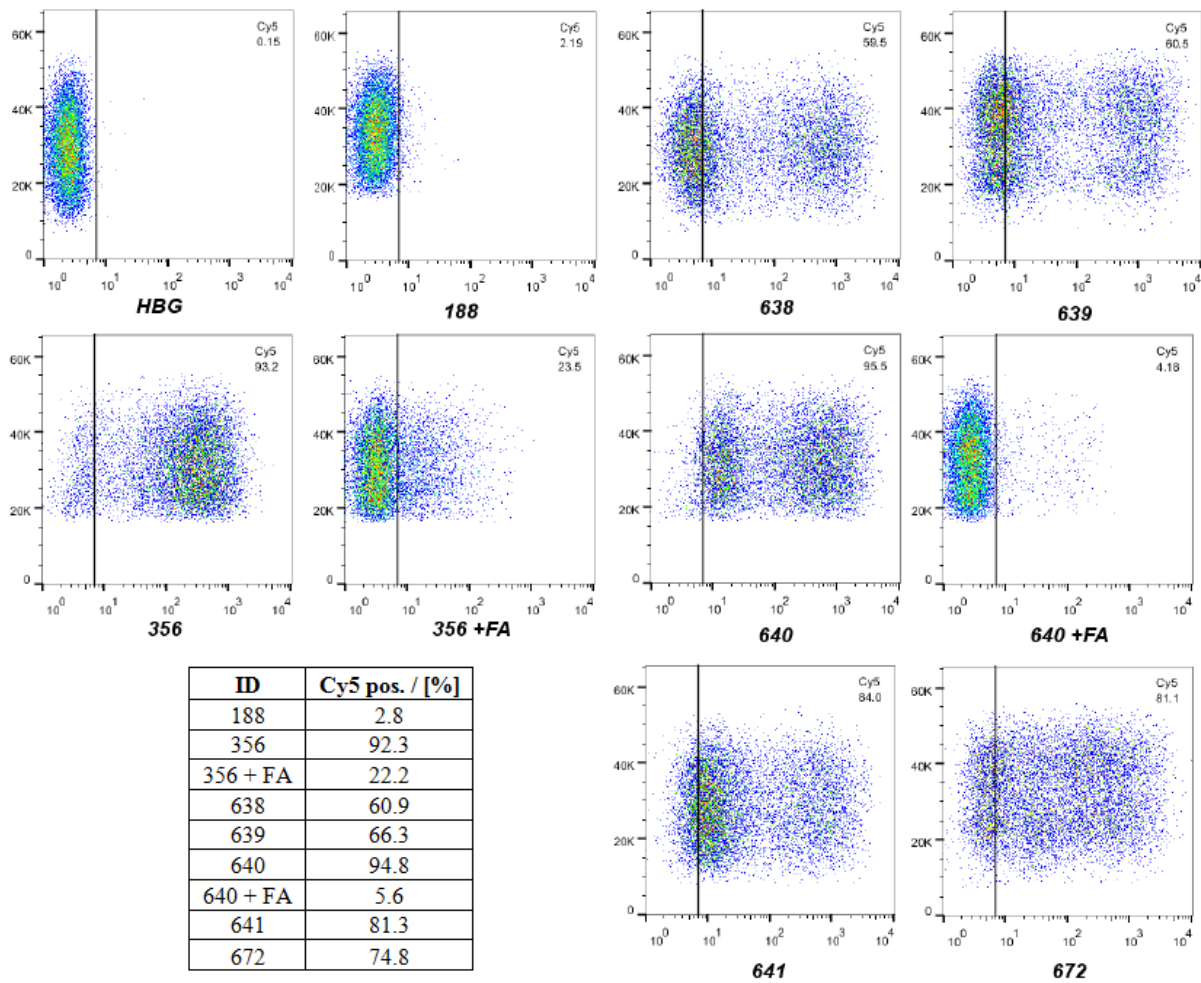
Supplementary Figure 1. (A) Quantification of folic acid receptor positive cells by flow cytometry using fluorescently labeled anti-FR antibody. (B) Qualitative confirmation of reduced folate carrier expression in KB cells by immunofluorescence microscopy using primary anti-RFC antibody and fluorescently labeled secondary antibody.



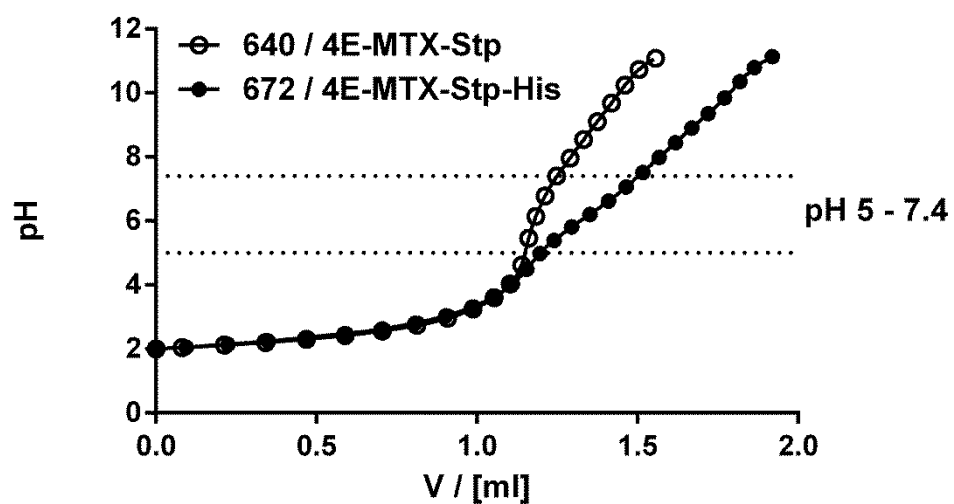
Supplementary Figure 2. Toxicity of oligomer *188* (A-Stp) in KB cells determined by MTT assay. Dose-response curve was approximated by four-parameter logistic model.



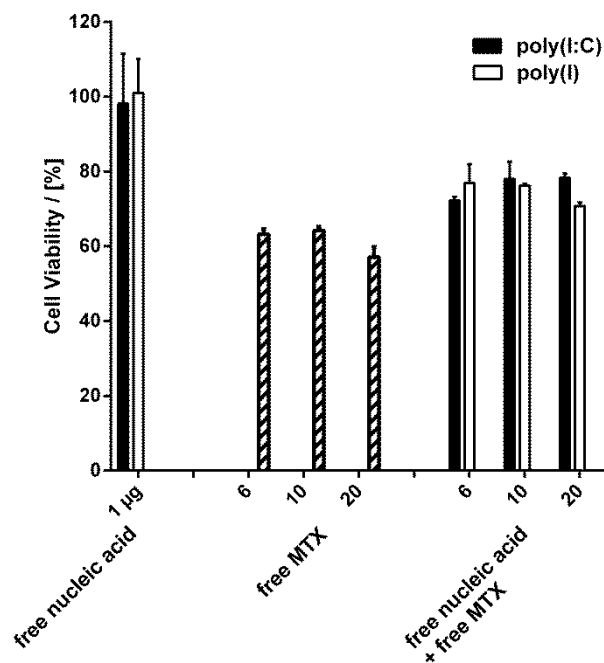
Supplementary Figure 3. MTX conjugate toxicity in KB cells determined by MTT assay. Dose-response curves were approximated by four-parameter logistic models.



Supplementary Figure 4. Representative flow cytometry results of cellular uptake of poly(I:C) polyplexes of various oligomers (ID number indicated) and containing 20% Cy5 labeled poly(I:C). HBG: mock-treated cells, treated with HEPES buffered glucose.



Supplementary Figure 5. Acidimetric titrations of oligomer solutions. Solutions containing 1 μmol of oligomer in 50 mM NaCl were acidified to pH 2 with HCl. Solutions were titrated with 50 mM NaOH solution until pH 11 was reached.



Supplementary Figure 6. Cell viability of KB cells after treatment with free poly(I:C), free poly(I), free MTX or combinations determined by MTT assay. Same conditions like in poly(I:C) transfections were used. Per well 1 µg of nucleic acids was used. The molar amount of free MTX at the values 6, 10, 20 corresponds to the amount of MTX-conjugate in transfections with polyplexes at N/P 6, 10 or 20.

Particle Size and Zeta Potential

Polyplexes comprising 10 μ g poly(I:C) or poly(I) and oligomer at N/P ratio 10 were prepared in 50 μ l HBG as described. Subsequently the solution was diluted to a total volume of 1 ml in 20 mM HEPES (pH 7.4) and measured in a folded capillary cell (DTS1061) with laser light scattering using a Zetasizer Nano ZS with backscatter detection (Malvern Instruments, Worcestershire, UK). For size measurements, the following parameters were chosen: equilibrating time 0 min, temperature 25 °C, refractive index 1.330, viscosity 0.8872 mPa/s. Single exponentials were fit to the correlation function to determine the number mean diameter and polydispersity index (PDI). Each sample was measured three times with 10 subruns of 10 s. For the determination of zeta potential three measurements with 10 to 30 subruns of 10 s at 25 °C were done. Data analysis was carried out with use of the Smoluchowski equation.

Supplementary Table 1. Hydrodynamic diameter (number mean) and zeta potential of oligomer/poly(I:C) polyplexes at N/P 10 (n=3 for each sample).

	Poly(I:C)			Poly(I)		
	Size [nm]	PDI	Zeta-Potential [mV]	Size [nm]	PDI	Zeta-Potential [mV]
638 / MTX-Stp	469 \pm 43	0.114 \pm 0.057	7.6 \pm 0.1	489 \pm 26	0.156 \pm 0.037	9.2 \pm 0.1
639 / 2E-MTX-Stp	597 \pm 36	0.085 \pm 0.092	8.0 \pm 0.4	554 \pm 20	0.072 \pm 0.058	9.1 \pm 0.6
640 / 4E-MTX-Stp	386 \pm 45	0.167 \pm 0.056	6.0 \pm 0.1	546 \pm 17	0.367 \pm 0.065	5.2 \pm 0.2
641 / 6E-MTX-Stp	627 \pm 16	0.119 \pm 0.056	8.7 \pm 0.3	551 \pm 7	0.067 \pm 0.052	7.8 \pm 0.3

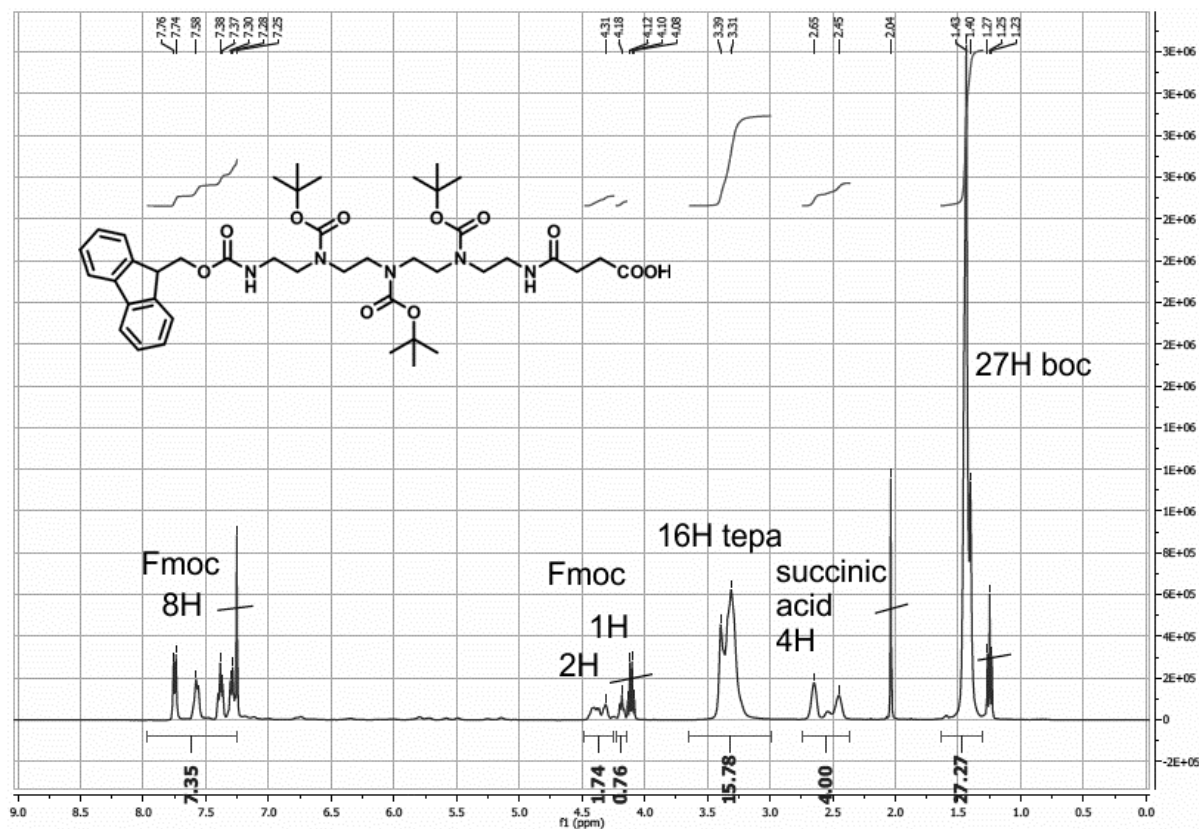
RP-HPLC

Synthesized oligomers were analyzed by RP-HPLC using a Waters HPLC system equipped with a Waters 600E multisolvent delivery system, Waters 996 PDA detector and Waters 717plus autosampler. All structures were analyzed using a Waters Sunfire C18 column (5 μ m, 4.6 x 150 mm) and a water/acetonitrile gradient (5 to 100 % MeCN in 20 min) containing 0.1 % TFA. Detection wavelength was 214 nm.

Proton NMR Spectroscopy

^1H NMR spectroscopy was carried out using a Jeol JNMR-GX 400 (400 MHz) or JNMR-GX 500 (500 MHz). Chemical shifts were calibrated to residual proton signal of the solvent. Spectra were analyzed using MestReNova LITE (Ver. 5.2.5-5780, Mestrelab Research). Integrals were normalized to succinic acid peaks.

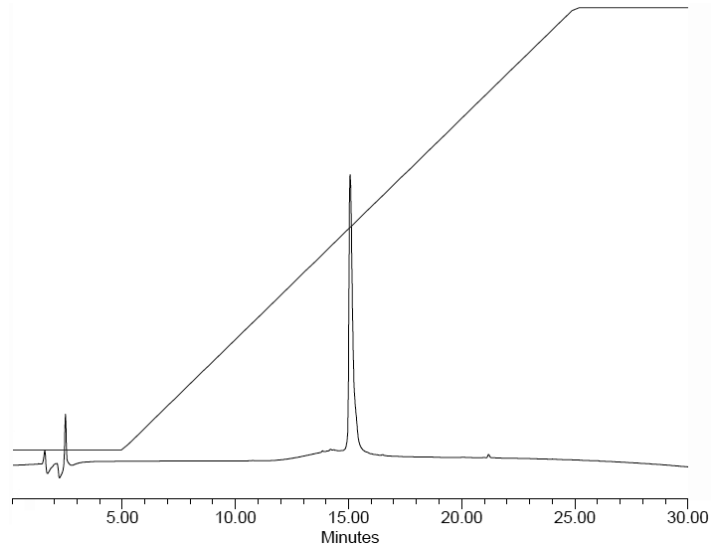
Fmoc-Stp(boc)₃-OH



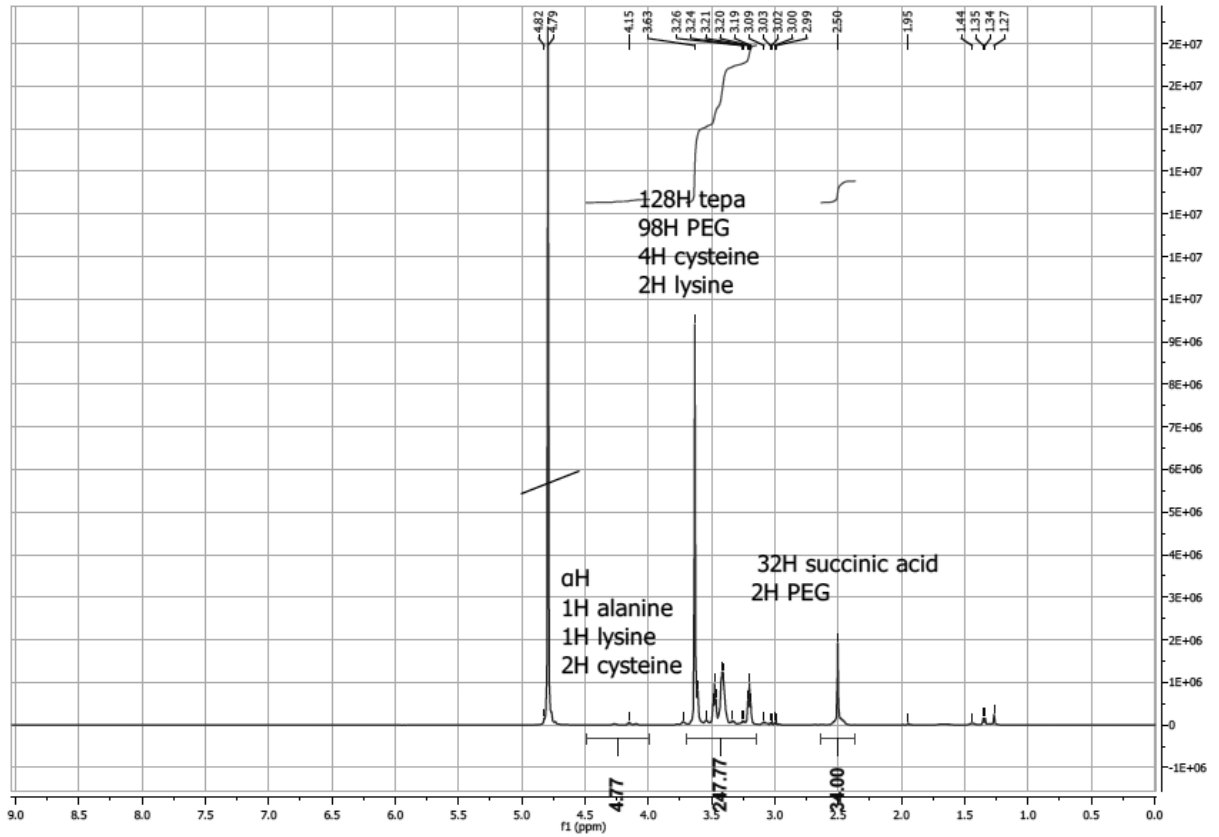
^1H -NMR spectrum in CDCl_3

188 (A-Stp)

Sequence (C->N): A-dPEG₂₄-K(Stp₄-C)₂



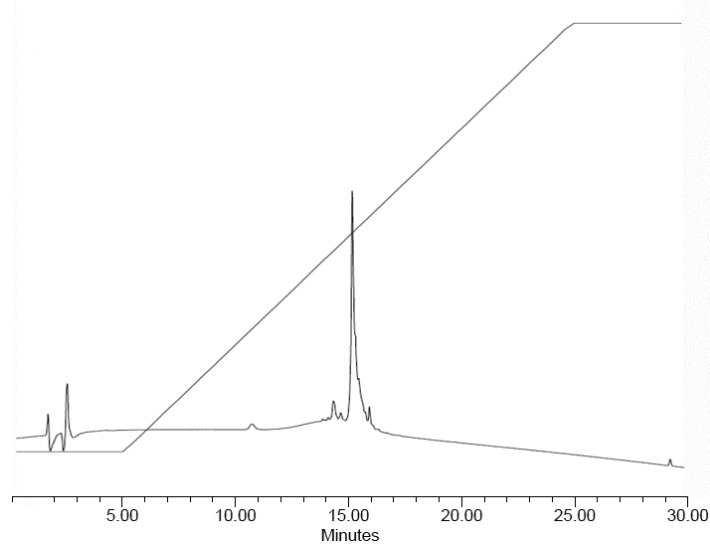
RP-HPLC chromatogram



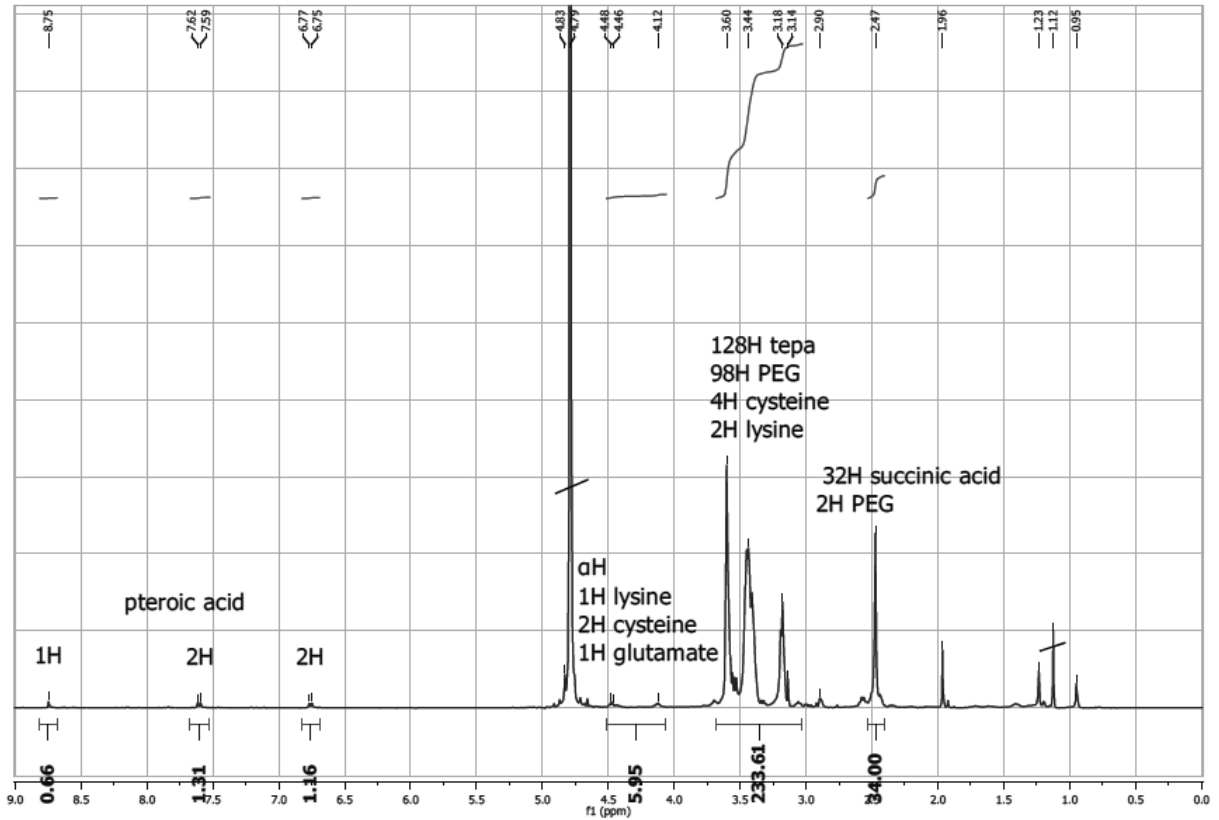
¹H-NMR spectrum in D₂O

356 (FolA-Stp)

Sequence (C->N): C-Stp₄-K(Stp₄-C)-dPEG₂₄-FolA



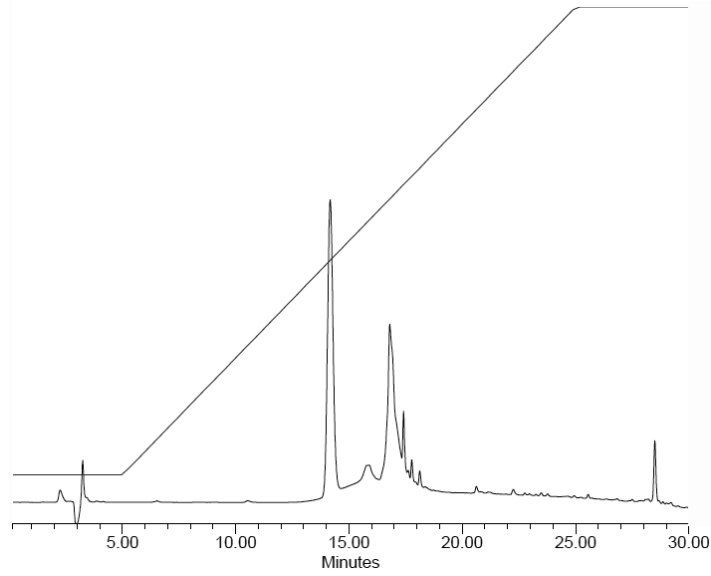
RP-HPLC chromatogram



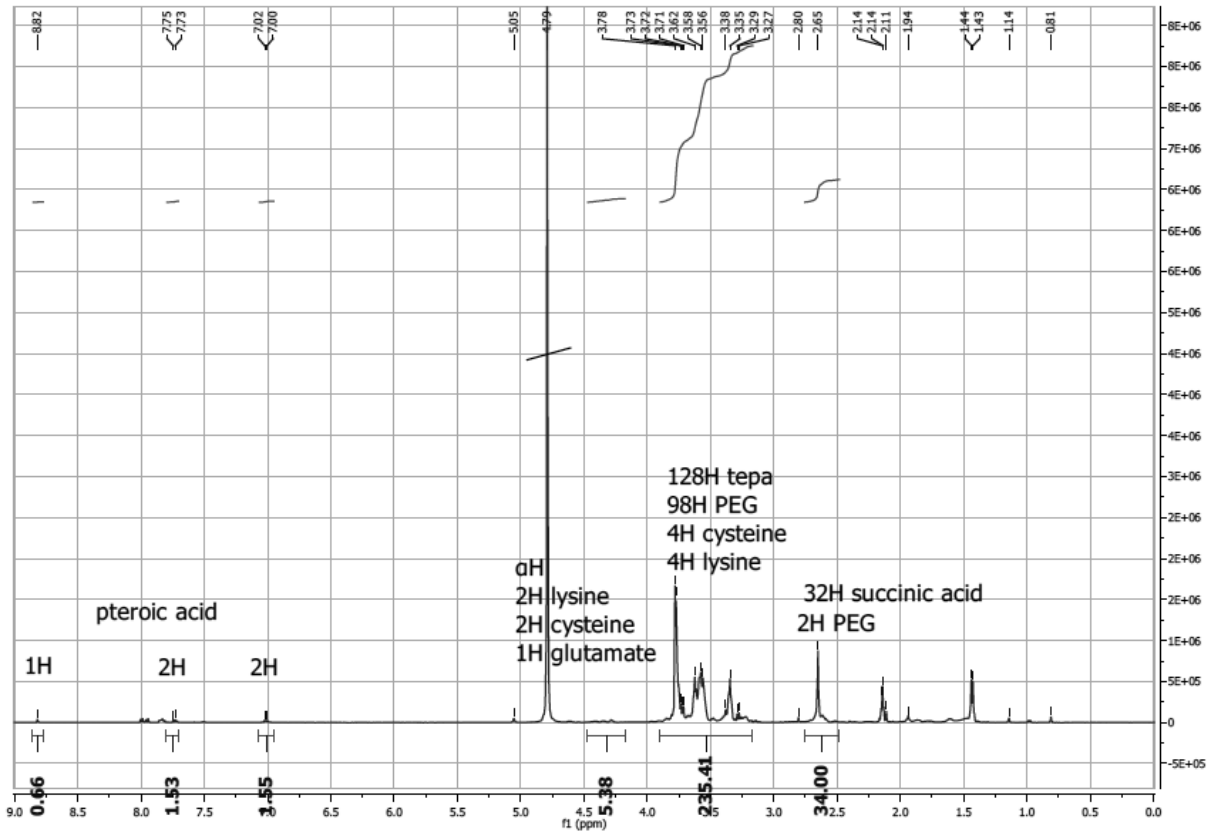
¹H-NMR spectrum in D₂O

638 (MTX-Stp)

Sequence (C->N): K(dPEG₂₄-MTX)-K(Stp₄-C)₂



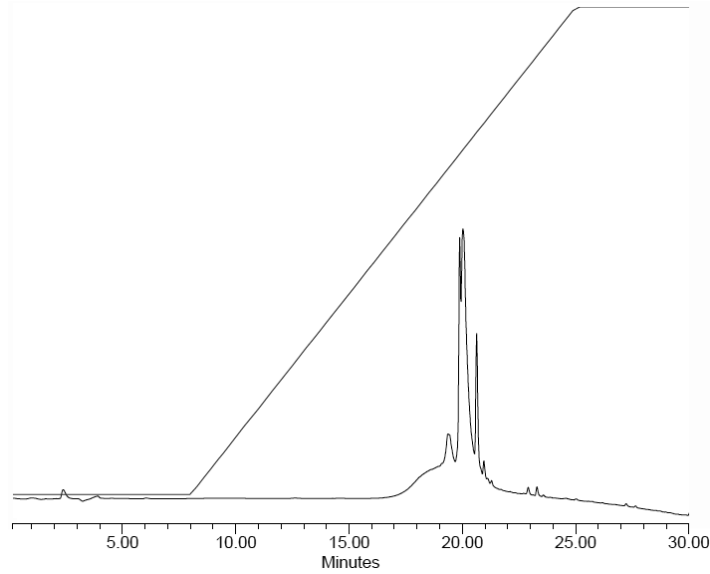
RP-HPLC chromatogram



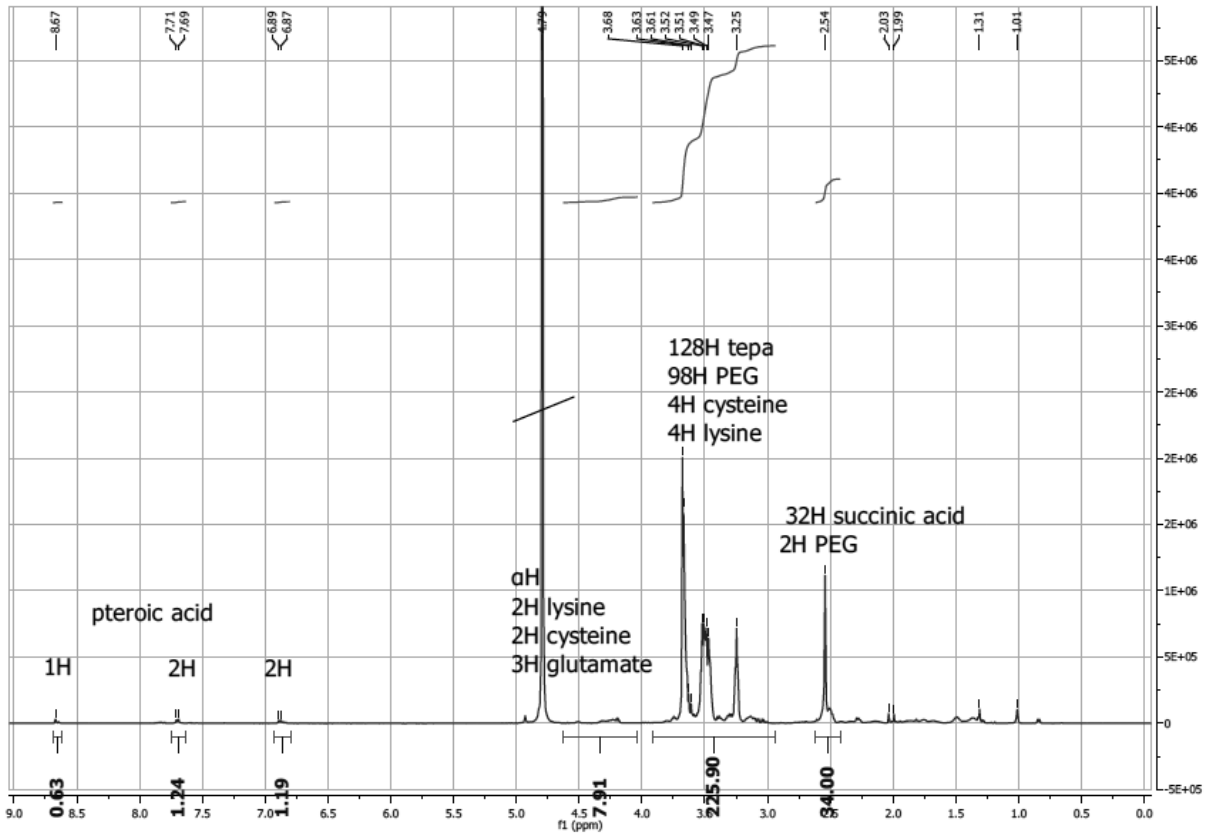
¹H-NMR spectrum in D₂O

639 (2E-MTX-Stp)

Sequence (C->N): K(dPEG₂₄-E₂-MTX)-K(Stp₄-C)₂



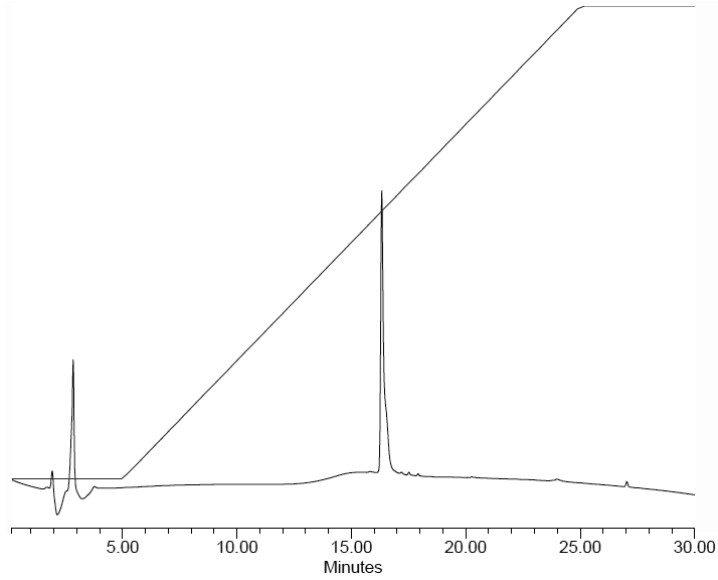
RP-HPLC chromatogram



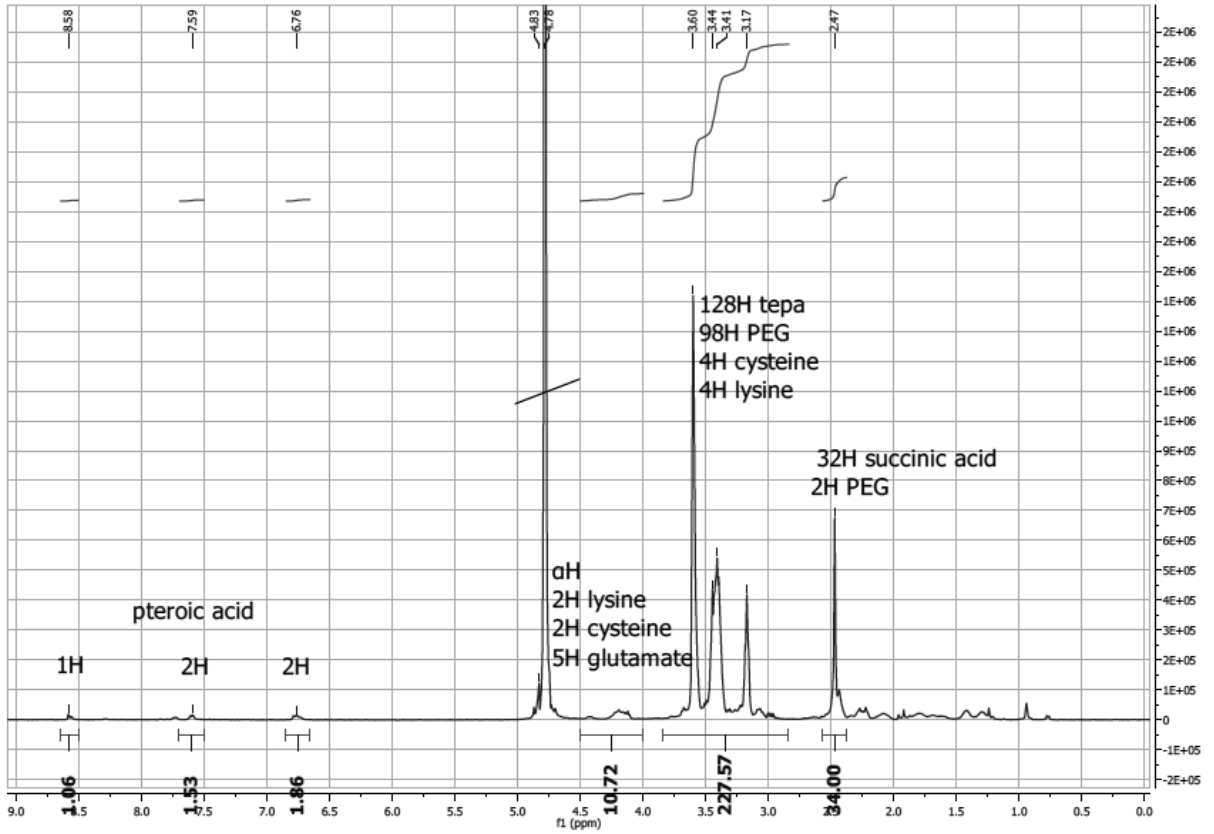
¹H-NMR spectrum in D₂O

640 (4E-MTX-Stp)

Sequenze (C->N): K(dPEG24-E₄-MTX)-K(Stp₄-C)₂



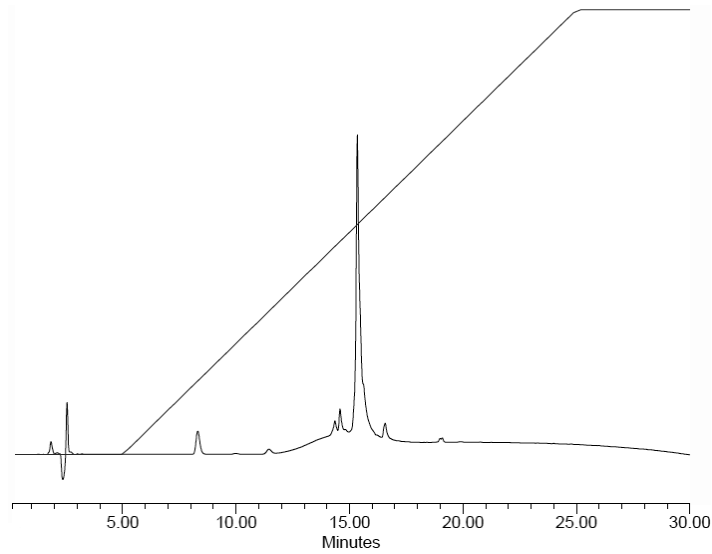
RP-HPLC chromatogram



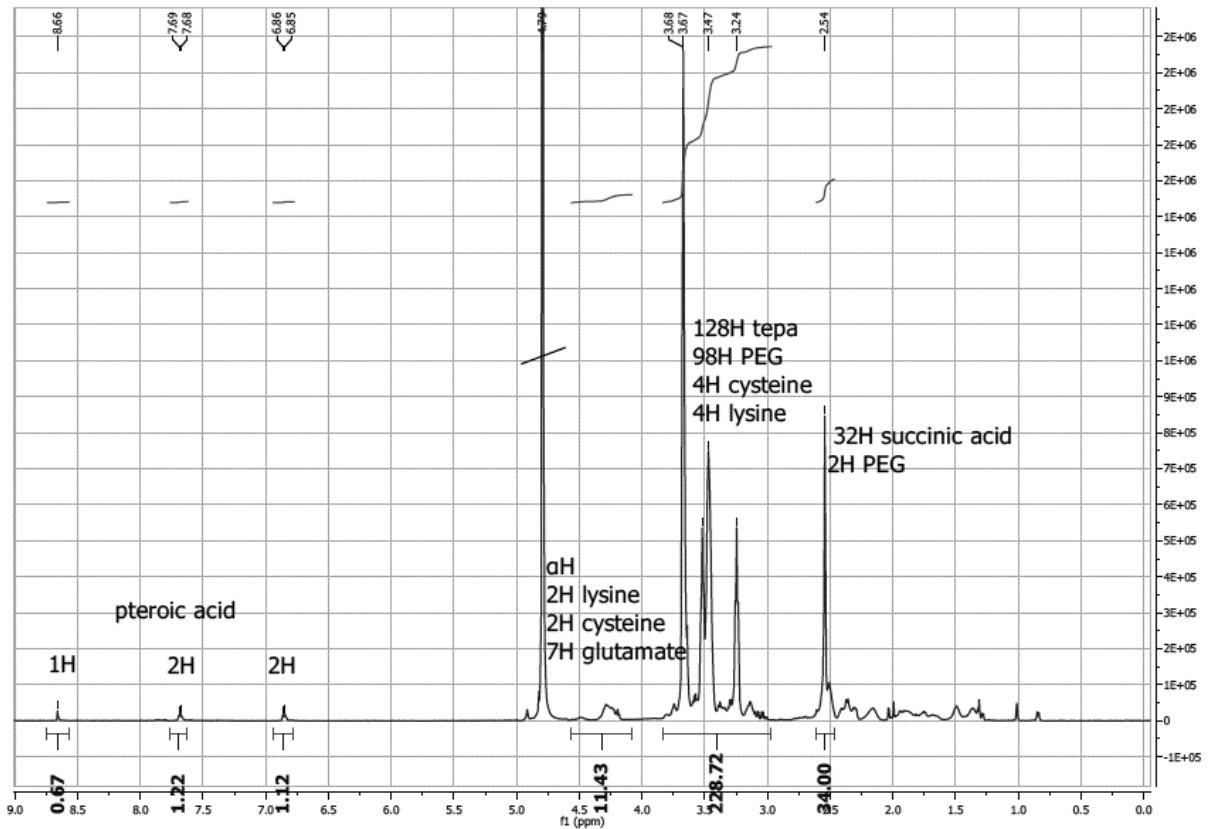
¹H-NMR spectrum in D₂O

641 (6E-MTX-Stp)

Sequence: K(dPEG₂₄-E₆-MTX)-K(Stp₄-C)₂



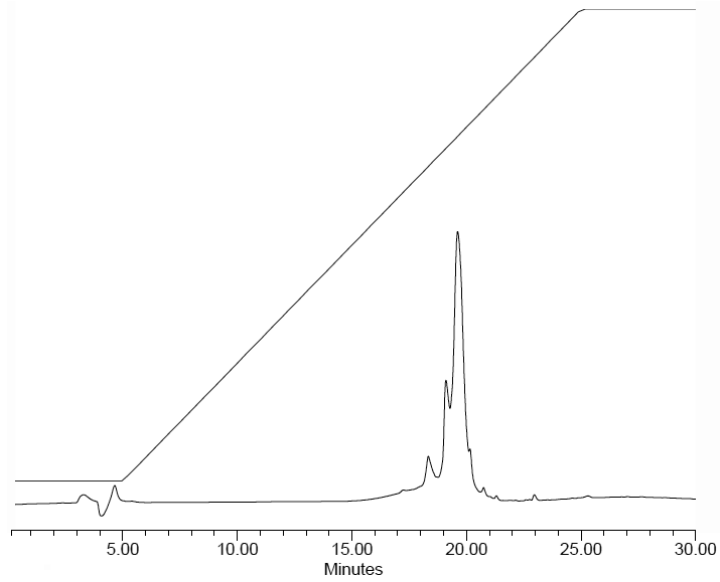
RP-HPLC chromatogram



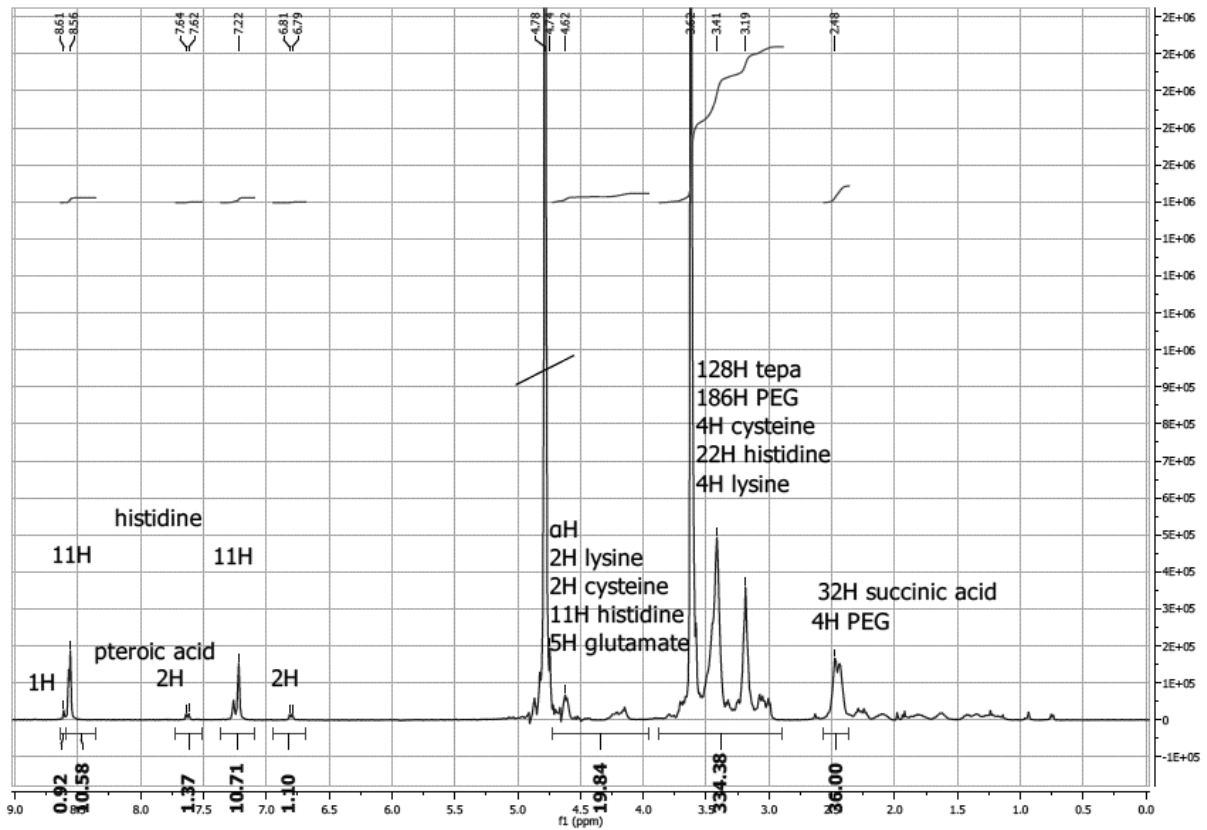
¹H-NMR spectrum in D₂O

672 (4E-MTX-Stp-His)

Sequence: K[(dPEG₂₄)₂]-HK[H-(Stp-H)₄-C]₂



RP-HPLC chromatogram



¹H-NMR spectrum in D₂O