Delivery of Inorganic Polyphosphate into Cells using Amphipathic Oligocarbonate Transporters

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Supplementary Information

Supplementary Figures

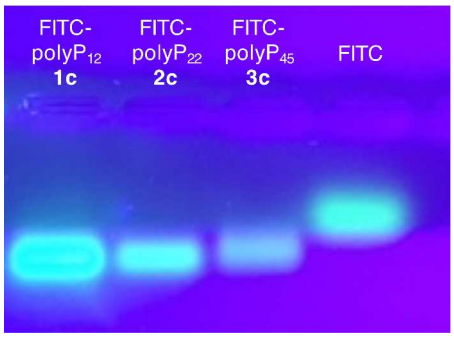
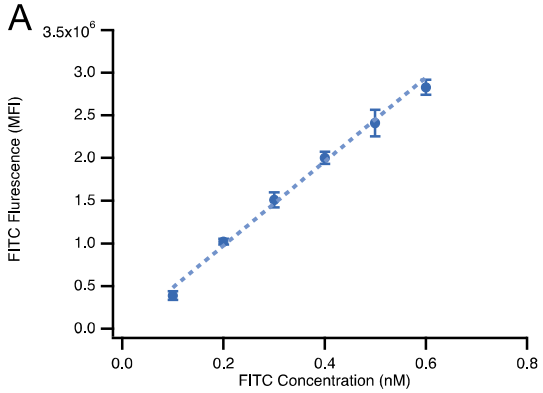


Figure S1. Gel-shift assay of the complexation of varying lengths of polyP-FITC complexed with D_7:G_7 **6** demonstrating fluorescent end-labeling.

A



B

	% FITC labeling	
	^{1}H NMR	Fluorescence
FITC-polyP$_{12}$	21	19
FITC-polyP$_{22}$	11	13
FITC-polyP$_{45}$	33	32

Figure S2. Quantification of FITC end-labeling of polyP. A) Standard curve for FITC fluorescence. B) Amounts of FITC-incorporation determined by ^{1}NMR and fluorescence comparison to FITC standard curve.

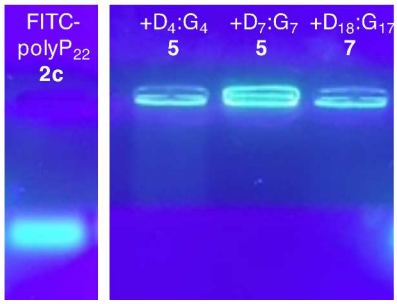


Figure S3. Complexation of FITC-polyP$_{22}$ by guanidinium-rich amphipathic transporters D$_4$:G$_4$ **5**, D$_7$:G$_7$ **6**, and D$_{18}$:G$_{17}$ **7.**

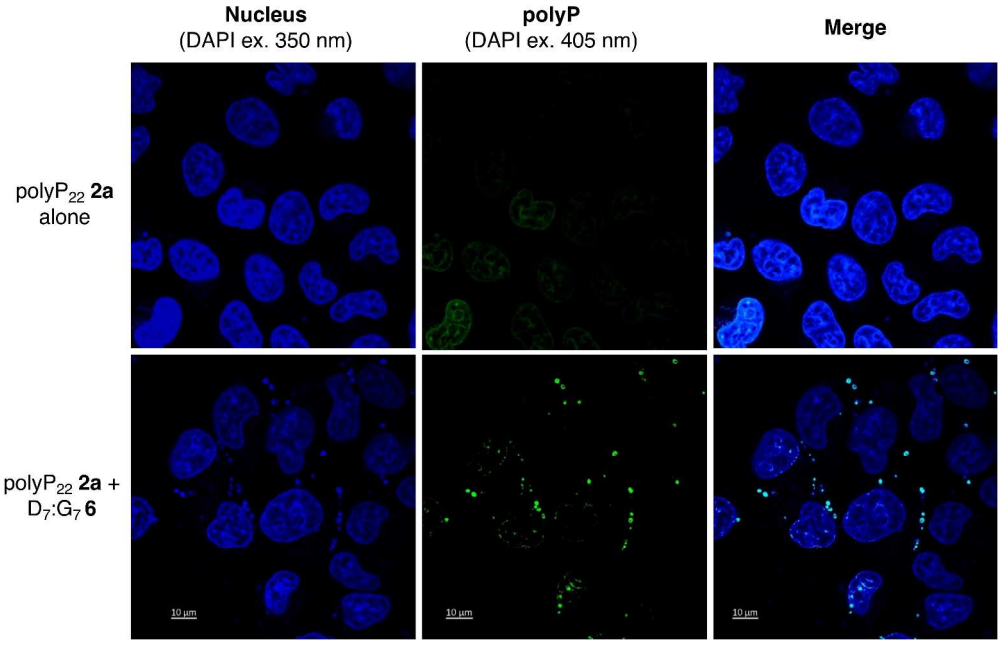


Figure S4. PolyP-specific DAPI staining of cells treated with $polyP_{22}$ **2a** complexed with D_7:G_7 **6** showing the presence of intracellular polyp. Cells were imaged after 4 hours.

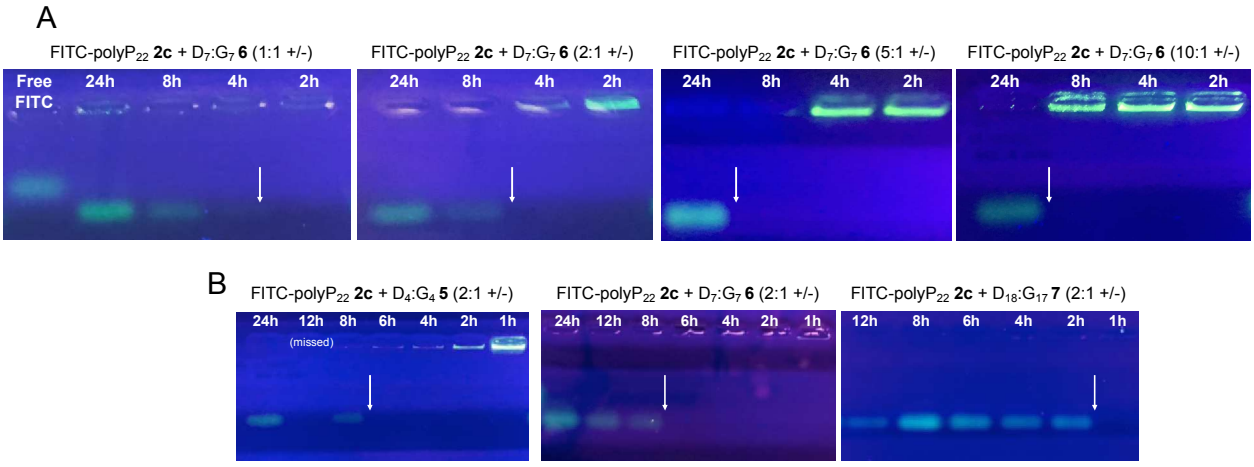


Figure S5. Release of FITC-polyP$_{22}$ **2c** by oligocarbonate transporters over time. A) Release time-dependence on charge ratio of D$_7$:G$_7$ **6** to FITC-polyP$_{22}$ **2c**. B) Release time dependence on length of oligocarbonate transporter used. Arrow indicates first timepoint where free polyP-FITC band becomes visible.

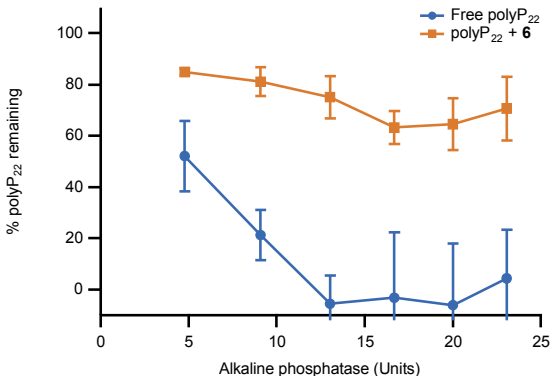


Figure S6. Stability of free polyP$_{22}$ **2a** or complexes of polyP **2a** and oligocarbonate transporter D$_7$:G$_7$ **6** to varying doses of alkaline phosphatase, demonstrating that oligocarbonate transporters protect polyphosphate from phosphatase degradation.

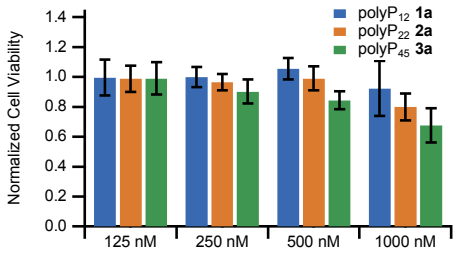


Figure S7. Cell viability data for HeLa cells treated with varying lengths of polyP complexed with D_7:G_7 **6**, as determined using an MTT assay. Values are normalized to untreated cells.

EXPERIMENTAL SECTION

Materials. Inorganic polyphosphates $polyP_{12}$ and $polyP_{22}$ were obtained from Innophos as "Vitrafos® SHMP Crushed" for $polyP_{12}$ (CAS: 68915-31-1) and "Vitrafos® SHMP Long Chain Crushed" for $polyP_{22}$ (CAS: 68915-31-1). $polyP_{45}$ was obtained from Aldrich as "Sodium Phosphate Glass, Type 45". The lengths of all polyphosphates were confirmed by ^{31}P NMR endgroup analysis prior to use. Fluorescein isothiocyanate (FITC) was obtained from Aldrich. Oligocarbonate transporters were prepared as previously described.[1]

Preparation of amine-labeled polyP-NH_2 (1b, 2b, 3b). PolyP (**1a**, **2a**, or **3a**) was dissolved in 1M MES buffer to a final concentration of 50 mM with respect to polymer. 2,2′-(Ethylenedioxy)bis(ethylamine) (20 equiv.) was added to the polyP solutions followed by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) (20 equiv.) and the pH was adjusted to 6.5 with 1M HCl. The mixture was heated to 60°C for 2 hours, after which time the pH of the reaction was increased to 10 with 1M NaOH and the crude product was purified by precipitation into 45 mL ethanol (3x). The precipitate was collected by centrifugation (6 min at 1100 x g) and lyophilized to dryness (SI).

Preparation of fluorescein-labeled polyP (polyP-FITC 1c, 2c, and 3c). polyP-NH_2 (**1b**, **2b**, or **3b**) was dissolved in 0.1 M carbonate buffer (pH 9.0) to a final concentration of 230 mM. Fluorescein isothiocyanate (4 equiv.) was added and the reaction was stirred for 18 hours at room temperature. The crude product was purified by precipitation into 45 mL of ethanol (3x) and collected by centrifugation (15 min at 1100 x g), dissolved in alkaline water (pH ~ 8.0) and then further purified by preparative size exclusion chromatography (Sephadex 25). Samples were lyophilized for 2 days and then evaluated for FITC content by NMR endgroup analysis and fluorimetry relative to a standard curve (SI).

polyP-FITC/transporter Uptake by Flow Cytometry. Hela cells were seeded at 40,000 cells/well in a 24-well plate for 24 h at 37 °C. PolyP-FITC/D_7:G_7 complexes were formed at the indicated charge ratio by mixing varying amounts of a 1.25 mM stock solution of transporter in PBS with the corresponding amount of a 1 mM stock solution of polyP-FITC in PBS into an additional amount of PBS to bring the final polyP concentration to 10 μM. Charge ratios were calculated based on the number of cationic charges on the transporter (4, 7, or 17 for **5**, **6**, and **7**, respectively) and the anionic charges on the polyP conjugate (12, 22, or 45 for **1c**, **2c**, and **3c**, respectively). Polyplexes were incubated for 30 minutes at room temperature, after which time 10 μL of polyplex solutions was added to cell wells into a total volume of 200 μL for a final polyP concentration of 500 nM with respect to polymer chains and incubated for 4 hours. Cells were trypsinized with trypsin-EDTA (0.05%) for 10 min at 37 °C. Serum-containing DMEM was added, and the contents of each well centrifuged, the supernatant removed, and the pelleted cells were re-dispersed in PBS (125 μL) and transferred to FACS tubes, which were read on a flow cytometry analyzer (LSRII.UV, Stanford University). Results were analyzed using FlowJo software. The data presented are the mean fluorescent signals from 10,000 cells analyzed. Error expressed as ± standard deviation (SD) of at least three separate trials.

Gel Stability Assay. A 2% agarose gel was prepared by dissolving 4g of agarose into 200 mL of 1x TAE buffer. The solution was heated to 80°C, allowed to cool down to 60 °C and then set for 1 hour. PolyP/transporter polyplexes were prepared as noted above and then transferred to a heating block at 37 °C for varying amounts of time (0-24 h) before being loaded onto the gel along with 2.0 μL of a 50:50 glycerol/H_2O solution. The gel was run at 106 V for 1 h, after which band migrations were visualized with a UV illuminator and images captured using a CCD camera.

Confocal Microscopy. HeLa cells were plated 40,000 cells / well in a glass-bottom 24-well plate and allowed to incubate at 37°C for 24 hours. PolyP$_{22}$-FITC/D$_7$:G$_7$ complexes at charge ratio 2:1 and final polyP concentration of 500 nM, were made as described for flow cytometry experiments and allowed to incubate at room temperature for 30 minutes. The cells were washed with 0.5 mL serum-free DMEM media, then 0.16 mL serum-free DMEM media was added to wells with untreated cells and 0.2 mL added to wells treated with polyplexes and polyP alone. Next, 40 μL of the polyplex solution was added to each well and the cells were incubated for 4 hours at 37°C. The media was then replaced with 1 mL of fresh, serum-containing DMEM media, and the cells were imaged immediately or after a further 18 hours of incubation. The cells were stained with Hoechst 33342 (5 μg/mL) for 20 minutes, then washed with PBS and then 0.5 mL of PBS was added to each well. Cells were imaged with a Leica TCS SP2 confocal microscope using an HCX APD 63X/0.90 water objective. Images were processed using ImageJ.

PolyP$_{22}$ was visualized intracellularly using DAPI staining as reported by Gomes et al. and Aschar-Sobbi et al.[2,3] Briefly, HeLa cells were incubated with polyP$_{22}$ + D$_7$:G$_7$, or polyP$_{22}$ alone for 4 hours in serum-free media. Then the cells were washed once with PBS and incubated with DAPI, 50 ug/mL, in complete medium, for 30 minutes. After that, the medium containing DAPI was removed, the cells were washed once with PBS and fresh media was added. The DAPI vound to PolyP$_{22}$ was evaluated under confocal microscopy using a 405 nm laser for excitation and emission between 500-600 nm.

Alkaline Phosphatase Assay. PolyP$_{22}$ **2a**/D$_7$:G$_7$ **6** complexes were formed as described for cell treatments except using 50 mM Tris-HCl (pH 9.5) instead of PBS. After complexes were formed (varying either charge ratio or incubation time as described in text), complexes were incubated with purified alkaline phosphatase enzyme (Sigma) ranging from 4 U to 23 U for 20

minutes at room temperature. For all evaluated conditions, polyP$_{22}$ alone, with or without the enzyme was used as positive and negative controls, respectively. The concentration of phosphate formed was quantified using a previously reported malachite green assay.[4] Briefly, a solution of 0.4 % w/v of malachite green in water was prepared and stirred for 30 minutes. Then, a solution of ammonium molybdate (4.2 % w/v) in 5 M HCl was added to malachite green solution (1:3 v/v). After filtering, 160 μL of the malachite green ammonium molybdate was added to a final volume of 40 uL of polyP$_{22}$ D_7:G_7 + enzyme and incubated. After 30 minutes the absorbance was read at 660 nm and free phosphate concentration was determined relative to a standard curve of monophosphate of concentrations from 12 mM to 0.05 mM.

Size Measurements and Zeta Potential for Polyplexes. Dynamic light scattering (DLS) was used to determine the size of the polyplexes. PolyP/D_7:G_7 and polyP-FITC/ D_7:G_7 complexes were formed at a ± charge ratio 2:1 and were prepared as described for flow cytometry experiments. Complexes were immediately transferred to a disposable clear plastic cuvette, with 125 uL of PBS and their size measured by DLS. Size measurements were taken at the initial time (0 min), 15 min, 30 min, 45 min, and 60 min. The sizes were reported as the z-average values, and are reported as the average of three different trial runs. Zeta potential measurements were taken by diluting the polyplexes formulated for DLS into 1000 μL of water, transferring to zeta cell (DTS1060), and measuring zeta potential. The values reported are the average of a minimum of three trial runs. Error is expressed as ± SD.

MTT Toxicity Assay. HeLa cells were seeded at 5,000 cells per well in a 96-well plate for 24 h at 37 °C. PolyP/D_7:G_7 complexes were formed at a charge ratio of 2:1 as above resulting in final polyP concentrations from 125 nM to 2000 nM. The complexes were allowed to incubate at room temperature for 30 min. Cells were washed with serum-free DMEM medium, and 200 μL

of serum-free DMEM medium was added to the untreated wells and 195 μL of serum-free DMEM medium was added to the wells treated with polyplexes. Then, 5 μL polyP/D_7:G_7 complexes or polyP alone was added to each respective well followed by a serial dilution of 100 μL into 100 μL across 4 wells. The cells were incubated with the compounds for 4 h at 37 °C, and then the medium was removed and replaced with 100 μL of fresh, serum-containing DMEM medium. The cells were incubated for a further 48 h. Viability was assayed by adding 10 μL of MTT solution (5 mg/mL in DMEM medium). After 2 h of incubation at 37 °C, 100 μL of solubilizing solution (10% Triton-X-100, 90% 0.1 N HCl in isopropanol) was added to each well, and colorimetry data was obtained on a plate reader. Percent viability was determined by dividing the average colorimetric value obtained for a treated sample by the average colorimetric value obtained for untreated cells. The values reported are the average of a minimum of three different trial runs ($n \geq 3$). Error is expressed as ± SD.

Additional Characterization Data:

PolyP$_{12}$-bis(2,2-(ethylenedioxy)bis(ethylamine)) **1b**
^{1}H NMR (D_2O, 500 MHz): δ: 3.76 (m, 16H, -CH_2O-), 3.24 (m, 4H, -CH_2NH_2), 3.19 (m, 4H, -CH_2NHP).
^{13}C NMR (D_2O, 500 MHz): δ: 71.16, 69.67, 69.47, 66.71, 41.00, 39.23.
^{31}P NMR (D_2O, 400 MHz): δ: -0.67 (d, 2P, end phosphates), -21.41-21.64 (20P, internal phosphates).

PolyP$_{22}$-bis(2,2-(ethylenedioxy)bis(ethylamine)) **2b**
^{1}H NMR (D_2O, 500 MHz): δ: 3.79 (m, 16H, -CH_2O-), 3.23 (m, 4H, -CH_2NH_2), 3.11 (m, 4H, -CH_2NHP).
^{13}C NMR (D_2O, 500 MHz): δ: 71.20, 69.70, 69.50, 66.68, 41.01, 39.25.
^{31}P NMR (D_2O, 400 MHz): δ: -1.90(d, 2P, end phosphates), -22.82-22.96 (s, 20P, internal phosphates).

PolyP$_{45}$-bis(2,2-(ethylenedioxy)bis(ethylamine)) **3b**
^{1}H NMR (D_2O, 500 MHz): δ: 3.76 (m, 16H, -CH_2O-), 3.24 (m, 4H, -CH_2NH_2), 3.19 (m, 4H, -CH_2NHP).
^{13}C NMR (D_2O, 500 MHz): δ: 71.24, 69.70, 69.46, 66.66, 41.17, 39.26.
^{31}P NMR (D_2O, 400 MHz): δ: -0.60 (d, 2P, end phosphates), -21.57 (20P, internal phosphates).

FITC-PolyP$_{12}$-bis(2,2-(ethylenedioxy)bis(ethylamine)) **1c**
^{1}H NMR (500 MHz, D_2O) δ 7.56 (m, 0.43H), 7.42 (m, 0.40H), 7.21 (m 0.43H), 7.11 (m, 0.95H), 6.48 (m 1.67H), 3.63-3.49 (m, 18H), 3.07-2.95 (m, 8H).
^{31}P NMR (162 MHz, D_2O) δ -1.79, -22.54.

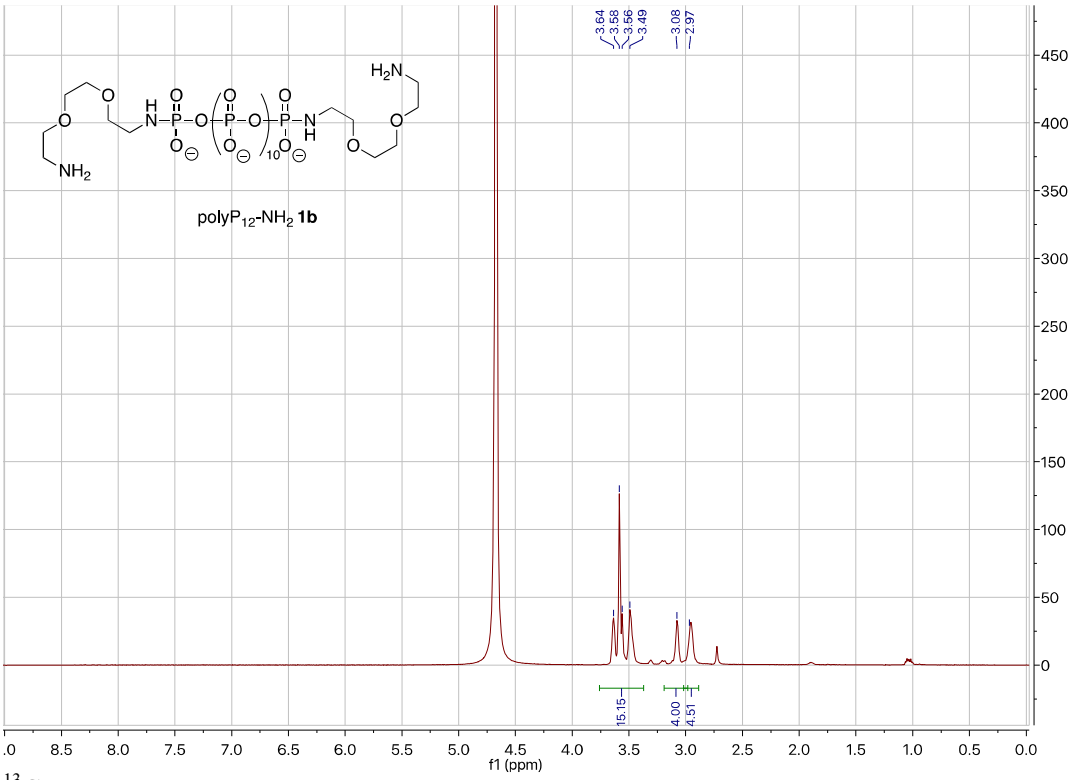
FITC-PolyP$_{22}$-bis(2,2-(ethylenedioxy)bis(ethylamine)) **2c**
^{1}H NMR (400 MHz, D_2O) δ 7.56 (m, 0.2H), 7.41 (m, 0.24H), 7.20 - 7.10 (m, 0.72H), 6.46 (m, 0.83H), 3.56- 3.47 (m, 18H), 2.95 (m, 8H).
^{31}P NMR (162 MHz, D_2O) δ -1.71, -22.69.

FITC-PolyP$_{45}$-bis(2,2-(ethylenedioxy)bis(ethylamine)) **3c**
^{1}H NMR (400 MHz, D_2O) δ 7.54 (m, 0.49H), 7.41 (m, 0.53H), 7.19 (m, 0.75H), 7.09 (m, 1.67H), 6.46 (m, 2.80H), 3.57-3.44 (m, 18H), 2.92 (m, 8H).
^{31}P NMR (162 MHz, D_2O) δ 0.16, -9.04, -22.67.

Compiled Spectra of polyP conjugates:

PolyP$_{12}$-bis(2,2-(ethylenedioxy)bis(ethylamine)) **1b**

^{1}H NMR:



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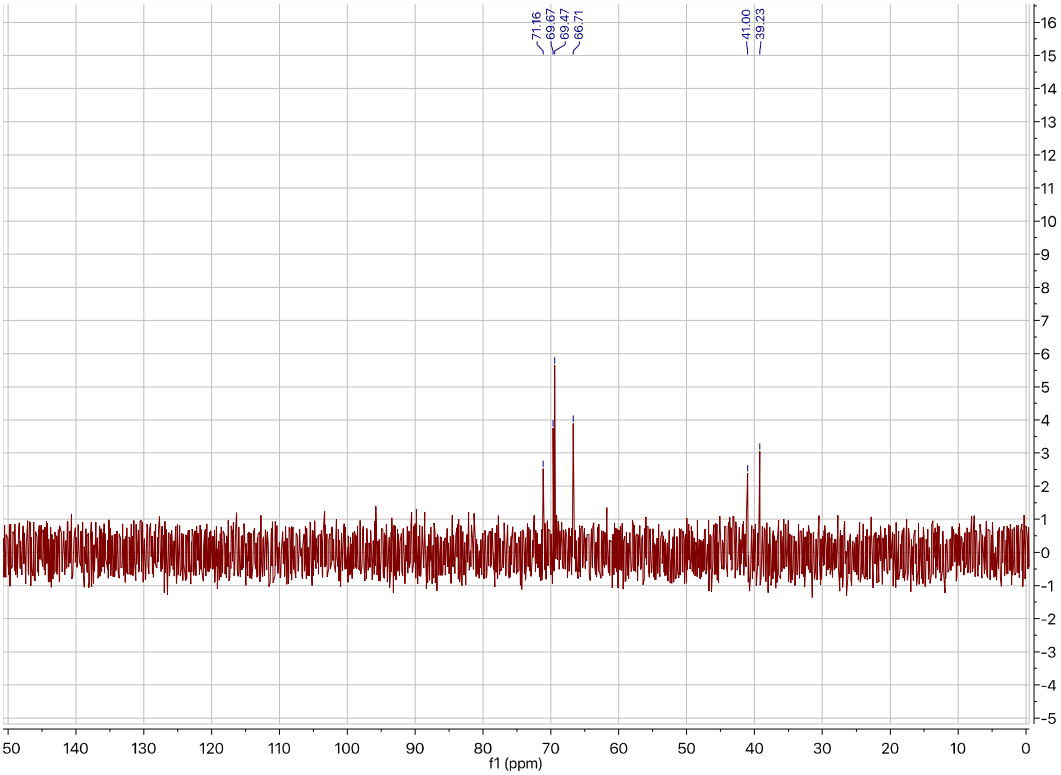

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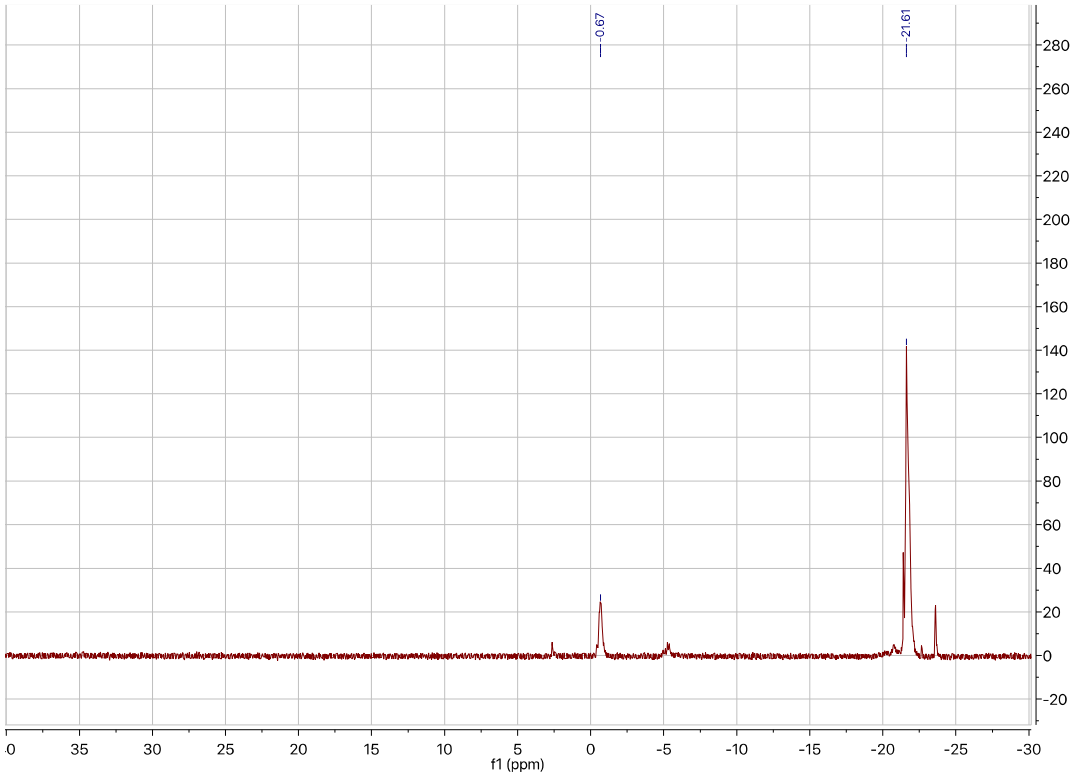
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260
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220
200
180
160
140
120
100
80
60
40
20
0
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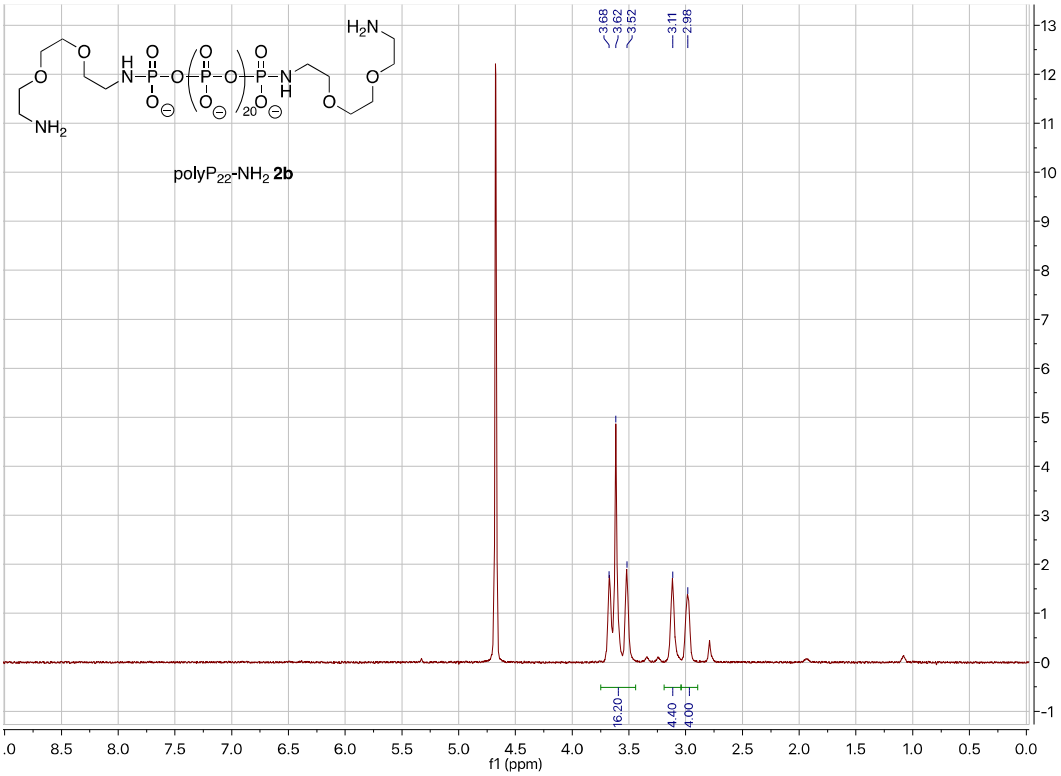
35
30
25
20
15
10
5
0
-5
-10
-15
-20
-25
-30
f1 (ppm)

PolyP$_{22}$-bis(2,2-(ethylenedioxy)bis(ethylamine)) **2b**

^{1}H NMR:



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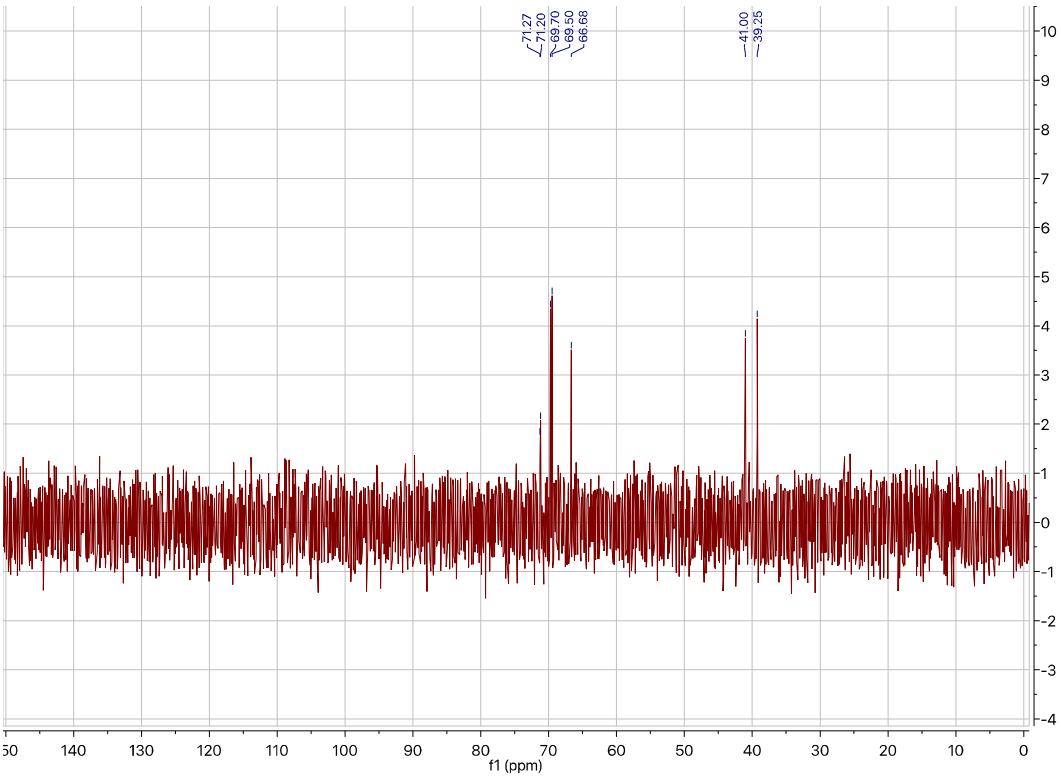

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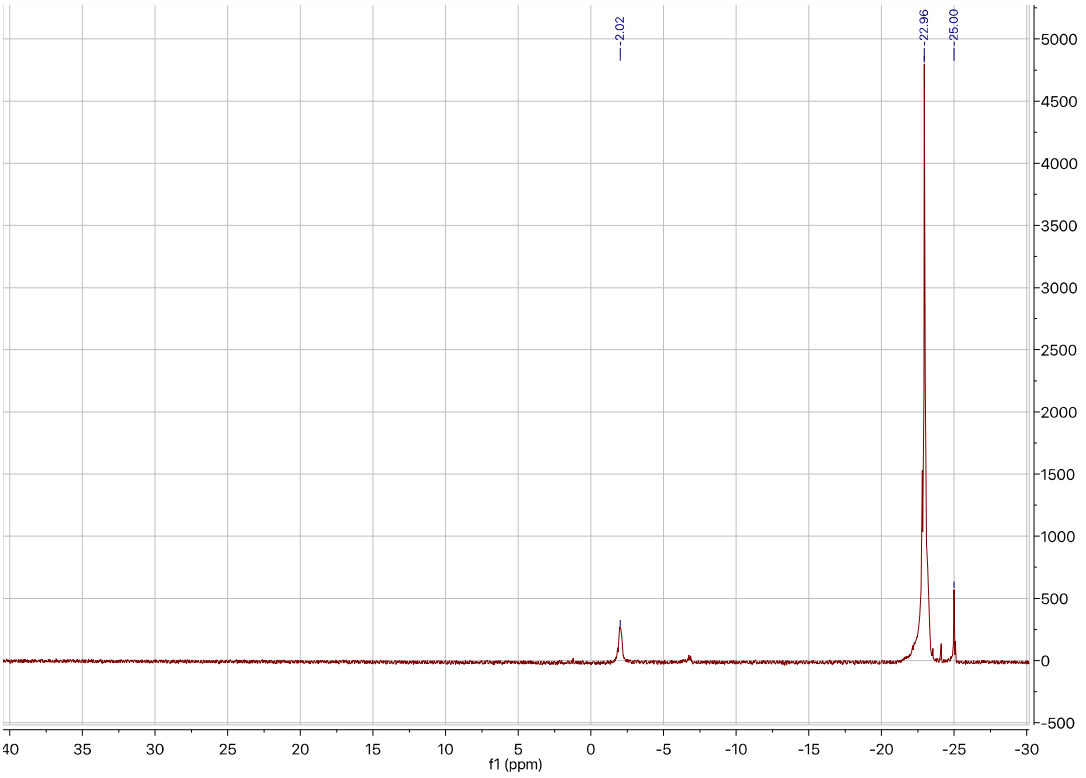
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25.00
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4500
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3500
3000
2500
2000
1500
1000
500
0
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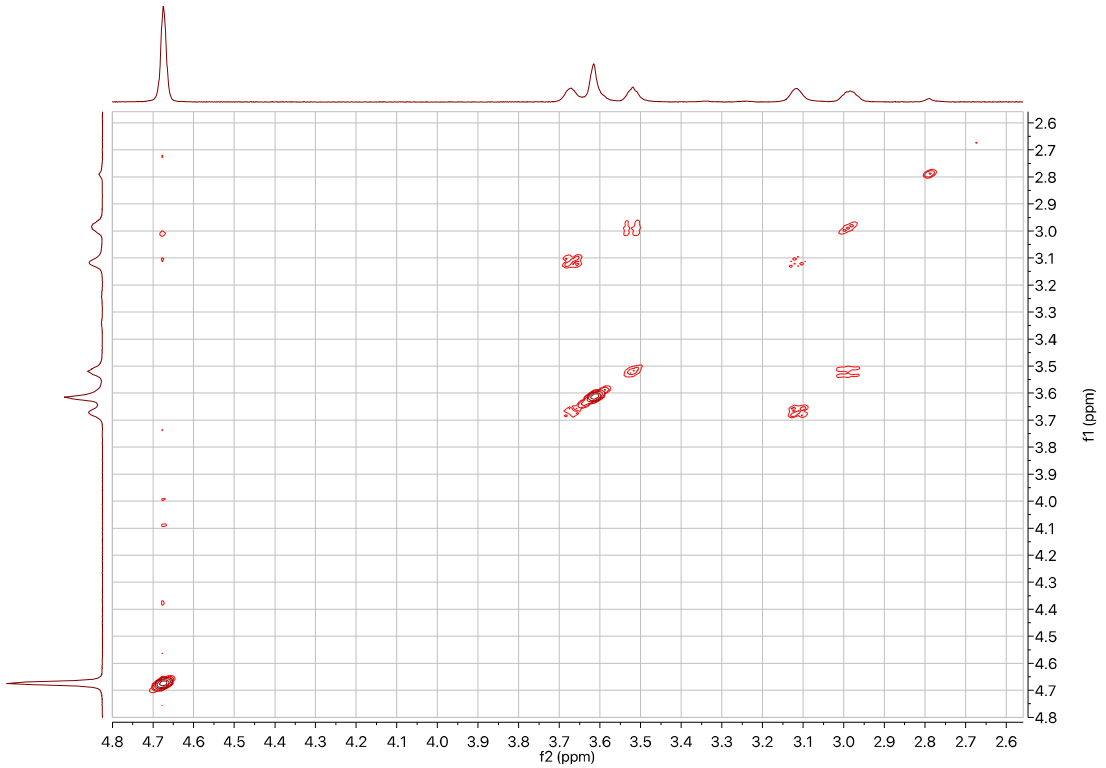
40 35 30 25 20 15 10 5 0 -5 -10 -15 -20 -25 -30
f1 (ppm)

COSY: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f1 (ppm)
4.8 4.7 4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0 2.9 2.8 2.7 2.6
f2 (ppm)

PolyP$_{45}$-bis(2,2-(ethylenedioxy)bis(ethylamine)) **3b**

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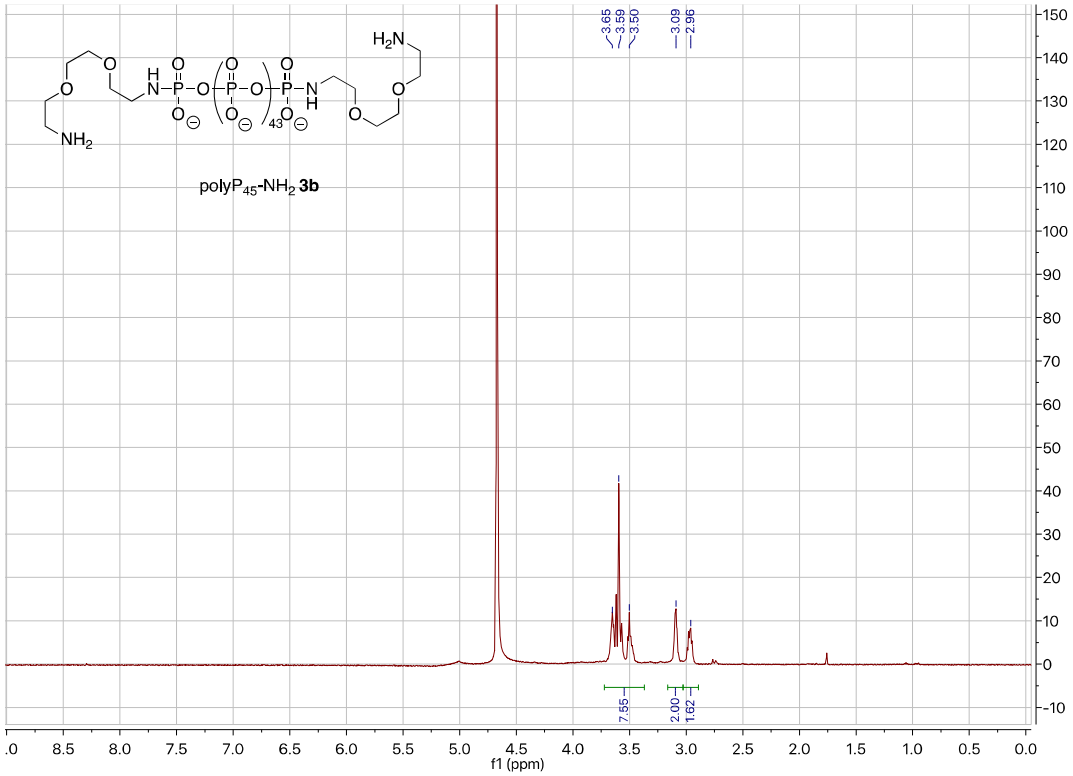

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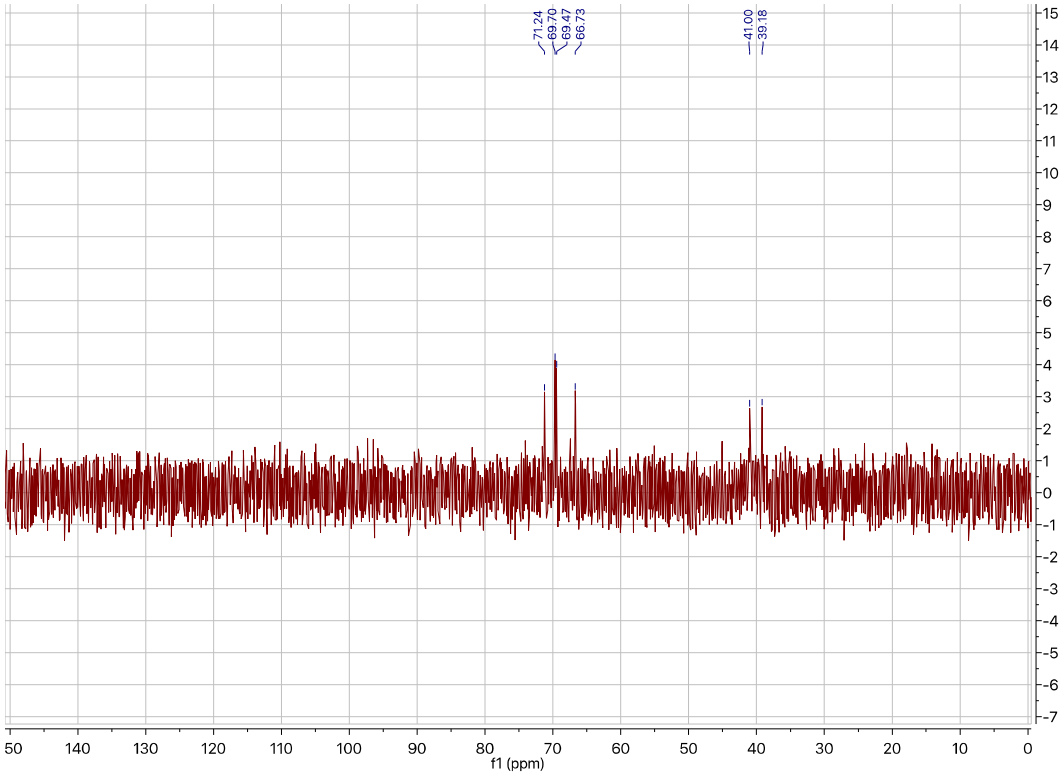

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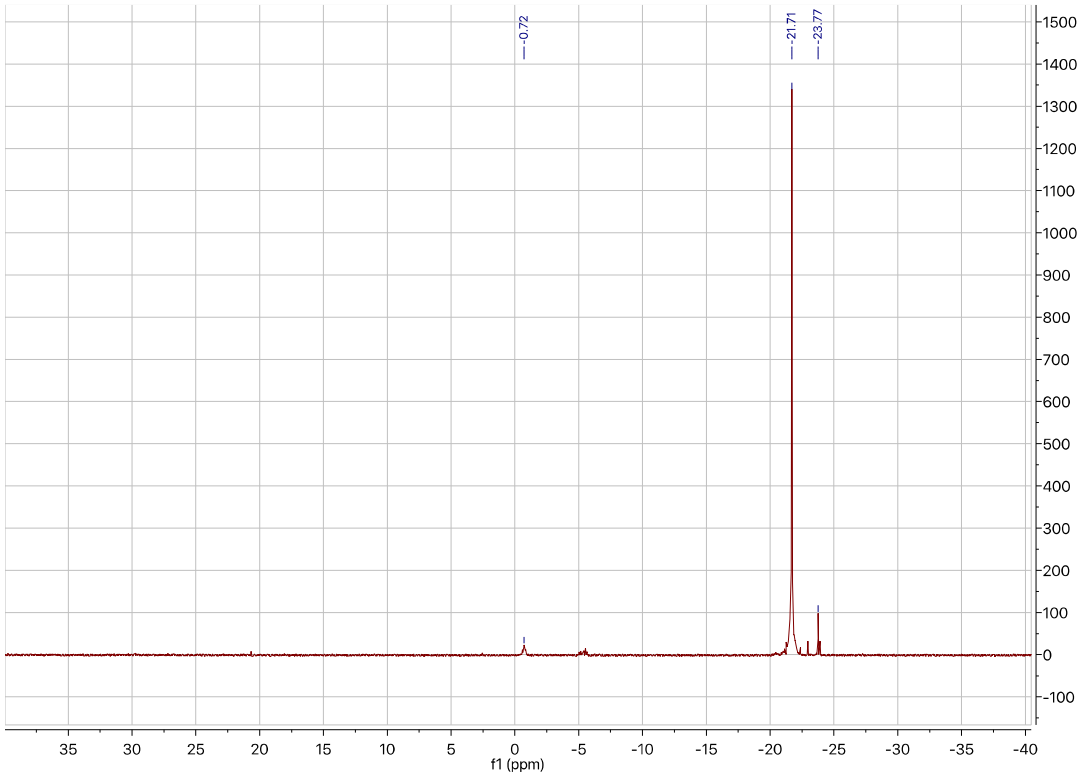
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1400
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1200
1100
1000
900
800
700
600
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300
200
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0
-100
35
30
25
20
15
10
5
0
-5
-10
-15
-20
-25
-30
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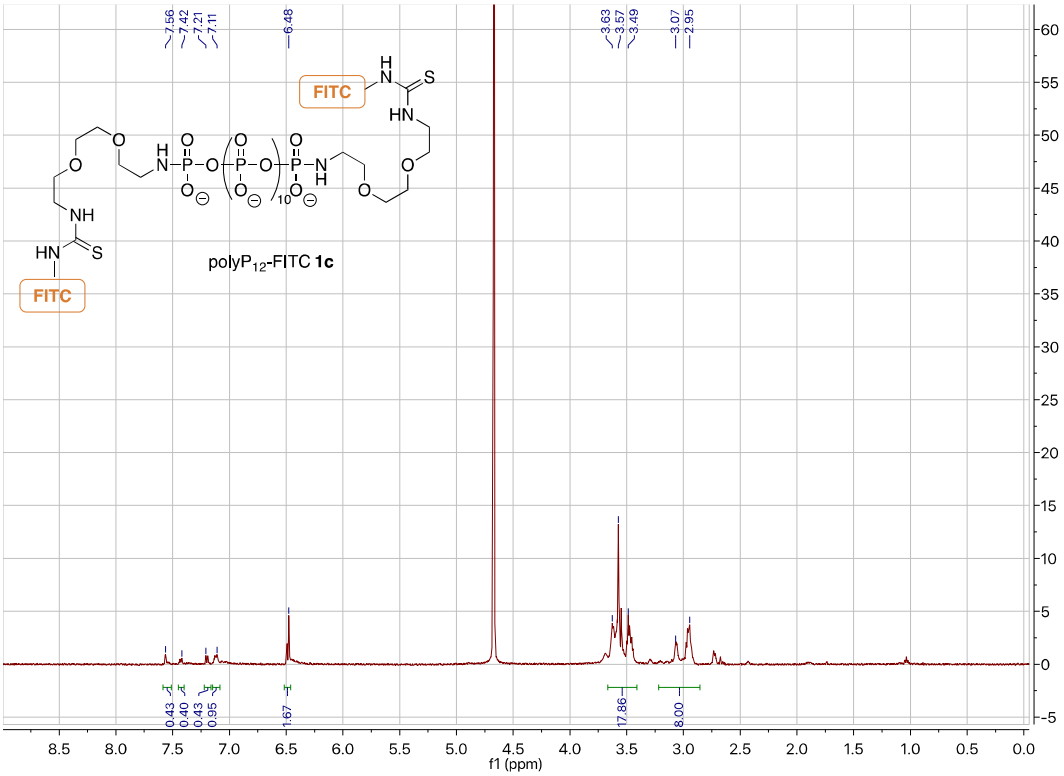
-40
f1 (ppm)

FITC-PolyP$_{12}$-bis(2,2-(ethylenedioxy)bis(ethylamine)) **1c**

^{1}H NMR:



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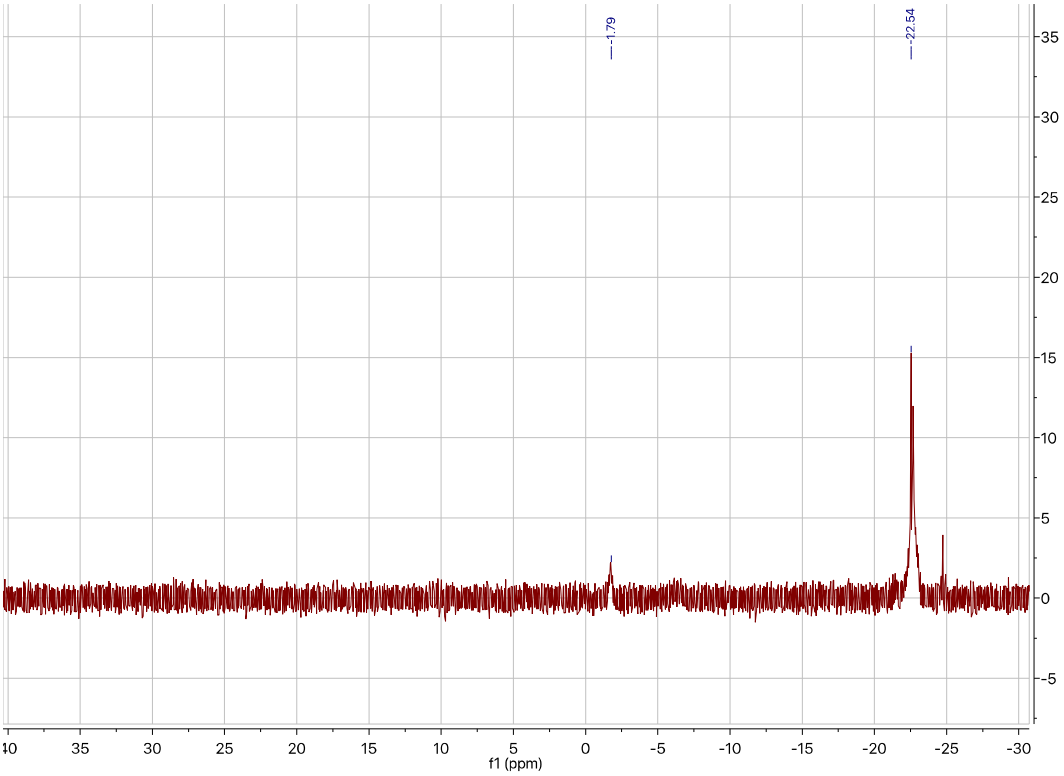

FITC-PolyP$_{22}$-bis(2,2-(ethylenedioxy)bis(ethylamine)) **2c**

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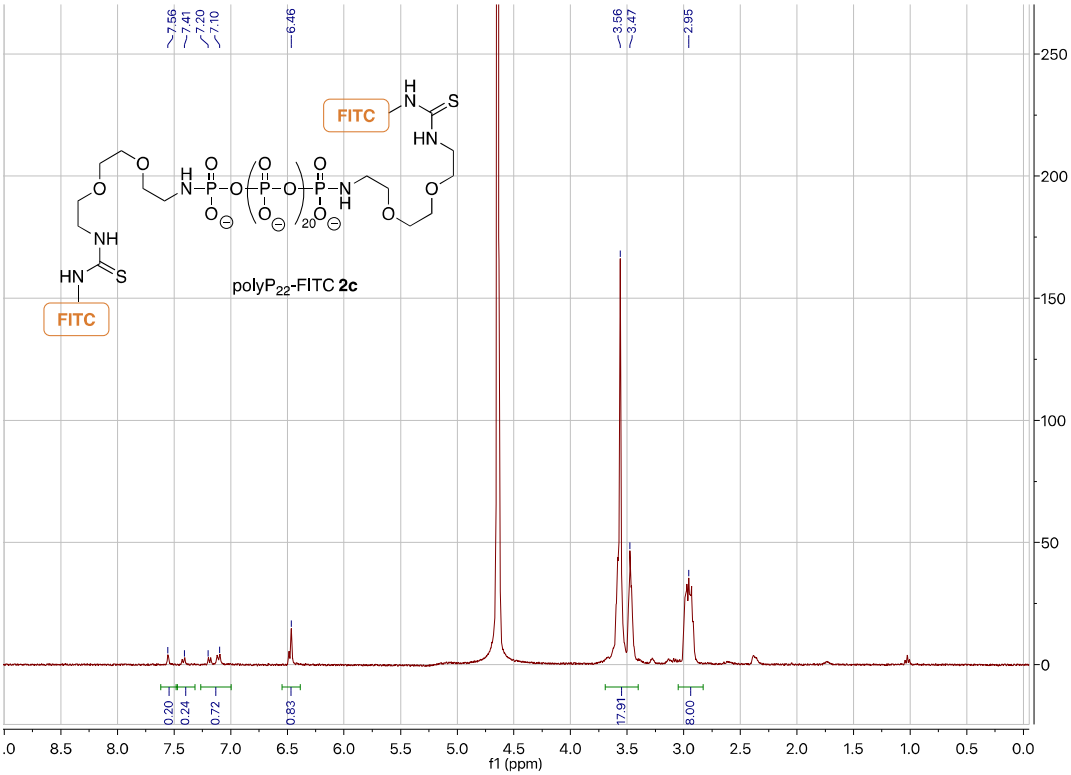

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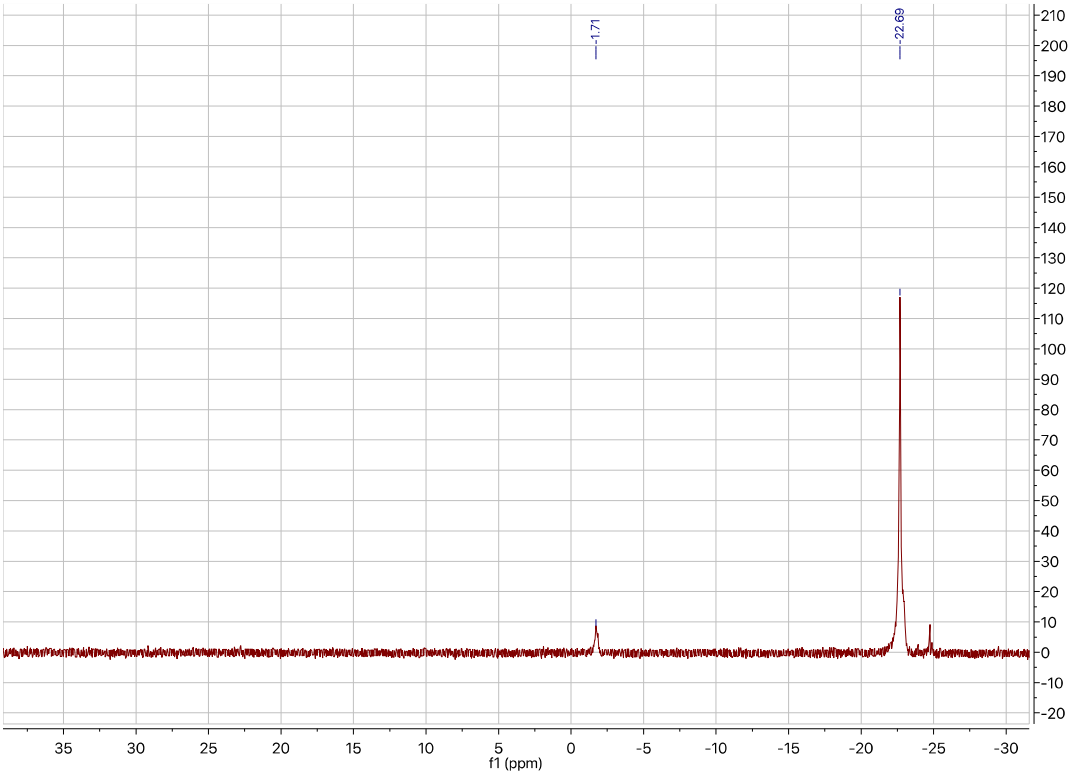

FITC-PolyP$_{45}$-bis(2,2-(ethylenedioxy)bis(ethylamine)) **3c**

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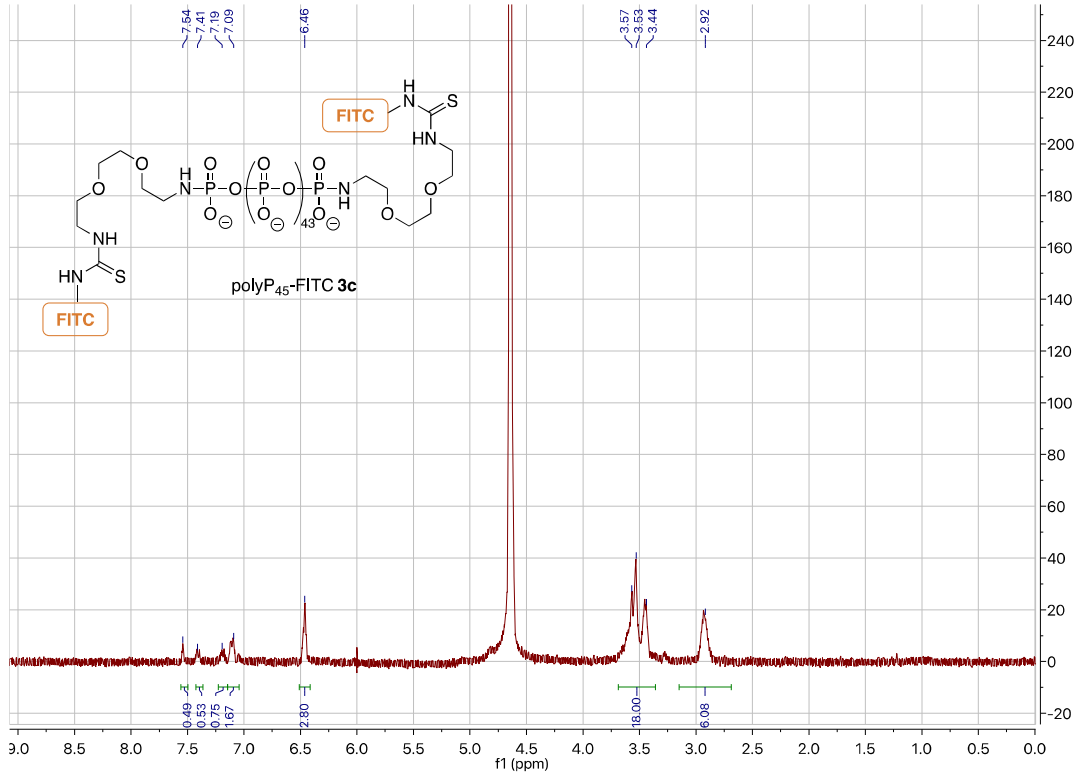

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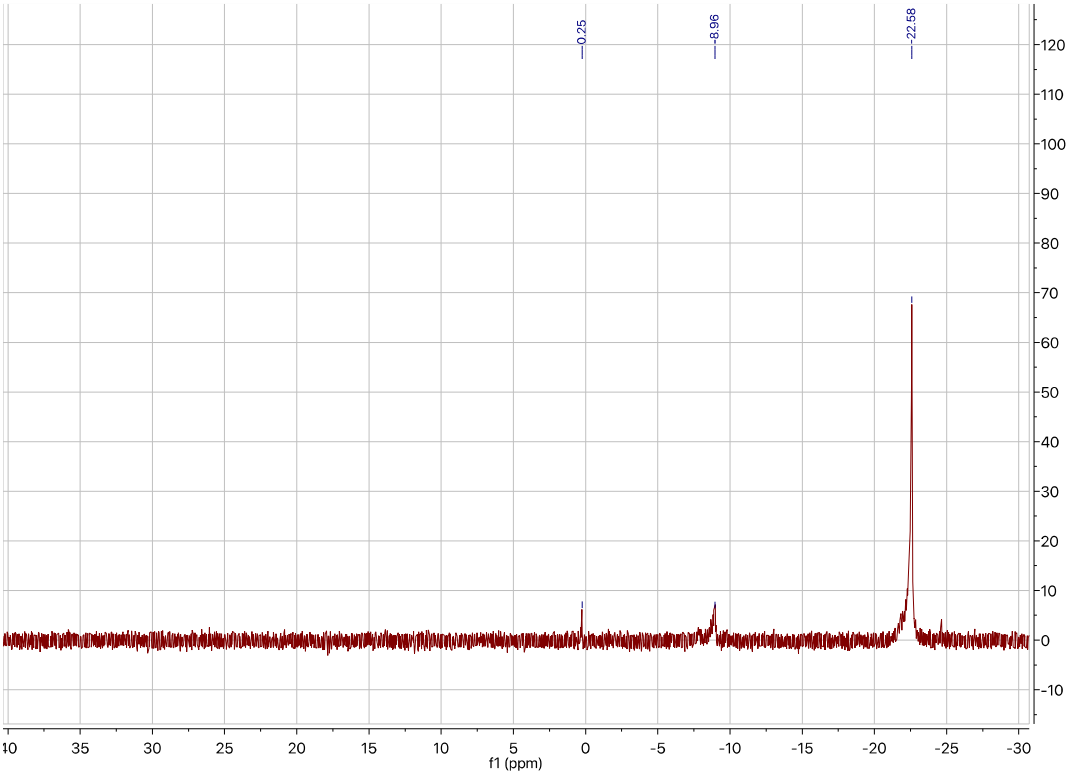

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(1) Geihe, E. I.; Cooley, C. B.; Simon, J. R.; Kiesewetter, M. K.; Edward, J. A.; Hickerson, R. P.; Kaspar, R. L.; Hedrick, J. L.; Waymouth, R. M.; Wender, P. A. Designed Guanidinium-Rich Amphipathic Oligocarbonate Molecular Transporters Complex, Deliver and Release SiRNA in Cells. *Proc. Natl. Acad. Sci.* **2012**, *109* (33), 13171–13176.

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