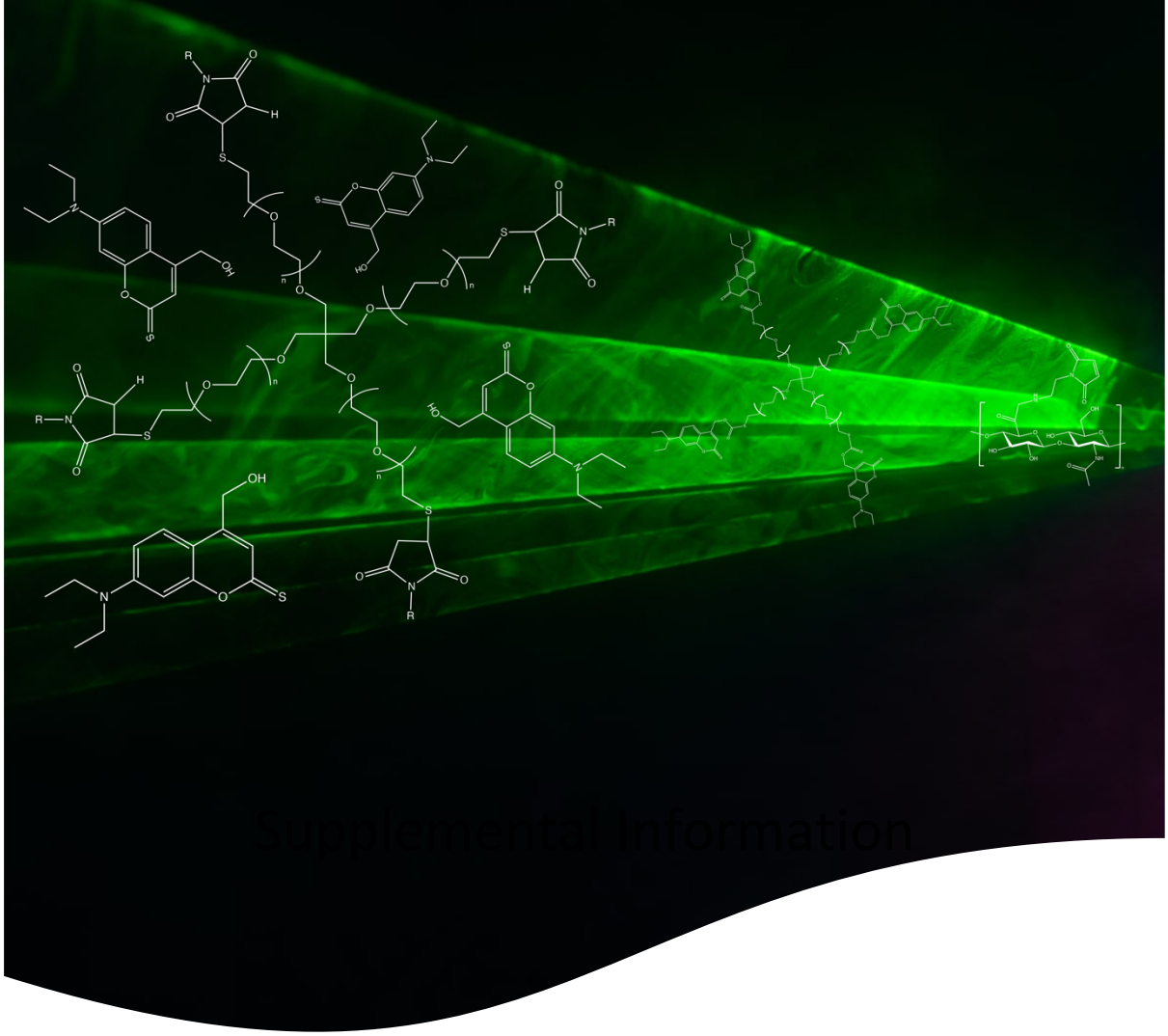


Supporting Information

Cleaving to Connect:
Non-Free Radical Photopolymerization for Orthogonal Multi Material Volumetric Bioprinting of Hydrogels

Michaël Hanna

Table of Contents

1 Synthesis protocols

1.1 Compound 1

*Compound **1*** was synthesized according to a protocol by Klimek *et al.*[1] To a solution of 5 g (21.62 mmol, 1 eq) of *7-Diethylamino-4-methylcoumarin* in 40 mL DMF, 4.31 mL (32.43 mmol, 1.5 eq) DMF-DMA was added under stirring. The reaction mixture was refluxed at 155 °C. After 8 hours the heating element was switched off, allowing for slow cooling down to room temperature. The mixture was continued to stir overnight. After 14 hours, yellow precipitation was visible. The precipitate was filtrated and kept separately. The filtrate was added to 400 mL of ice water, giving further yellow/brown precipitation. This solution was filtrated several times, yielding more precipitate with each filtration.[a] This precipitate was combined with the earlier separately kept precipitate. The combined precipitate was washed with 500 mL distilled water and subsequently dried over a Büchner filter. A yellow/brown solid was obtained, 4.61 g (75% yield). The structure was confirmed by NMR: ^{1}H NMR (400 MHz, cdcl3) δ 7.52 (d, *J* = 9.0 Hz, 1H), 7.24 (d, *J* = 12.3 Hz, 1H), 6.60 – 6.46 (m, 2H), 5.86 (s, 1H), 5.22 (d, *J* = 13.0 Hz, 1H), 3.40 (qd, *J* = 7.1, 4.2 Hz, 4H), 2.99 (s, 6H), 1.19 (td, *J* = 7.1, 3.1 Hz, 6H). 13C NMR (101 MHz, cdcl3) δ 163.99, 156.80, 152.88, 150.57, 147.15, 125.28, 108.57, 108.36, 98.09, 93.76, 87.87, 45.09, 12.87.

[a]*When no new precipitate remained on the filter, the next step was done. Since the filtrate was still slightly cloudy, it was put in the refrigerator at 4 °C for overnight to see if more precipitation or larger crystals would be formed. However, when filtrating this solution, no new precipitate remained on filter.*

1.2 Compound 2

Compounds **2** and ***3*** were synthesized according to a protocol by Weinrich *et al.*[2] To a solution of 2 g (6.98 mmol, 1 eq) of *compound 1* in 20 mL THF/H_2O (1:1), 4.49 g (20.95, 3 eq) $NaIO_4$ was added. The reaction mixture was stirred at room temperature for 1.5 hours. The precipitate was filtered off and was washed with ethyl acetate. Half of the solvent was removed by rotary evaporation. Extraction of the resulting solution was done with concentrated $NaHCO_3$ aqueous solution, followed by three repeated extractions of the aqueous phase with DCM. Both organic layers (ethyl acetate and total amount of DCM) were combined and dried with $MgSO_4$. The solvent was removed by rotary evaporation and further dried under N_2. A red resin was obtained, 1.69 g (98% yield). The structure of *compound **2*** was confirmed by NMR: ^{1}H NMR (400 MHz, $cdcl_3$) δ 10.03 (s, 1H), 8.31 (d, *J* = 9.2 Hz, 1H), 6.65 (dd, *J* = 9.2, 2.6 Hz, 1H), 6.54 (d, *J* = 2.6 Hz, 1H), 6.46 (s, 1H), 3.43 (q, *J* = 7.1 Hz, 4H), 1.22 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (101 MHz, $cdcl_3$) δ 192.63, 161.94, 157.48, 150.99, 144.02, 127.20, 117.66, 109.84, 98.00, 45.09, 12.54.

1.3 Compound 3

A solution of 1.69 g (6.89 mmol, 1 eq) of *compound **2*** in 20 mL THF was cooled to 0 °C, and 0.52 g (13.78 mmol, 2 eq) $NaBH_4$ was added. The mixture was stirred at room temperature for 5 hours. 20 mL of concentrated $NaHCO_3$ was added and the organic phase was separated. The aqueous layer was extracted with 20 mL DCM, three times. The organic phases were combined and dried with $MgSO_4$. The solvent was removed by rotary evaporation and further dried under N_2 airflow. Brown solid crystals were obtained, 1.54 g (91% yield). The structure was confirmed by NMR: ^{1}H NMR (400 MHz, cdcl3) δ 7.34 (dd, *J* = 24.9, 9.0 Hz, 1H), 6.57 (td, *J* = 9.6, 2.6 Hz, 1H), 6.49 (d, *J* = 2.6 Hz, 1H), 6.29 – 6.23 (m, 1H), 5.93 (d, *J* = 1.2 Hz, 1H), 4.82 (d,

J = 1.4 Hz, 2H), 3.40 (qd, *J* = 7.1, 3.7 Hz, 4H), 1.20 (td, *J* = 7.1, 2.7 Hz, 6H). ^{13}C NMR (101 MHz, cdcl3) δ 162.76, 156.30, 154.85, 150.64, 124.51, 108.56, 106.44, 105.58, 98.34, 61.11, 44.85, 12.58.

1.4 Compound 4

*Compounds **4**, **5**,* and ***6*** were synthesized according to a protocol by Fournier *et al.*[3] A solution of 2.4891 g (10.07 mmol, 1 eq) of *compound **3**,* 1.4850 g (12.08 mmol, 1.2 eq) DMAP, and 0.6908 mL (12.08 mmol, 1.2 eq) acetic acid in 165 mL dry DCM was cooled to 0 °C and flushed with N_2 for 10 minutes. Under stirring 2.4922g (12.08 mmol, 1.2 eq) of DCC was added. For 15 hours at room temperature in the dark under N_2, the mixture was stirred. The mixture was filtrated, and the filtrate was washed with 200 mL 1.2 M HCL. The organic phase was washed with 200 mL concentrated $NaHCO_3$. The organic phase was dried with Na_2SO_4. The solvent was removed by rotary evaporation and further dried under N_2 airflow. An orange powder was obtained, 2.6645 g (91% yield). The structure was confirmed by NMR: ^{1}H NMR (400 MHz, cdcl3) δ 7.39 (t, *J* = 8.9 Hz, 1H), 7.28 (d, *J* = 9.0 Hz, 1H), 6.51 (d, *J* = 2.6 Hz, 2H), 6.13 (s, 1H), 5.21 (d, *J* = 1.3 Hz, 1H), 3.41 (d, *J* = 7.1 Hz, 4H), 2.33 (d, *J* = 1.1 Hz, 3H), 1.20 (t, *J* = 7.2 Hz, 6H). ^{13}C NMR (101 MHz, cdcl3) δ 170.72, 162.33, 156.72, 151.12, 149.83, 125.95, 124.82, 109.13, 106.92, 106.48, 98.33, 61.80, 45.22, 34.40, 25.40, 21.21, 12.88.

1.5 Compound 5

A solution of 2.37 g of *compound **4*** and 2.23 g of Lawesson's reagent in 300 mL dry Toluene was refluxed at 100 °C in the dark and under N_2 for 16.5 hours. The solvent was removed by rotary evaporation and column chromatography was done for purification, using DCM as eluent. The solvent was removed by rotary evaporation and further dried under N_2 airflow. An orange powder was obtained, 1.31 g (47% yield). The structure was confirmed by NMR: ^{1}H NMR (400 MHz, $cdcl_3$) δ 7.34 (d, *J* = 8.8 Hz, 1H), 7.06 (t, *J* = 1.1 Hz, 1H), 6.70 – 6.62 (m, 2H), 5.18 (d, *J* = 1.2 Hz, 2H), 3.43 (q, *J* = 7.1 Hz, 4H), 2.19 (s, 3H), 1.22 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (101 MHz, $cdcl_3$) δ 197.02, 158.87, 150.84, 141.66, 124.27, 120.39, 110.12, 108.01, 97.30, 60.79, 44.77, 20.60, 12.23.

After measuring NMR, the tube was kept on the bench for 4 days under normal light conditions. After 4 days, it was measured again, yielding a similar spectrum.

1.6 Compound 6

To a solution of 0.1972 g (0.65 mmol, 1 eq) of *compound **5*** in 140 mL ethanol (absolute) was added 1.29 mL (1.61 mmol, 2.5 eq) of 1.25 M ethanolic HCl solution. The mixture was refluxed at 70 °C in the dark and under N_2 for 15 hours. The solvent was removed by rotary evaporation. The sample was dry loaded on a chromatography column. DCM was used as the starting eluent to remove impurities. DCM/acetone in a gradient from 95/5 up until 50/50 (v/v) removed *compound **6*** from the column. This fraction was dried by rotary evaporation and further dried under N_2 airflow. A yellow/brown powder was obtained, 0.1458 g (86% yield). The structure was confirmed by NMR: ^{1}H NMR (400 MHz, acetone) δ 7.53 (d, *J* = 9.1 Hz, 1H), 7.12 (s, 1H), 6.81 (dd, *J* = 9.1, 2.6 Hz, 1H), 6.66 (d, *J* = 2.6 Hz, 1H), 4.80 (s, 2H), 4.64 (d, *J* = 5.8 Hz, 1H), 3.54 (q, *J* = 7.1 Hz, 4H), 1.23 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (101 MHz, acetone) δ 198.46, 159.79, 151.98, 149.27, 125.96, 120.32, 111.18, 109.15, 97.54, 60.23, 45.37, 12.80.

Wet loading and/or only using DCM/acetone in a 95/5 ratio as done by Fournier et al. gave lower yields (20-63%).

*After most syntheses and NMR measurements, compounds were transferred to 20 mL (light protective) storage vials. This was done by partially scraping most of the compounds out of the round bottom flasks and by dissolving the remaining parts with acetone and transferring them to the vials using a Pasteur pipette. Acetone was then evaporated under N_2 airflow, yielding dry compounds without transfer loss. Drying sometimes was slower than expected, especially after larger scale reactions. For example, after one synthesis of compound **4** yielding 3.3 g, the weight of the vial and compound kept decreasing even after two weeks of drying, although very slowly at the end. This means that small amounts of acetone may have remained present.*

1.7 Protected EDT

The protocol of San Miguel *et al.*[4] served as inspiration for the protection syntheses of both *EDT* and *4arm PEG 5000-SH*, replacing 3-(mercaptopropyl)triethoxysilane. Also, molar ratios and reaction times were adjusted. *EDT* and *4arm PEG 5000-SH* were synthesized in two-step reactions shown in Figure 1 and Figure 2.

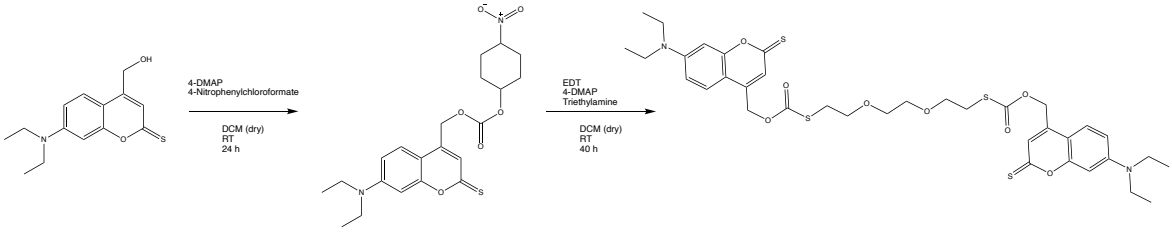


Figure 1: The protection synthesis of EDT by compound 6 in a two-step reaction.

In step 1, 35.5 mg (0.13 mmol, 2.5 eq) of *compound **6*** was dissolved in 4 mL dry DCM. 54.3 mg (0.44 mmol, 8.3 eq) 4-DMAP and 47.9 mg (0.24 mmol, 4.4 eq) 4-NPC were added, and the solution was stirred in the dark. After 18 hours, this mixture was spotted on TLC. The presence of *compound **6*** was still visible, as well as a new compound giving an orange color on TLC. After 24 hours total, another TLC spot was done, *compound **6*** was barely visible and step 2 of the reaction was started. 17.1 mg (0.14 mmol, 2.6 eq) 4-DMAP, 0.075 mL triethylamine (0.54 mmol, 10 eq), and 0.0088 mL (0.05 mmol, 1 eq) *EDT* were added. The mixture was stirred in the dark for 40 hours, after which the solvent was removed. The *protected EDT* was purified by silica gel chromatography using DCM/MeOH in a gradient from 99/1 to 90/10. The appropriate fraction was dried by rotary evaporation and further dried under N_2 airflow. The *protected EDT* was obtained as a dark brown sticky resin, 41.8 mg (quantitative yield).

1.8 Protected 4arm PEG Thiol

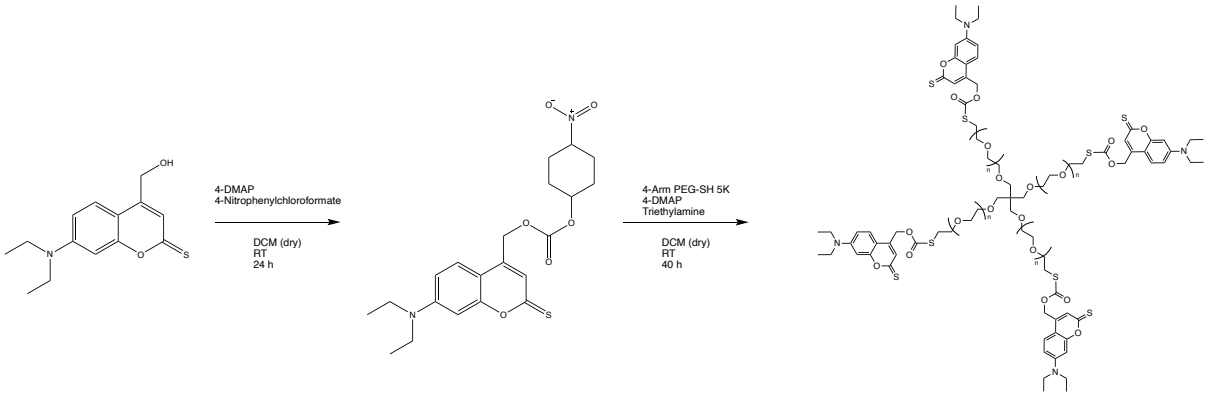


Figure 2: The protection synthesis of the 4arm PEG Thiol by compound 6 in a two-step reaction.

In step 1, 130.1 mg (5 eq, 0.49 mmol) of *compound **6*** was dissolved in 16 mL dry DCM. 206.3 (16.5 eq, 1.63 mmol) 4-DMAP, and 180.6 (8.9 eq, 0.89 mmol) 4-NPC were added, and the solution was stirred in the dark. After 24 hours, this mixture was spotted on TLC. The intermediary compound was present, with *compound **6*** not visible anymore. In step 2, 35.5 mg (2.6 eq, 0.29 mmol) 4-DMAP, 0.1376 mL (10 eq, 0.99 mmol) triethylamine, and 494.6 mg (1 eq, 0.1 mmol) of *4arm PEG 5000-SH* were added. 40 hours after starting step 2 the solvent was removed. The *protected 4arm PEG* was purified by silica gel chromatography. A DCM/MeOH gradient from 99/1 up until 90/10 separated the compounds. The appropriate fraction was dried by rotary evaporation and further dried under N_2 airflow. The *protected 4arm PEG* was obtained as an orange powder.

The structure and an 80% degree of protection were determined by ^{1}H NMR: integration of the methyl groups at 1.2 ppm belonging to the -CH_3 groups of the coumarin were determined at 4.82 (should be 6) protons against a set integral of 2 of the two triplets between 3.02-3.12 ppm, belonging the -CH_2 groups of the 4arm next to the thiols (Figure 12). Because of a wide single peak between 1.87-2.5 ppm, presumed to be H_2O, the sample was dissolved in Milli-Q and freeze-dried. The final yield obtained was 0.4876 g (85% yield).

1.9 HA-MAL

HA-MAL was synthesized according to a protocol from Yoo *et al.*[5] from HA and 1-(2-aminoethyl)maleimide by EDC/NHS coupling in buffer solution at pH 4.5, shown in Figure 3.

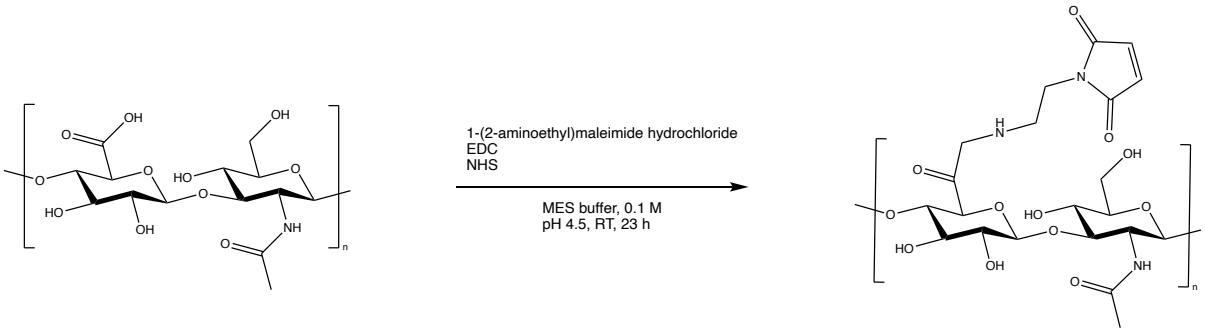


Figure 3: Functionalization of the carboxylic acids on HA with maleimide.

1.05 gram (2.48 mmol, 1 eq) *sodium hyaluronate containing 5% H_2O* was dissolved in 100 mL 0.1 M MES buffer at pH 4.5, taking 30 minutes under stirring. 0.58 g (2.97 mmol, 1.2 eq) EDC and 0.34 g (2.97 mmol, 1.2 eq) NHS were dissolved in 10 mL 0.1 M MES buffer at pH 4.5. After rapid solvation, this was added to the sodium hyaluronate solution. The mixture was stirred for 30 minutes at room temperature at 300 rpm. 0.53 g (2.97 mmol, 1.2 eq) of *1-(2-aminoethyl) maleimide HCl* was dissolved in 5 mL distilled water and then added dropwise to the reaction mixture. It was stirred for 23 hours at 300 rpm and then dialyzed (MWCO 12.000-14.000) in 5 L of water at pH 3.25 for three days at 100 rpm. The pH of pure solution was lowered to 3 and filtered through a 0.2 μm filter. The final solution was freeze-dried, yielding 1.04 g of *HA-MAL.* A 12% DoF was determined by ^{1}H NMR: integration of new maleimide peaks at 6.94 and 6.90 ppm was determined at 0.24 (should be 2 protons) against a set integral of 3 of the peak at 2.03 ppm, belonging to the N-acetyl group.
A second synthesis was done, with the only apparent difference of having the EDC and NHS already dissolved together for 30 minutes. This synthesis resulted in a 6.5% DoF.

2 Synthesis NMR Spectra

2.1 Starting compound

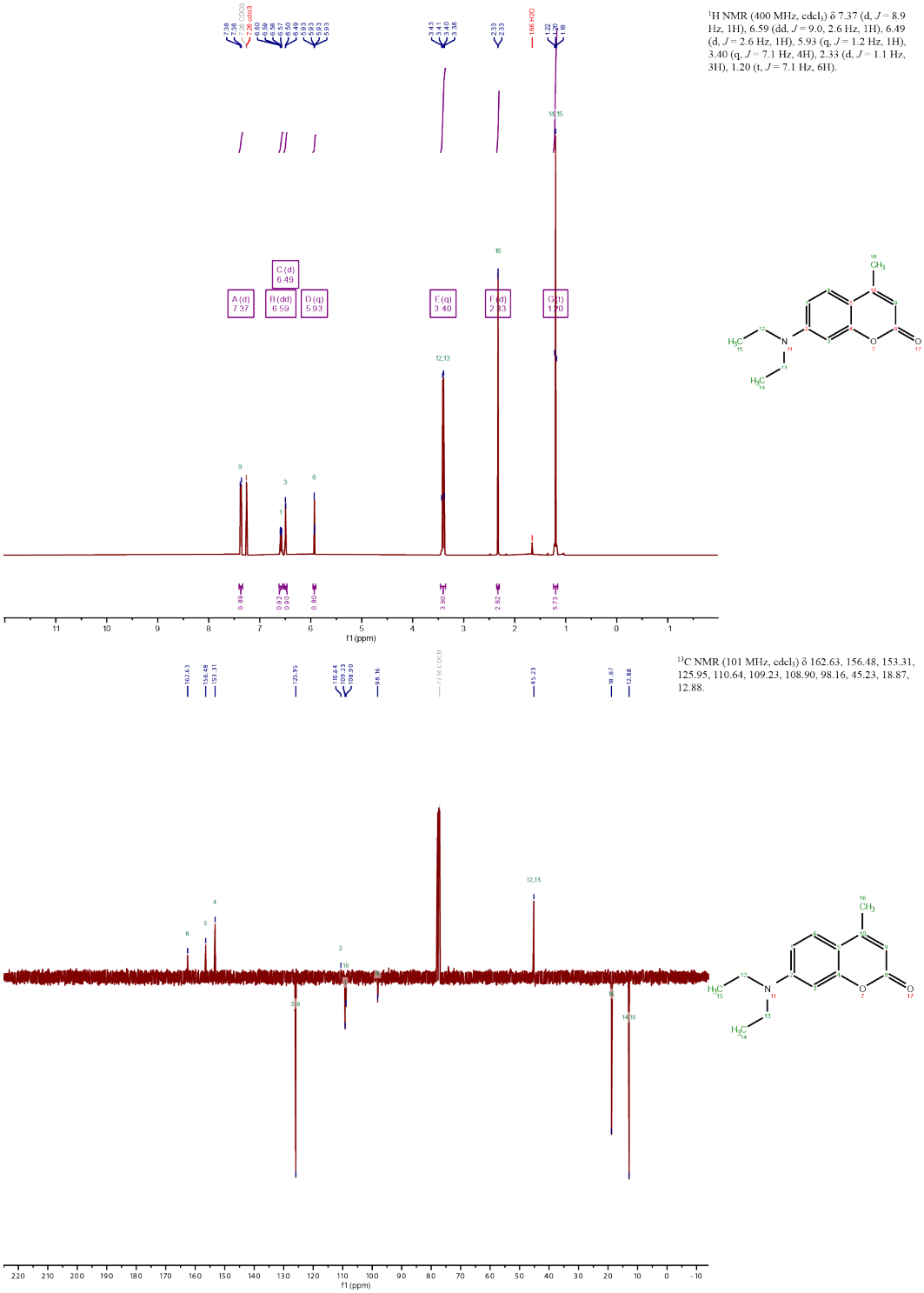


Figure 4: Starting coumarin NMR spectra in cdcl3.

2.2 Compound 1

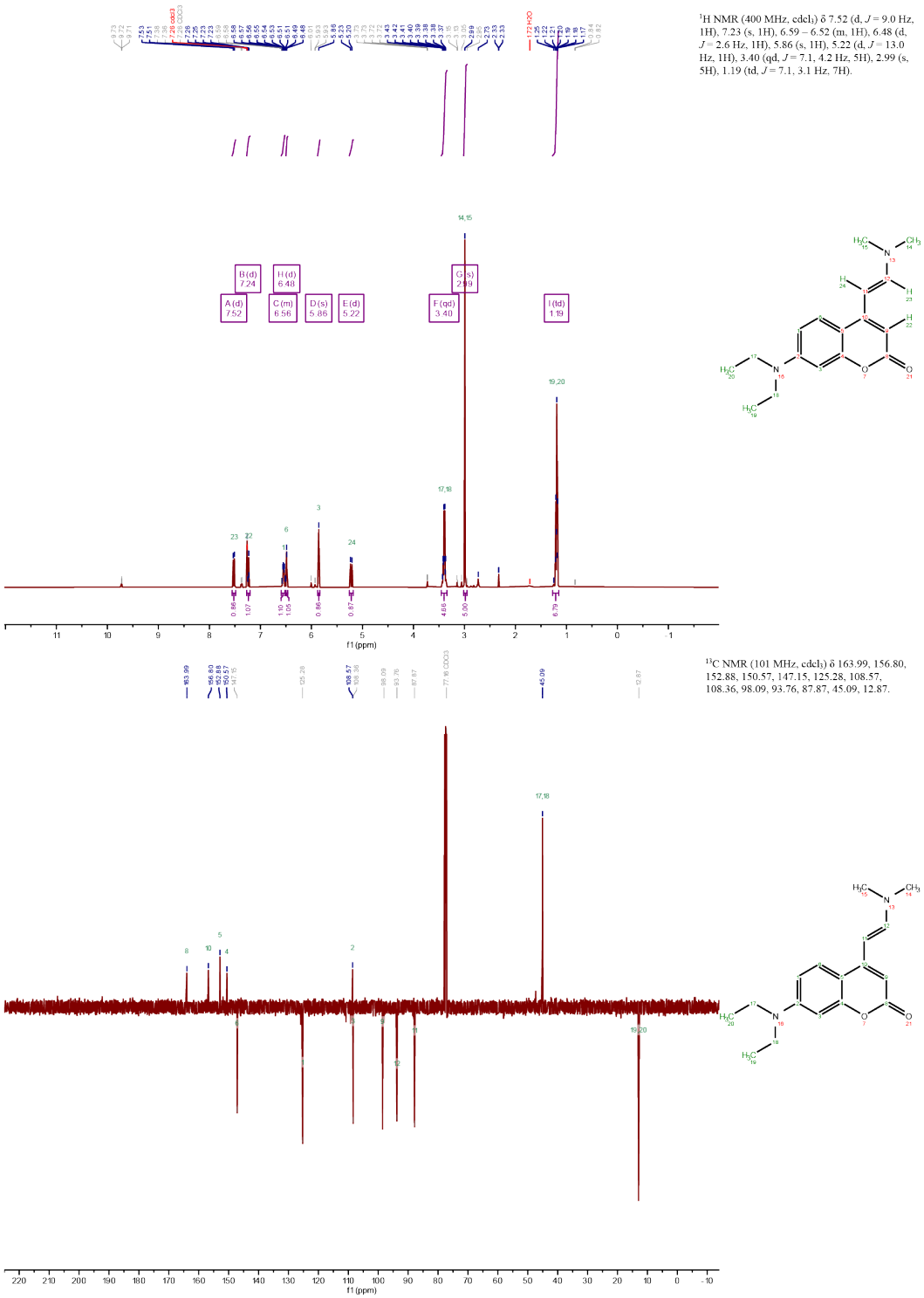


*Figure 5: Compound **1** NMR spectra in cdcl3. Of note, the peak at 2.33 ppm of the ^{1}H NMR spectrum likely belongs to a portion unreacted starting coumarin. On the ^{13}C NMR spectrum, C13 and C14 do not occur for an unknown reason, similar as in the reference.*

2.3 Compound 2

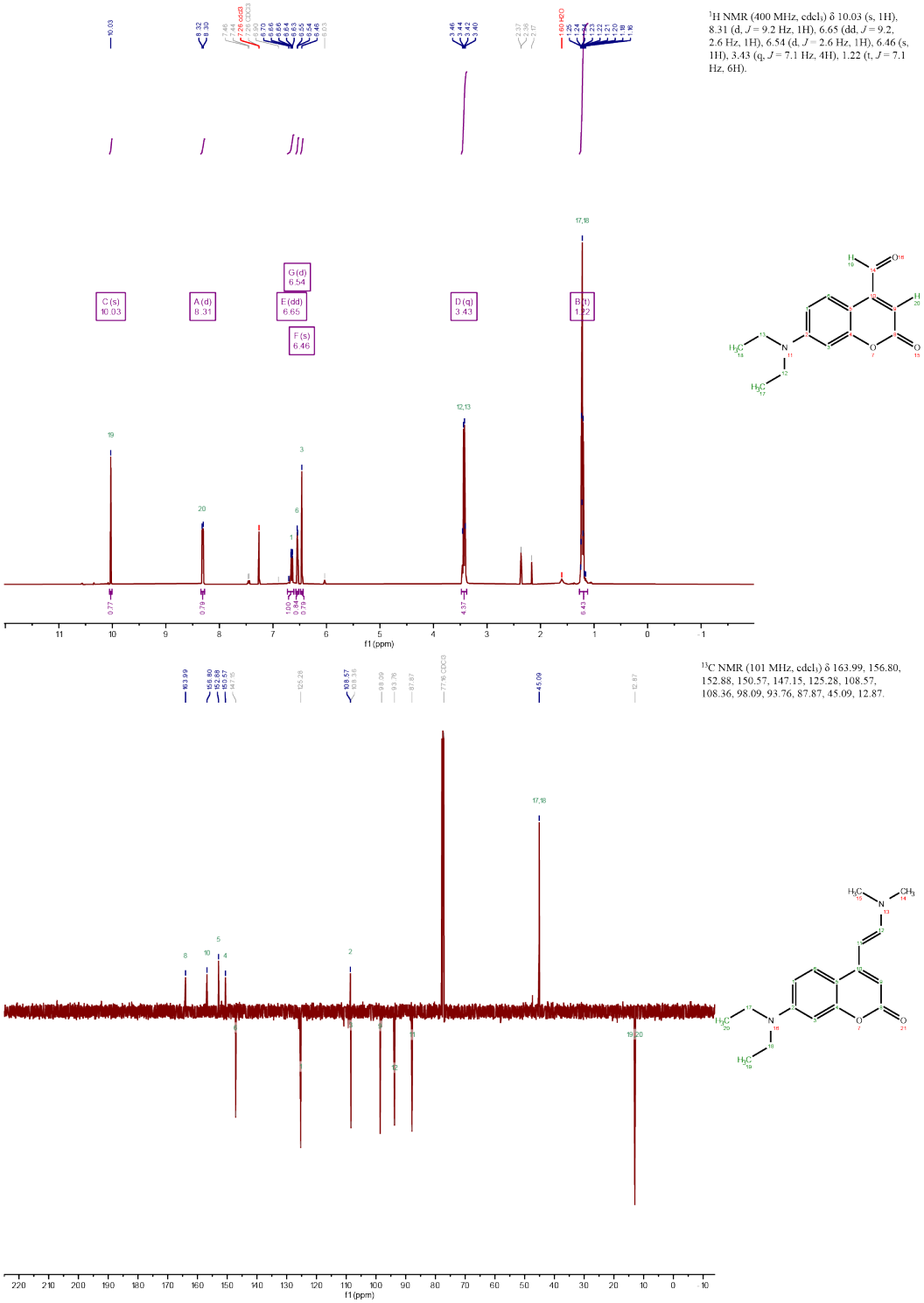


*Figure 6: Compound **2** NMR spectra in cdcl3.*

2.4 Compound 3

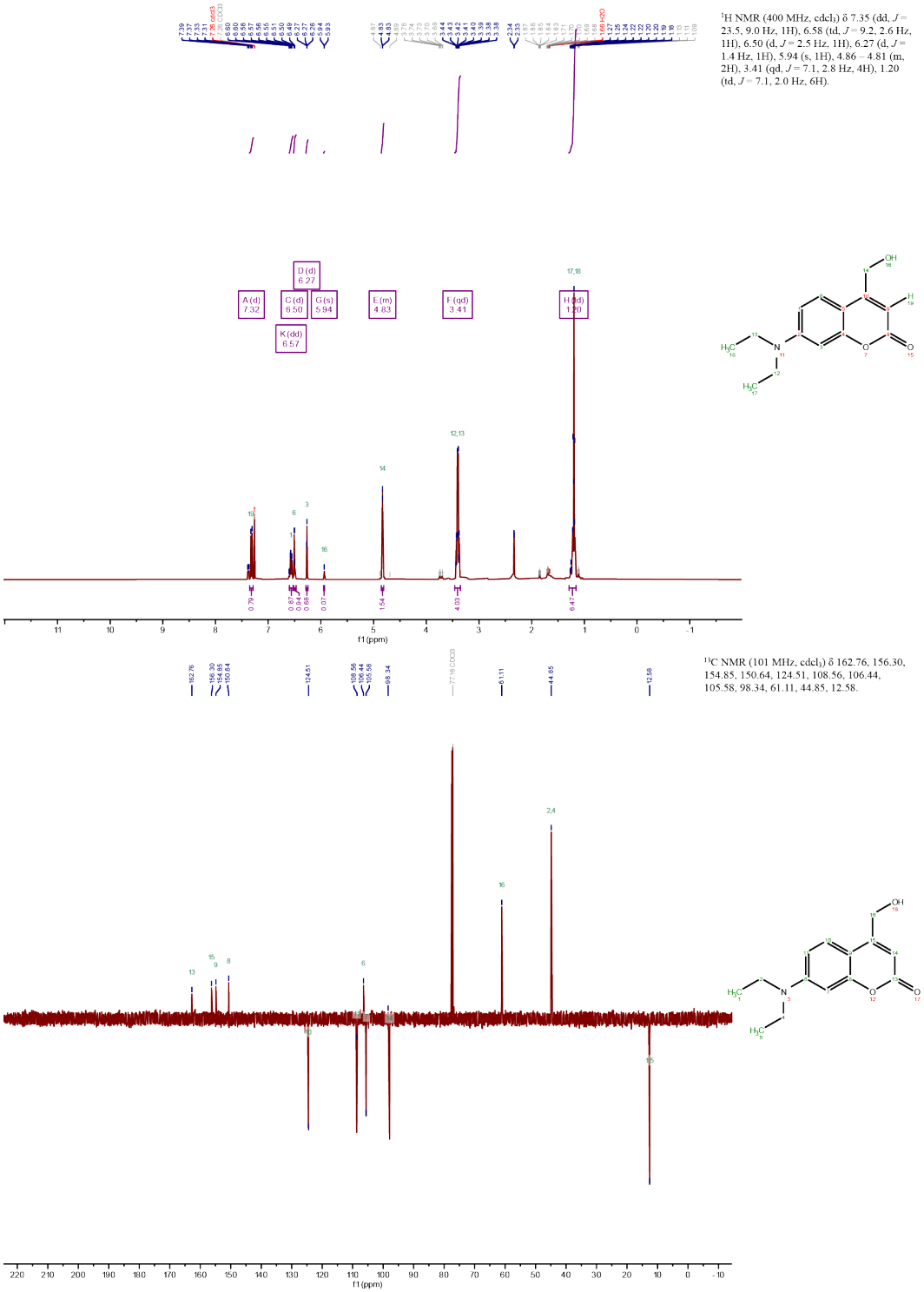


*Figure 7: Compound **3** NMR spectra in cdcl3.*

2.5 Compound 4

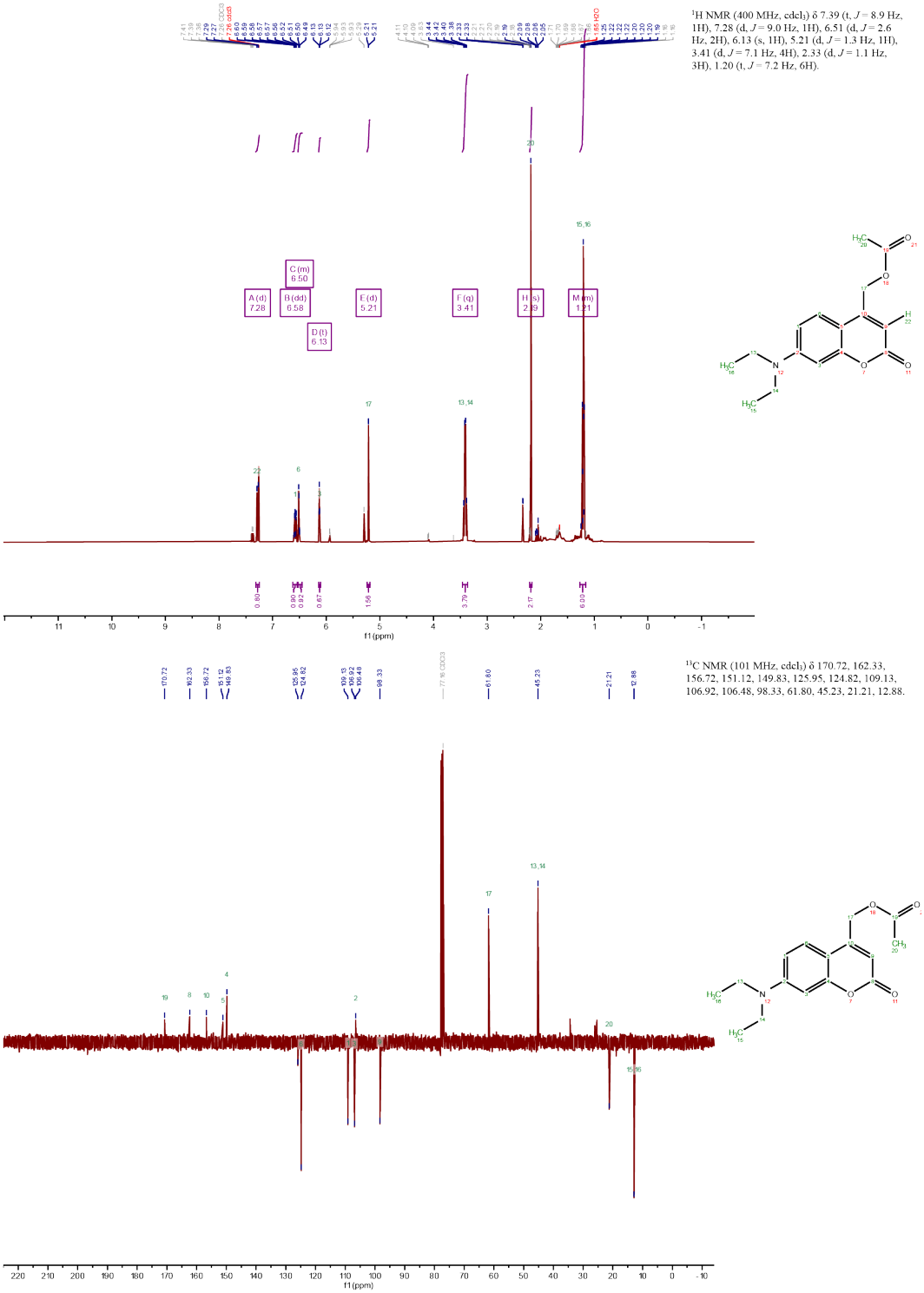


*Figure 8: Compound **4** NMR spectra in cdcl3. Of note, the respective peaks between 1 and 2 ppm for ^{1}H NMR and the peaks at 26 and 34 ppm for ^{13}C NMR correlate with DCC, one of the synthesis reactants.*

2.6 Compound 5

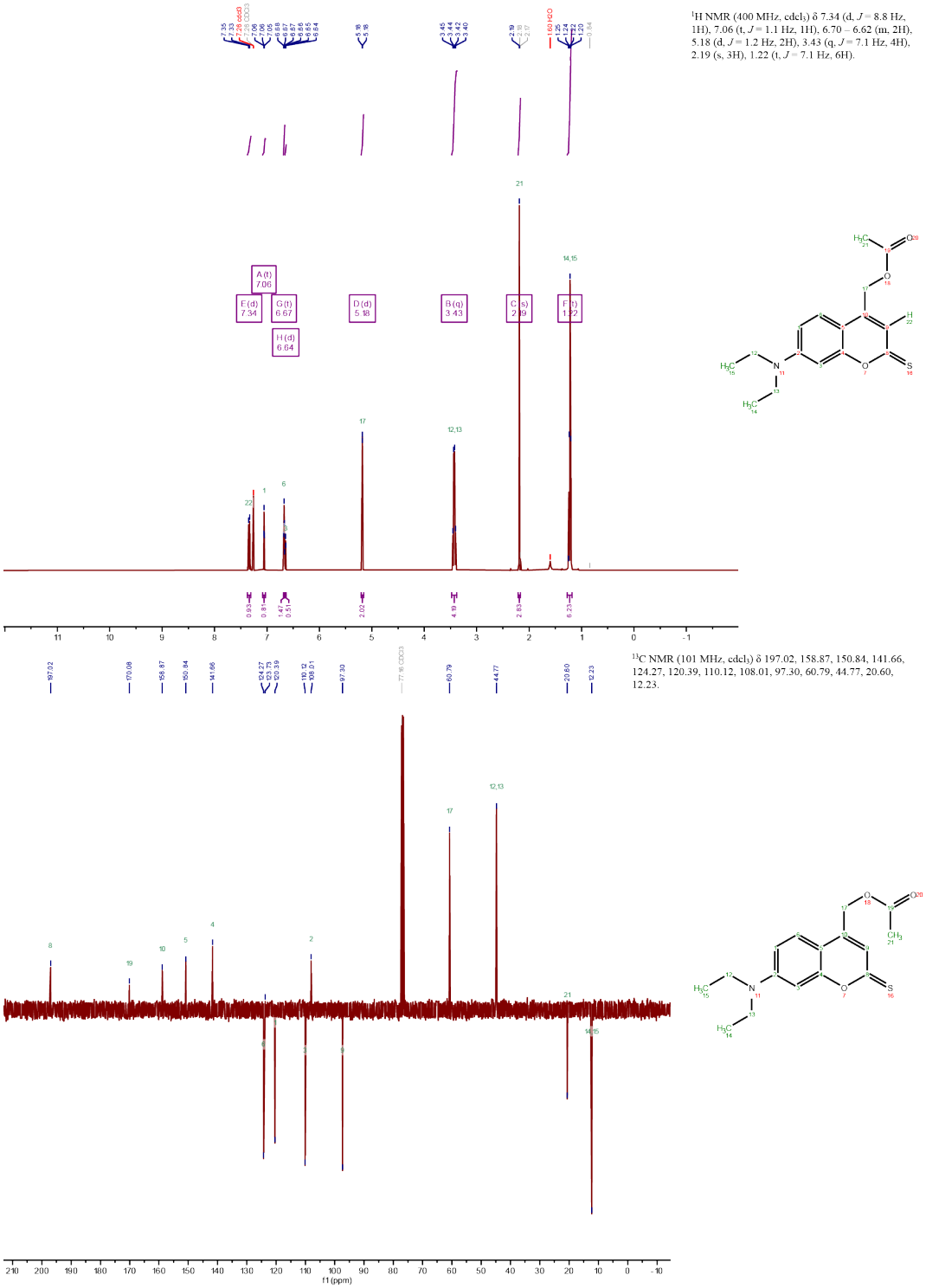


*Figure 9: Compound **5** NMR spectra in cdcl3.*

2.7 Compound 6

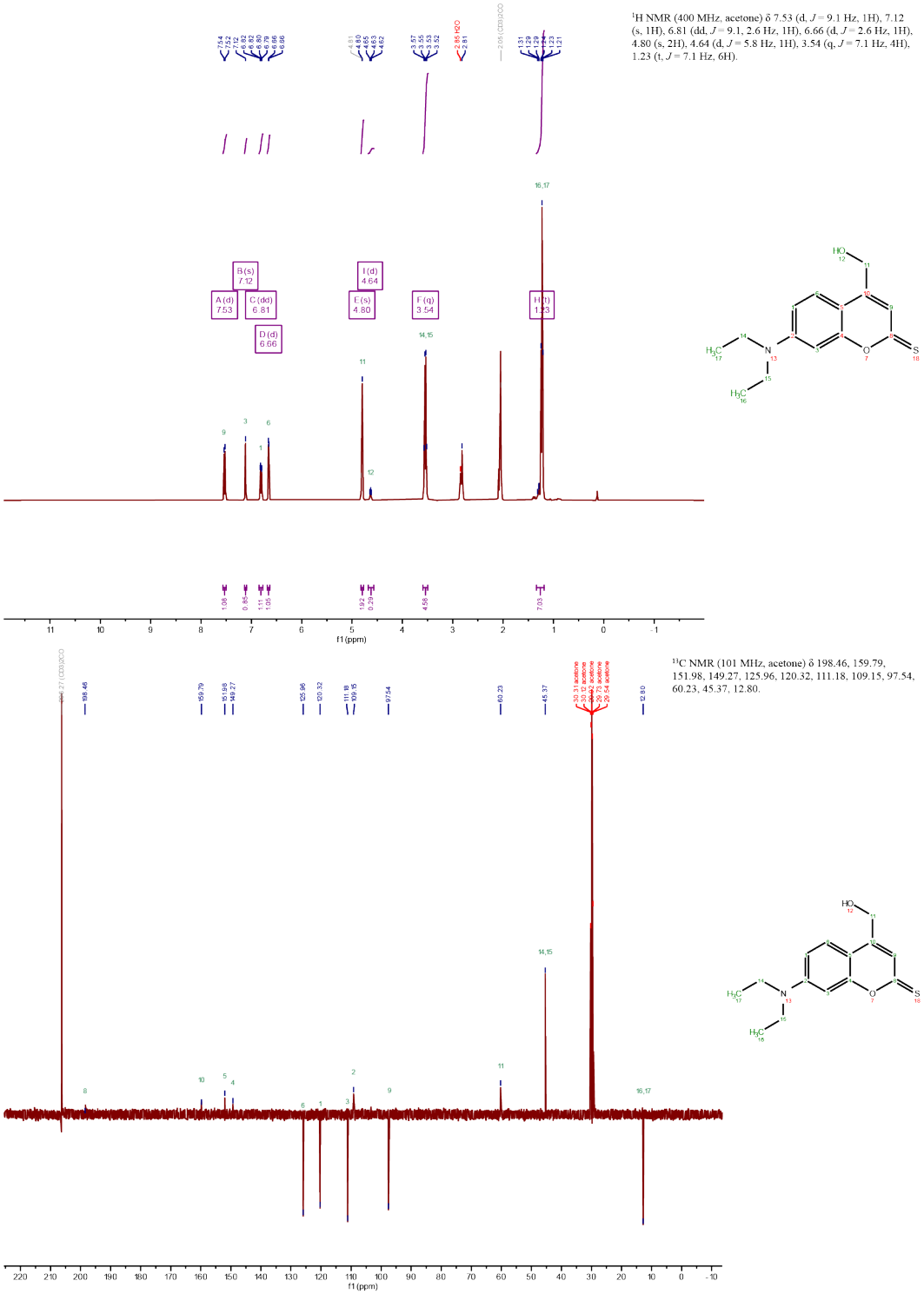


*Figure 10: Compound **6** NMR spectra in d-acetone.*

2.8 Protected EDT

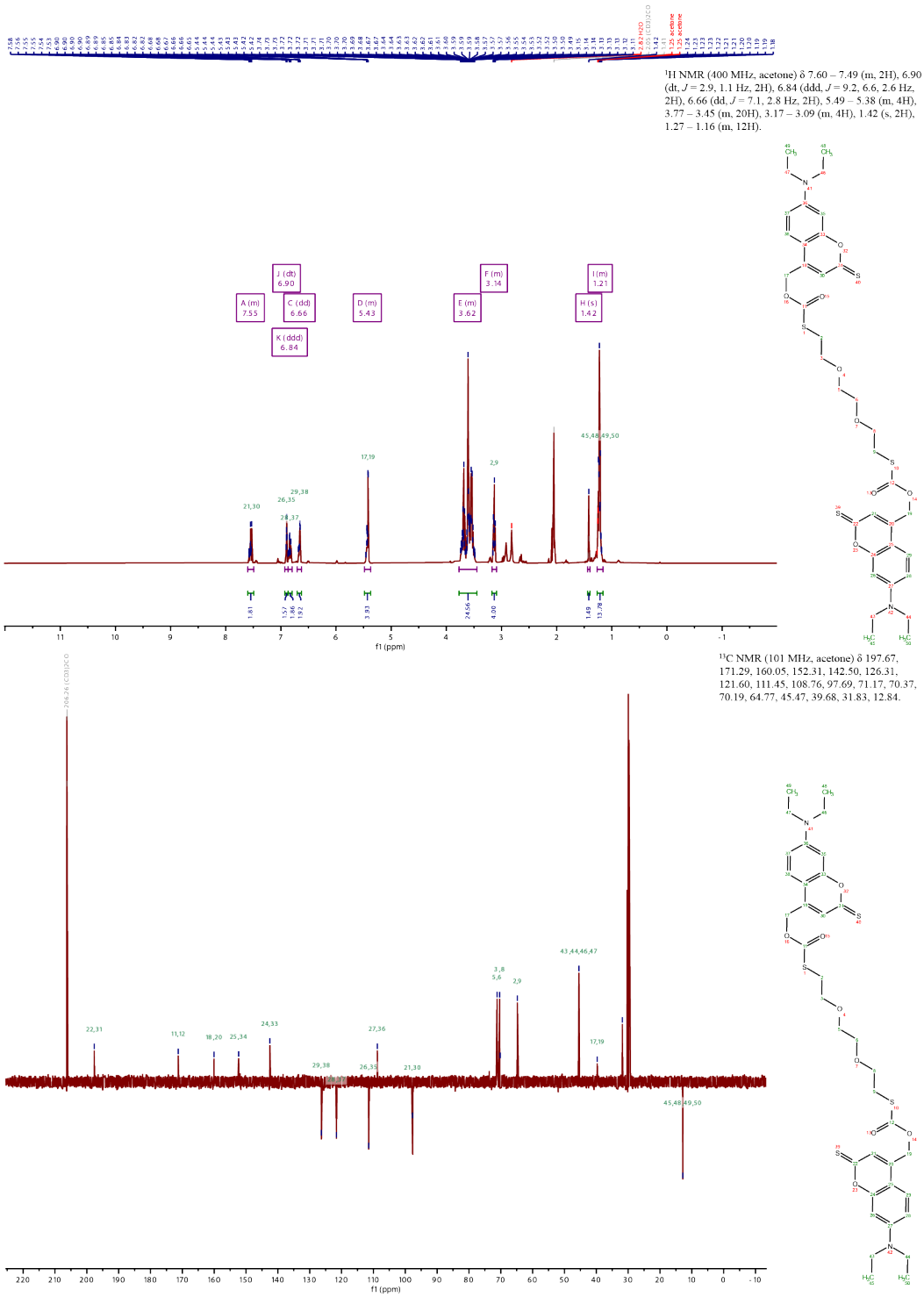


Figure 11: Protected EDT NMR spectra in d-acetone. For integration of the ¹H NMR spectrum peaks, protons on C2 and C9 were set as a reference of 4 protons. Increased proton estimations of the -CH_3 at 1.21 ppm (should be 12) and the PEG and coumarin -CH_2 (should be 22) may indicate some impurities.

2.9 Protected 4arm PEG

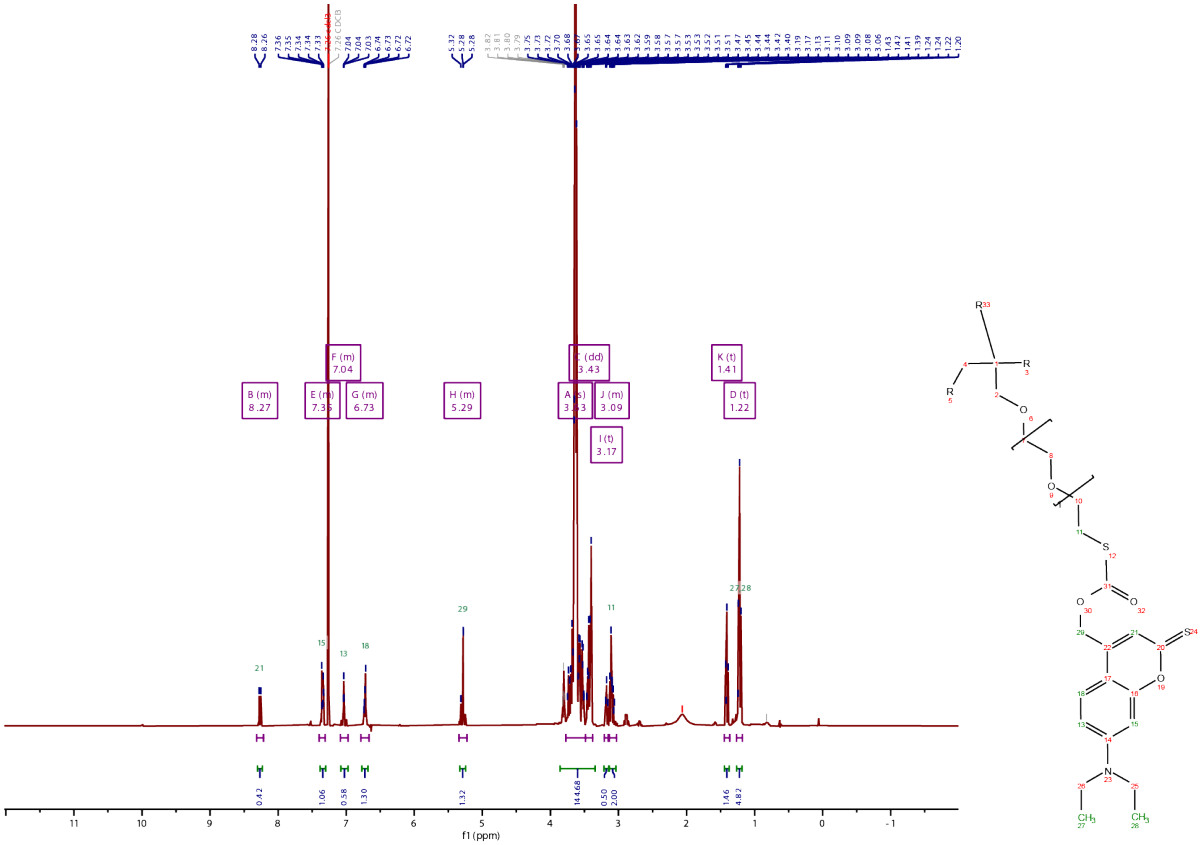


Figure 12: Protected 4arm PEG ^{1}H NMR spectrum. For simplicity, one of the identical four arms is schematized with the other three shown as R. Integration was done for one arm, the number of actual protons in the molecule is four times higher. Of note is the intensity of the PEG -CH_2 in the range of 3.4-3.5 ppm. For this reason, the ^{13}C NMR is absent due to absence of other peaks (not shown).

2.10 HA-MAL

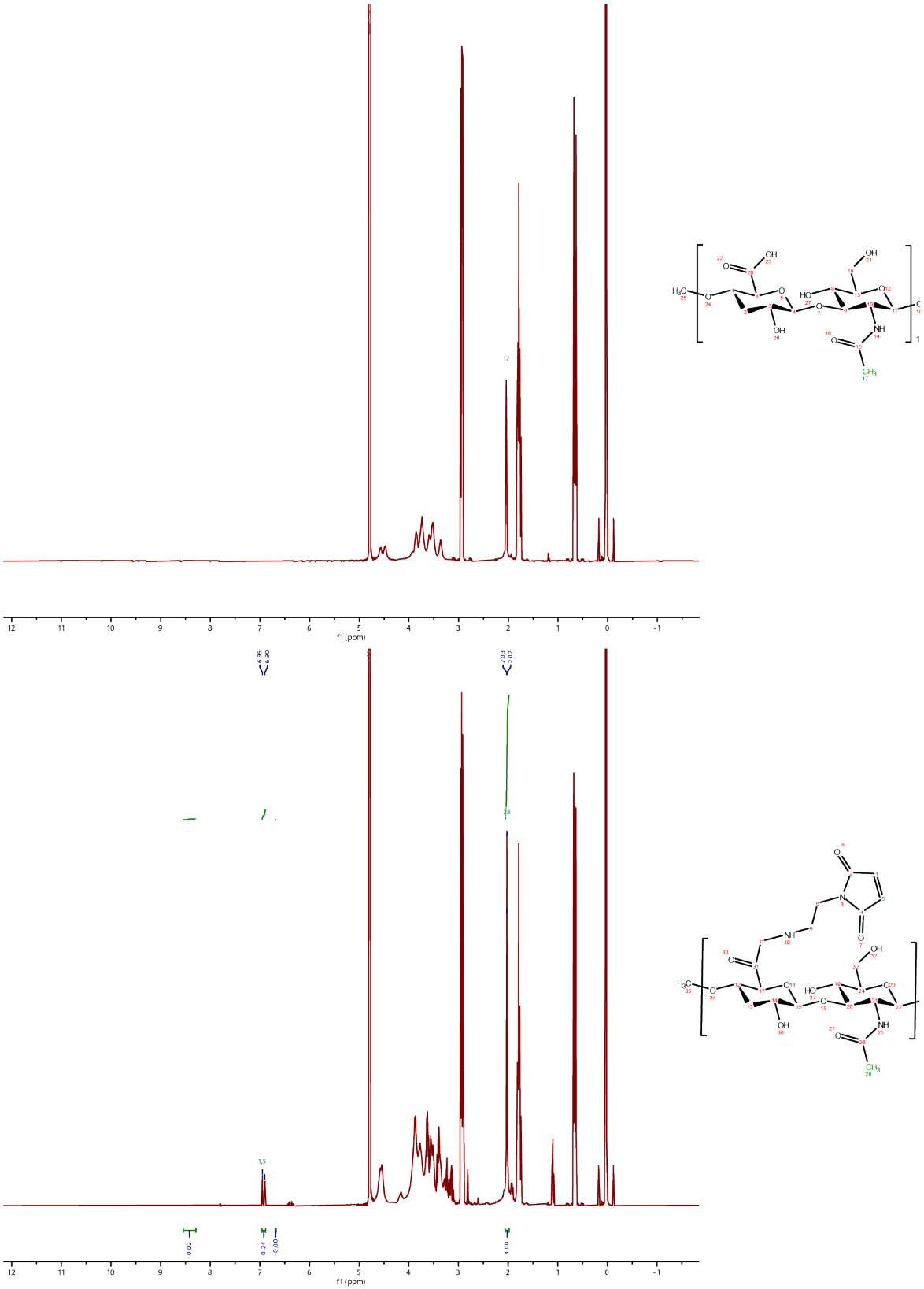


Figure 13: First synthesis HA-MAL. ^1H NMR spectra in D_2O. Top: Starting HA. Bottom: HA-MAL with 12% DoF.

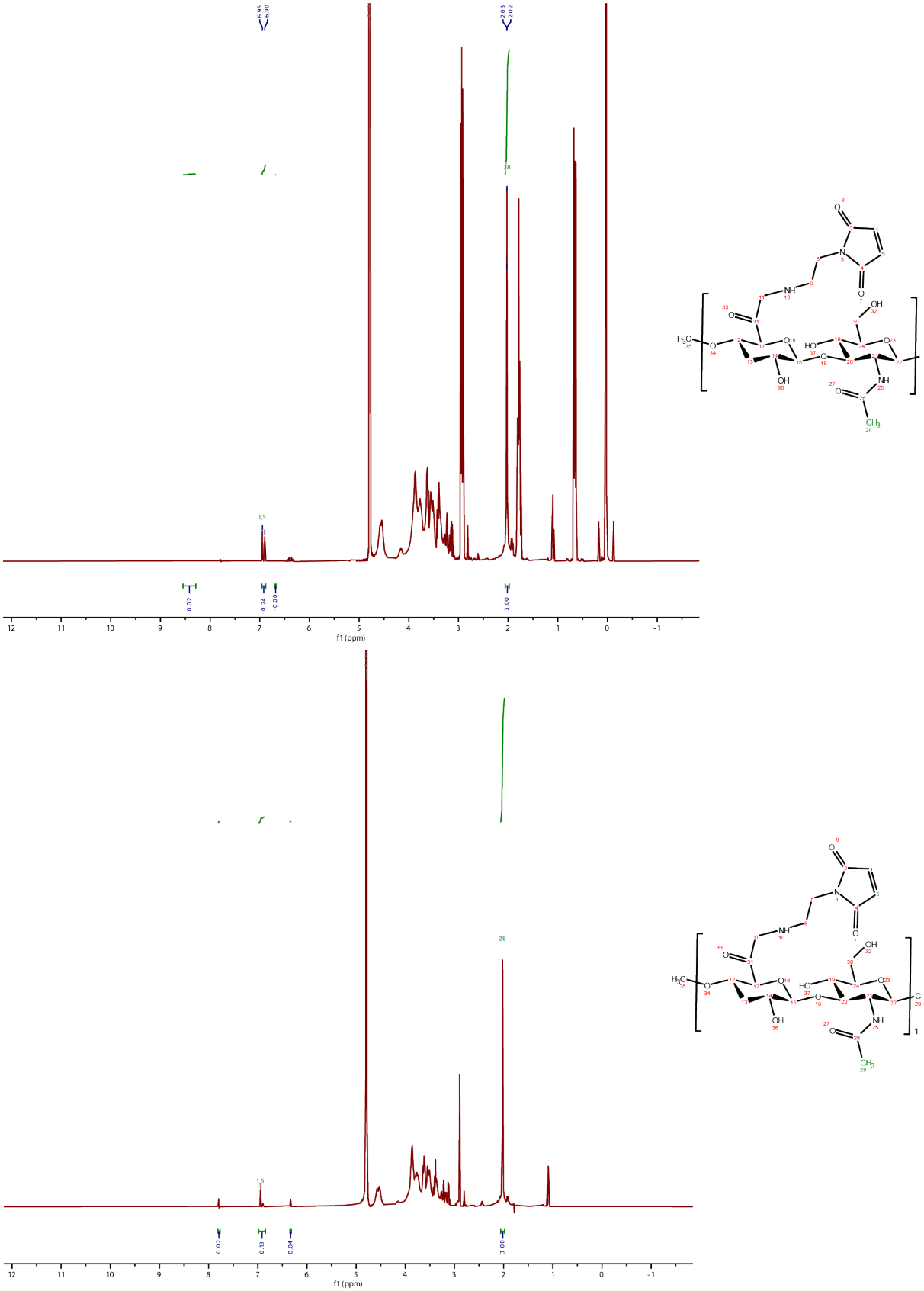


Figure 14: Second synthesis HA-MAL compared to first synthesis. ^{1}H NMR spectra in D_2O. Top: First synthesis result. Bottom: second synthesis of HA-MAL with 6.5% DoF. The main additional peaks of first synthesized HA-MAL can be attributed to the internal standard DSS present in the used D_2O, whereas in for the second batch another bottle of D_2O was used without internal standard. DSS is known to have resonance at 0, 0.63, 1.75, and 2.91 ppm.

3 GPC spectra

3.1 Overview first experiment

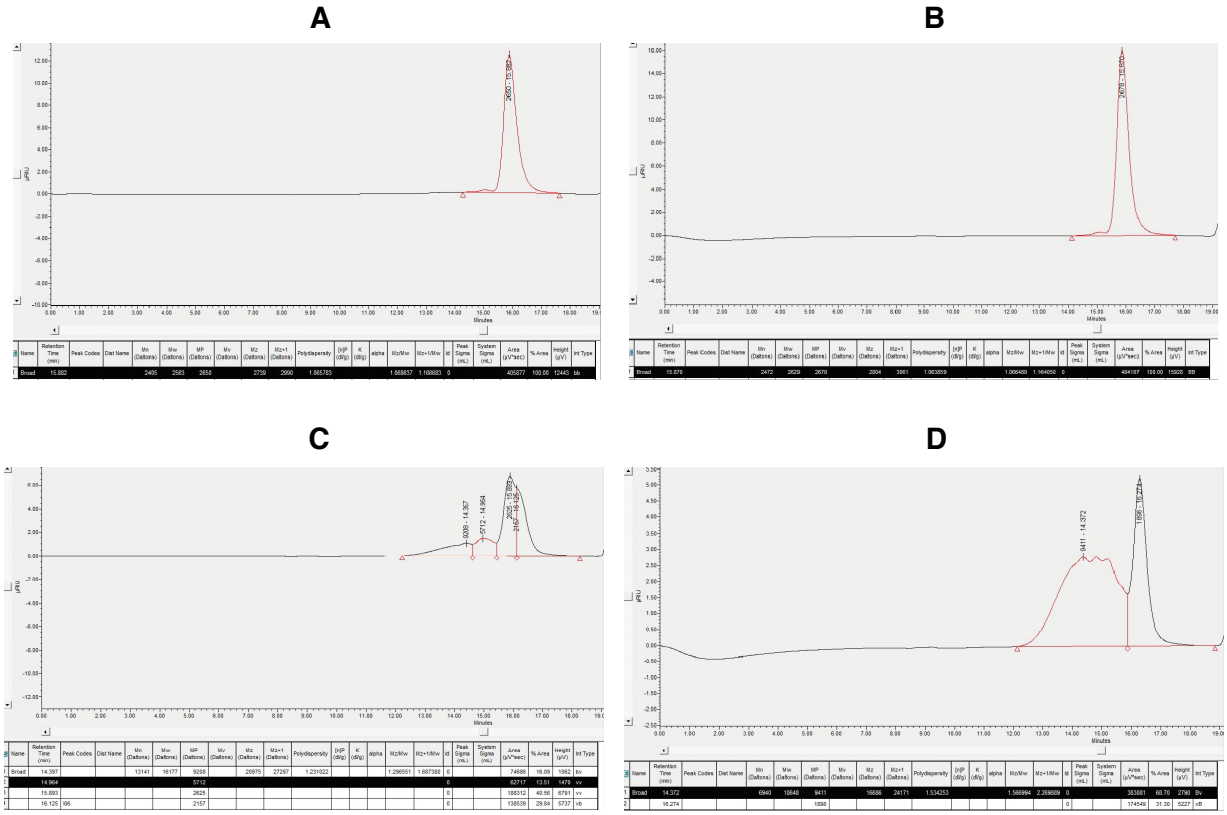


Figure 15: Linear PEG 2000 - EDT conjugation. A: PEG in PBS. Size estimated at 2650 Da. B: PEG in DMF. Size estimated at 2678 Da. C: PEG-EDT in PBS. Four populations distinguished: 2157 (29.84%), 2625 (40.56%), 5712 (13.51%), and 9208 (16.09%) Da. D: PEG-EDT in DMF. Two populations distinguished: 1898 (31.30%) and 9411 (68.70%) Da.

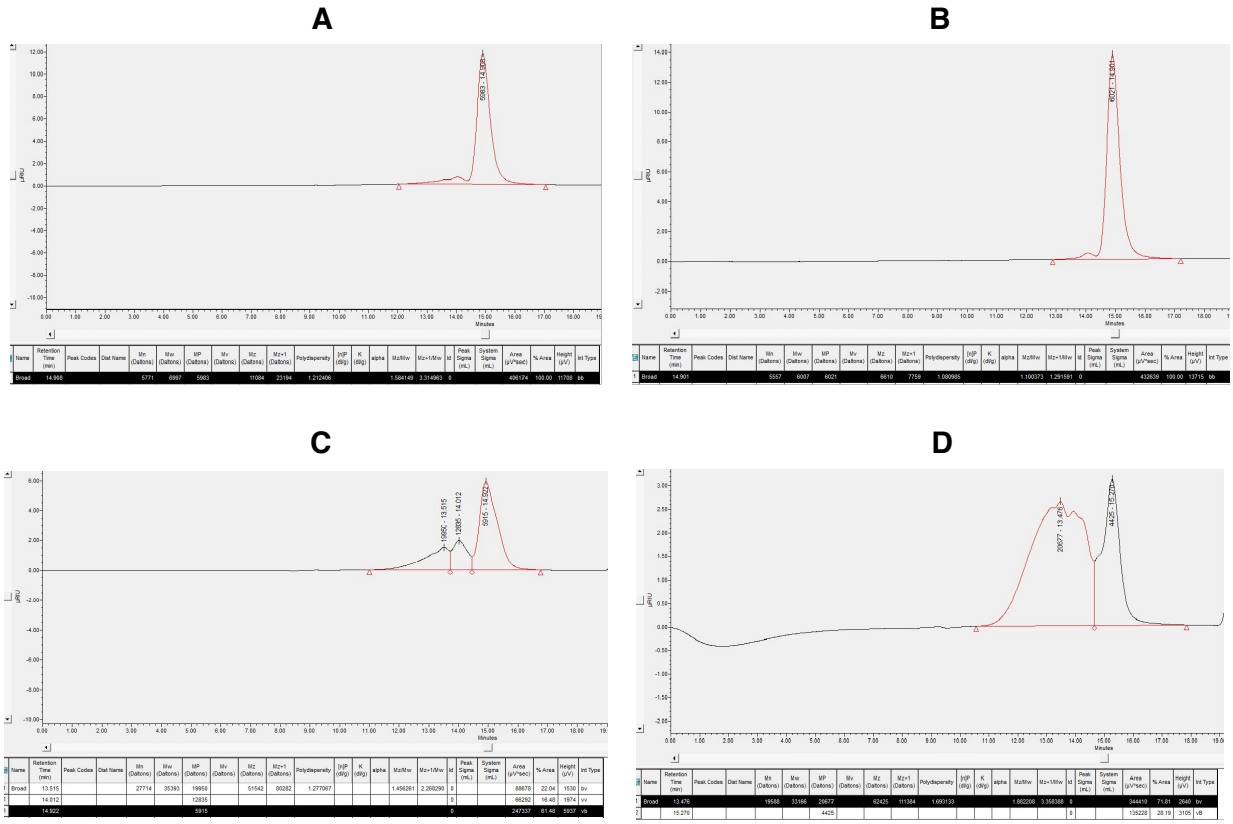
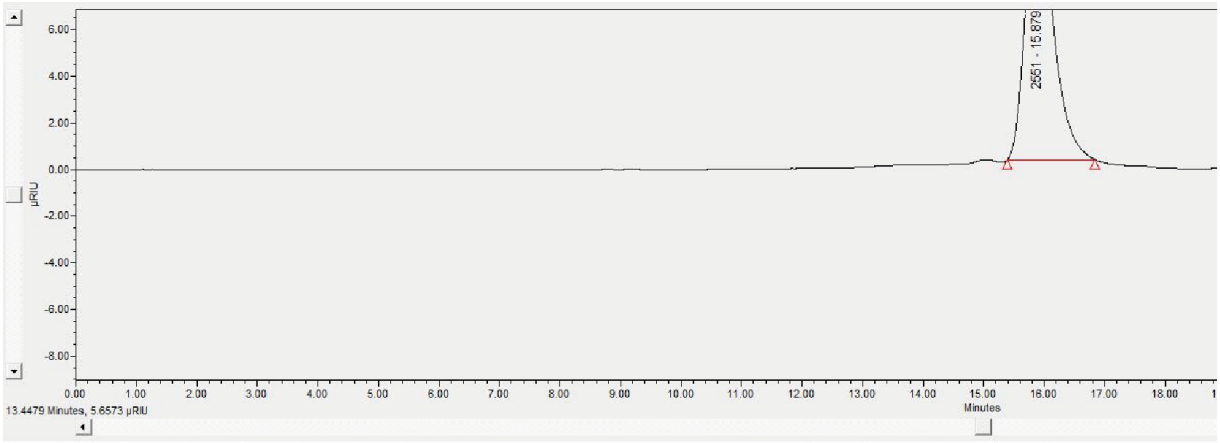
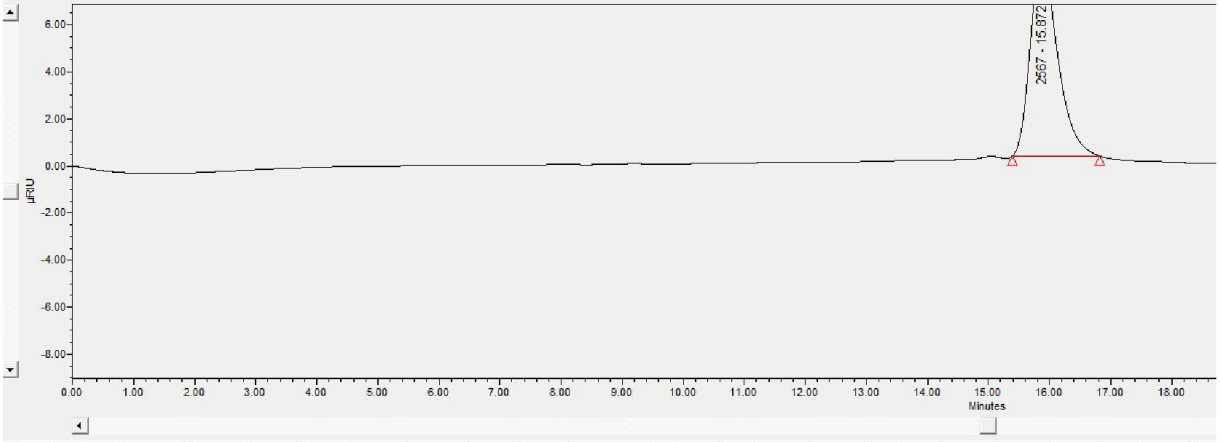


Figure 16: Linear PEG 5000 - EDT conjugation. A: PEG in PBS. Size estimated at 5983 Da. B: PEG in DMF. Size estimated at 6021 Da. C: PEG-EDT in PBS. Three populations distinguished: 5915 (61.48%), 12835 (16.48%), 19950 (22.04%) Da. D: PEG-EDT in DMF. Two populations distinguished: 4425 (28.19%) and 20677 (71.81%) Da.

3.2 Results of repetition of experiment in duplo

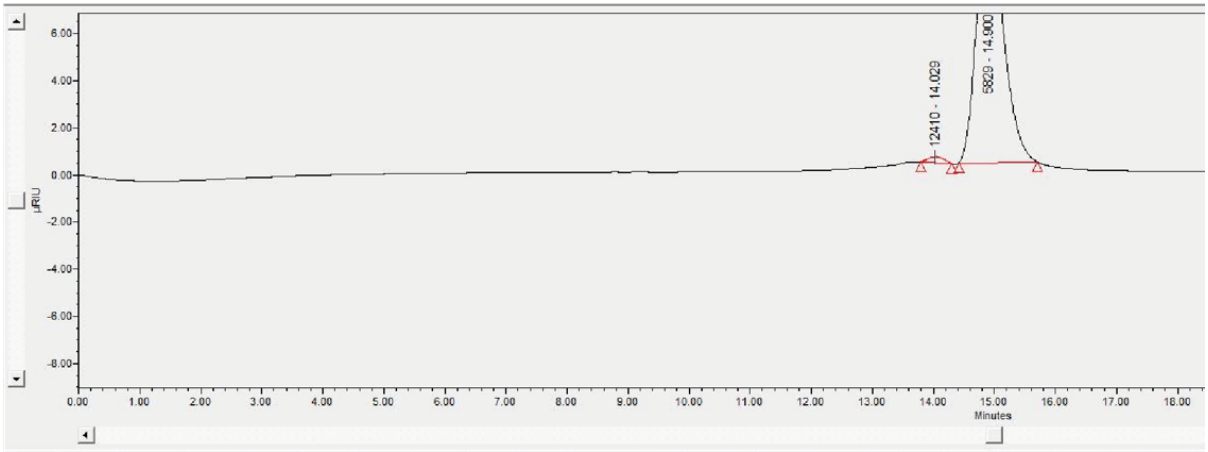


	Name	Retention Time (min)	Peak Codes	Dist Name	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mv (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Polydispersity	[n]P (dl/g)	K (dl/g)	alpha	Mz/Mw	Mz+1/Mw	Id	Peak Sigma (mL)	System Sigma (mL)	Area (µV*sec)	% Area	Height (µV)	Int Type
1	Broad	9.631															0						Missing
2	Peak3	15.879			2356	2441	2551		2519	2591	1.035997				1.032011	1.061552	5021			370724	100.00	12053	bb

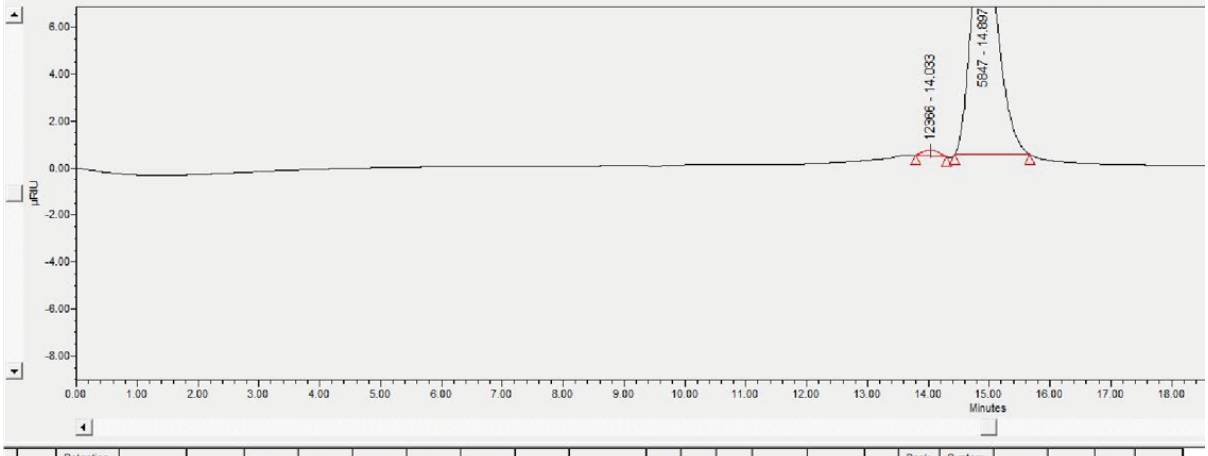


	Name	Retention Time (min)	Peak Codes	Dist Name	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mv (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Polydispersity	[n]P (dl/g)	K (dl/g)	alpha	Mz/Mw	Mz+1/Mw	Id	Peak Sigma (mL)	System Sigma (mL)	Area (µV*sec)	% Area	Height (µV)	Int Type
1	Broad	9.631															0						Missing
2	Peak3	15.872			2374	2459	2567		2536	2608	1.035513				1.031629	1.060837	5023			259088	100.00	8441	bb

Figure 17: Linear PEG 2000 in PBS. Size estimations were 2551 and 2567 Da.

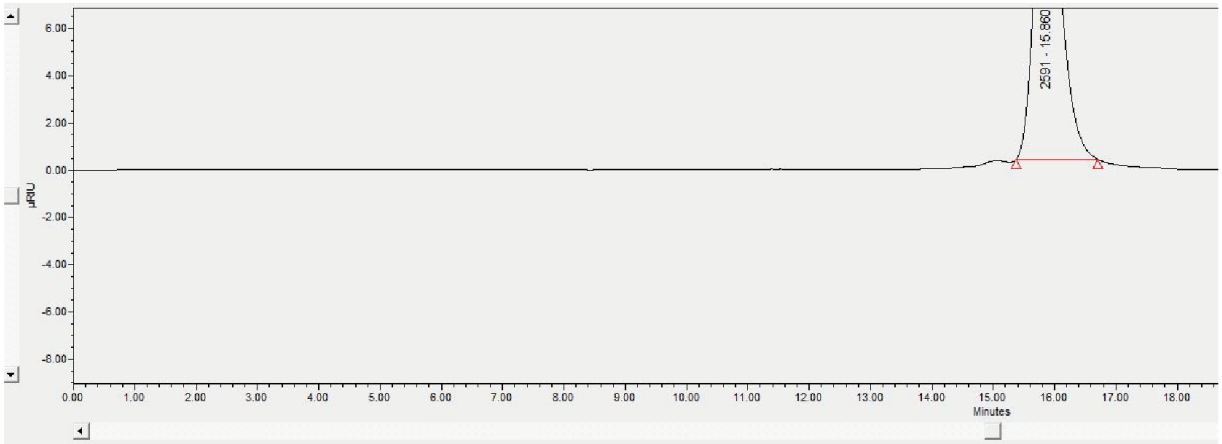


	Name	Retention Time (min)	Peak Codes	Dist Name	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mv (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Polydispersity	[η]P (dl/g)	K (dl/g)	alpha	Mz/Mw	Mz+1/Mw	Id	Peak Sigma (mL)	System Sigma (mL)	Area (μV*sec)	% Area	Height (μV)	Int Type
1	Broad	14.029			12171	12275	12410		12379	12484	1.008558				1.008529	1.017052	5025			4515	1.37	254	bb
2	Peak3	14.900			5505	5670	5829		5829	5980	1.030003				1.027908	1.054545	5026			324791	98.63	10984	bb

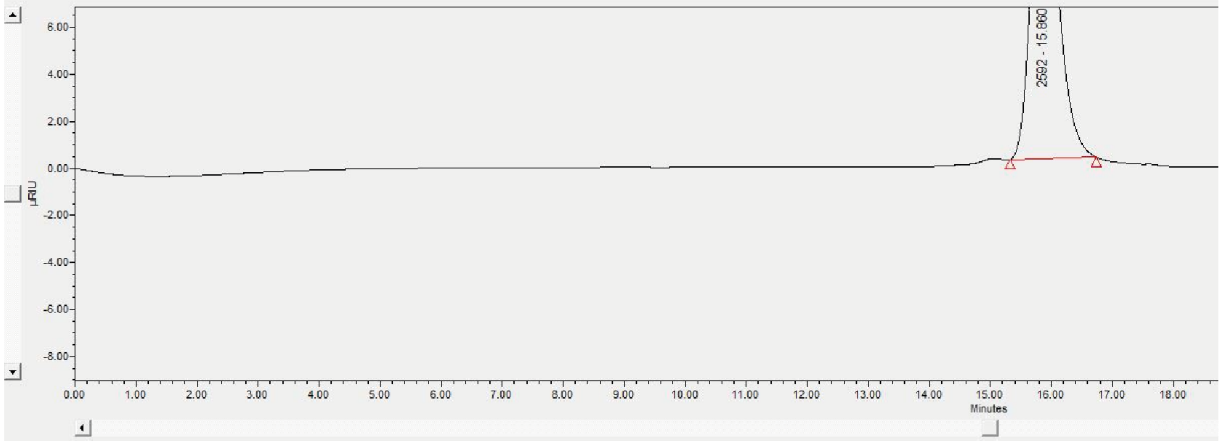


	Name	Retention Time (min)	Peak Codes	Dist Name	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mv (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Polydispersity	[η]P (dl/g)	K (dl/g)	alpha	Mz/Mw	Mz+1/Mw	Id	Peak Sigma (mL)	System Sigma (mL)	Area (μV*sec)	% Area	Height (μV)	Int Type
1	Broad	14.033			12176	12280	12366		12385	12490	1.008573				1.008543	1.017079	5028			4383	1.38	246	bb
2	Peak3	14.897			5530	5691	5847		5845	5992	1.028987				1.027062	1.052932	5029			312236	98.62	10656	bb

Figure 18: Linear PEG 5000 in PBS. Size estimations 5829 and 5847 Da. Also minor populations of larger compounds were present.

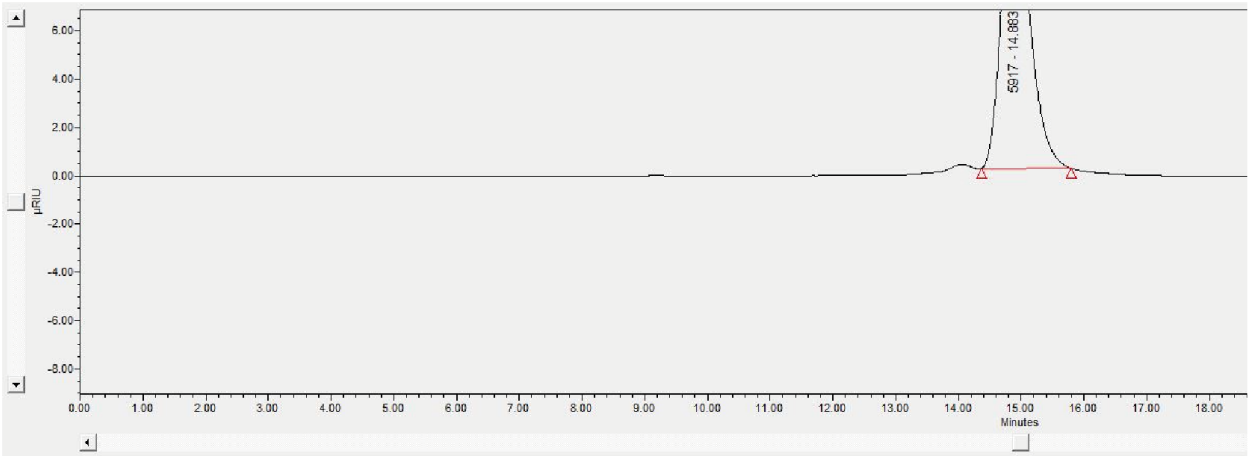


Name	Retention Time (min)	Peak Codes	Dist Name	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mv (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Polydispersity	[n]P (dl/g)	K (dl/g)	alpha	Mz/Mw	Mz+1/Mw	Id	Peak Sigma (mL)	System Sigma (mL)	Area (μV*sec)	% Area	Height (μV)	Int Type
Broad	*9.631*															*0*						*Missing*
Peak3	15.860			2446	2516	2591		2583	2646	1.028836				1.026521	1.051642	5033			426811	100.00	15080	bb

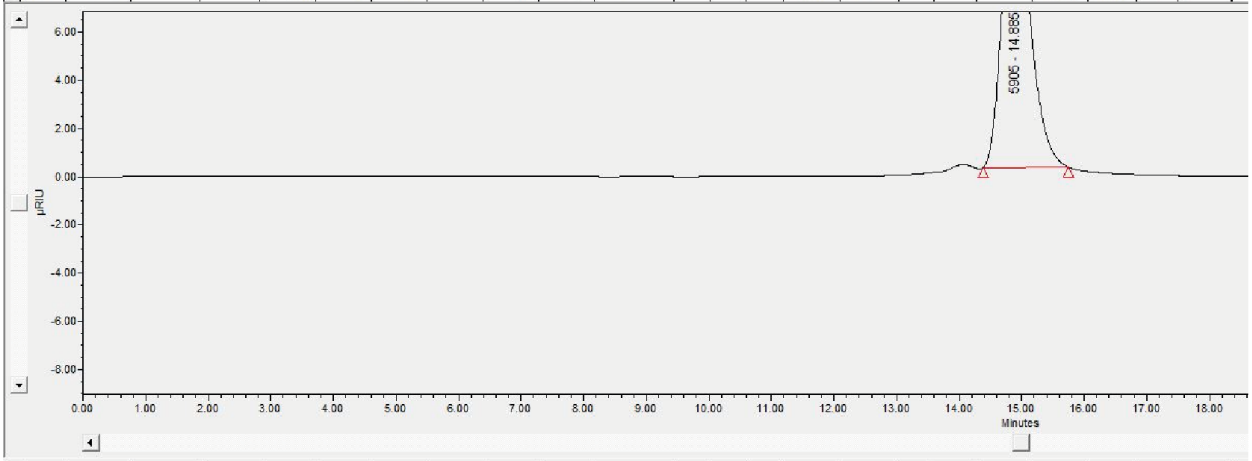


	Name	Retention Time (min)	Peak Codes	Dist Name	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mv (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Polydispersity	[n]P (dl/g)	K (dl/g)	alpha	Mz/Mw	Mz+1/Mw	Id	Peak Sigma (mL)	System Sigma (mL)	Area (μV*sec)	% Area	Height (μV)	Int Type
1	*Broad*	*9.631*															*0*						*Missing*
2	Peak3	15.860			2441	2515	2592		2585	2651	1.030359				1.027845	1.054233	5031			440426	100.00	15324	bb

Figure 19: Linear PEG 2000 in DMF. Size estimations were 2591 and 2592 Da.

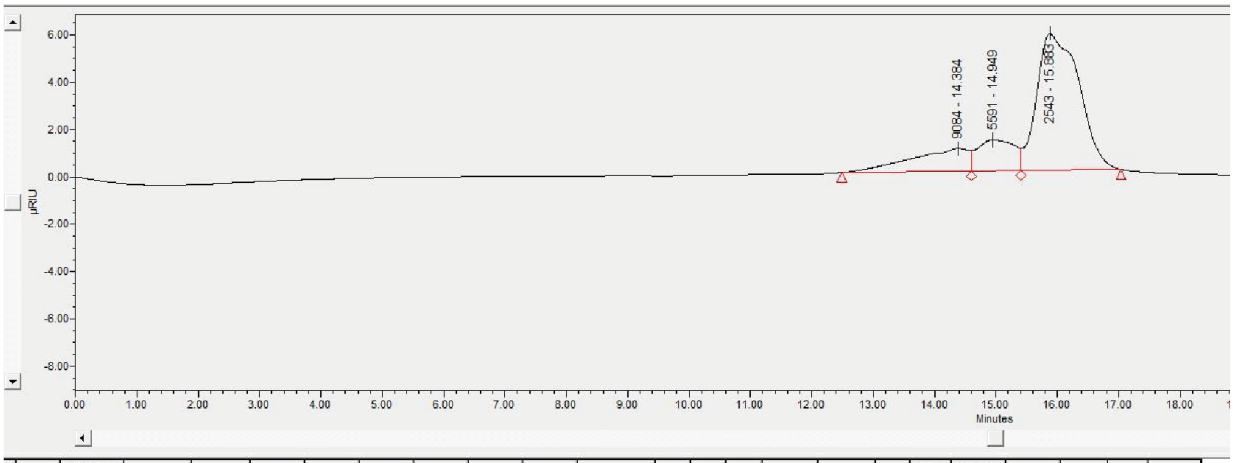


	Name	Retention Time (min)	Peak Codes	Dist Name	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mv (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Polydispersity	[n]P (dl/g)	K (dl/g)	alpha	Mz/Mw	Mz+1/Mw	Id	Peak Sigma (mL)	System Sigma (mL)	Area (µV*sec)	% Area	Height (µV)	Int Type
1	Broad	9.631															0						Missing
2	Peak3	14.883			5561	5739	5917		5907	6066	1.032084				1.029310	1.056990	5035			390350	100.00	13186	bb

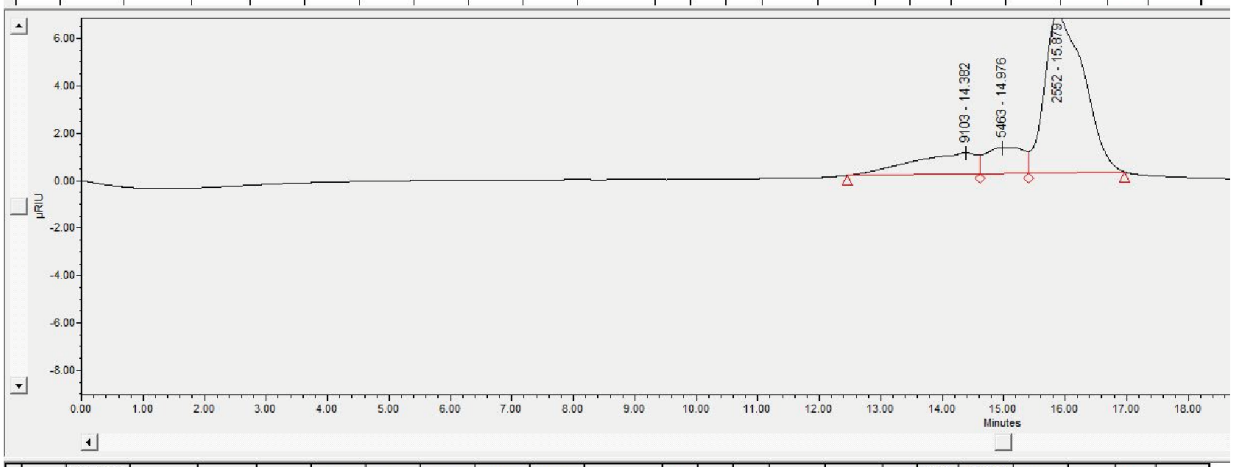


	Name	Retention Time (min)	Peak Codes	Dist Name	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mv (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Polydispersity	[n]P (dl/g)	K (dl/g)	alpha	Mz/Mw	Mz+1/Mw	Id	Peak Sigma (mL)	System Sigma (mL)	Area (µV*sec)	% Area	Height (µV)	Int Type
1	Broad	9.631															0						Missing
2	Peak3	14.885			5587	5739	5905		5903	6058	1.030896				1.028469	1.055500	5037			382731	100.00	13016	bb

Figure 20: Linear PEG 5000 in DMF. Size estimations were 5917 and 5905 Da.

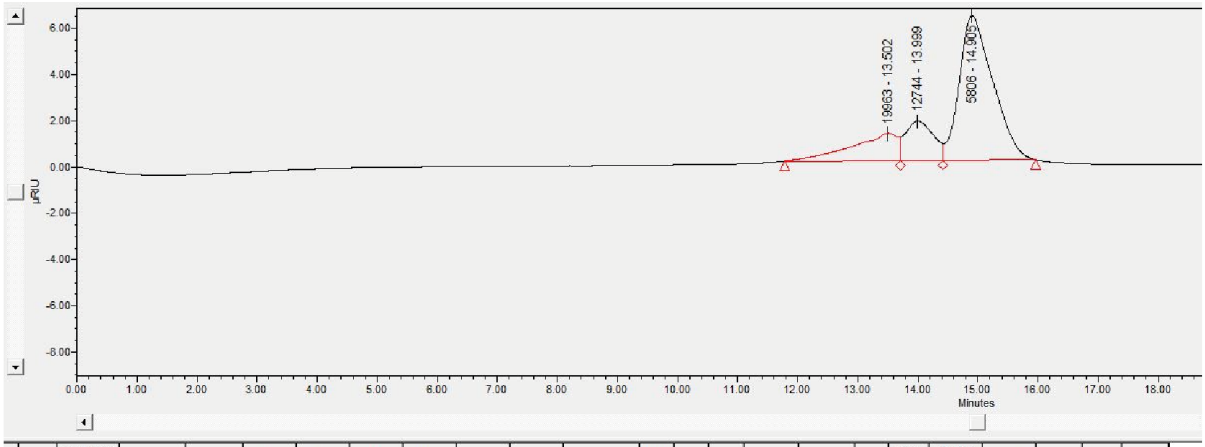


	Name	Retention Time (min)	Peak Codes	Dist Name	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mv (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Polydispersity	[n]P (dl/g)	K (dl/g)	alpha	Mz/Mw	Mz+1/Mw	Id	Peak Sigma (mL)	System Sigma (mL)	Area (µV*sec)	% Area	Height (µV)	Int Type
1	*Broad*	*9.631*															*0*						*Missing*
2	Peak2	14.384			12597	15043	9084		18615	23020	1.194223				1.237420	1.530221	5042			62687	15.94	968	bv
3	Peak3	14.949			5226	5415	5591		5608	5798	1.036251				1.035890	1.070761	5043			55197	14.04	1318	vv
4		15.883					2543										0			275377	70.02	5757	vb

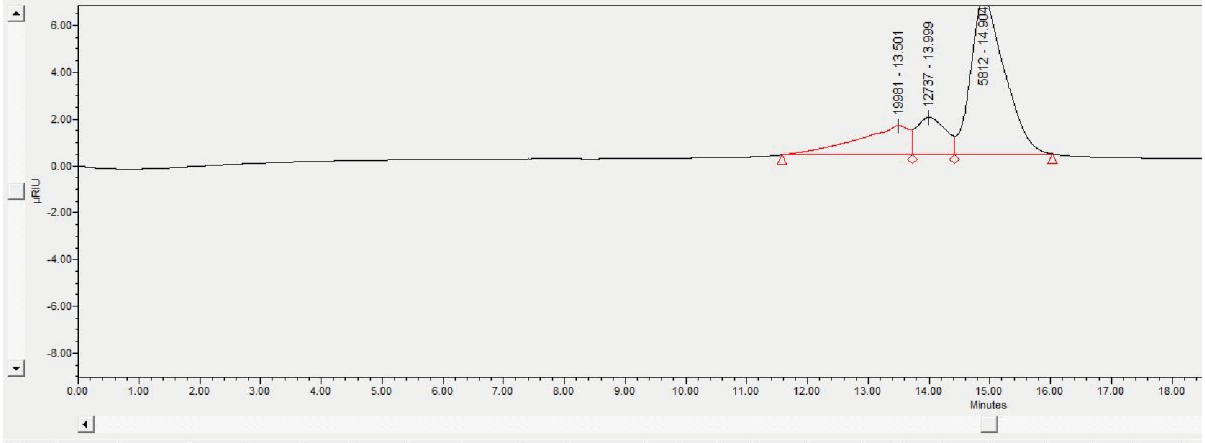


	Name	Retention Time (min)	Peak Codes	Dist Name	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mv (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Polydispersity	[n]P (dl/g)	K (dl/g)	alpha	Mz/Mw	Mz+1/Mw	Id	Peak Sigma (mL)	System Sigma (mL)	Area (µV*sec)	% Area	Height (µV)	Int Type
1	*Broad*	*9.631*															*0*						*Missing*
2	Peak2	14.382			13007	15817	9103		19820	24555	1.216052				1.253041	1.552389	5045			65450	15.90	890	bv
3	Peak3	14.976			5189	5355	5463		5545	5734	1.036004				1.035832	1.070768	5046			47647	11.58	1094	vv
4		15.879					2552										0			298415	72.52	6726	vb

Figure 21: Linear PEG 2000 and EDT in PBS. Three populations are visible.

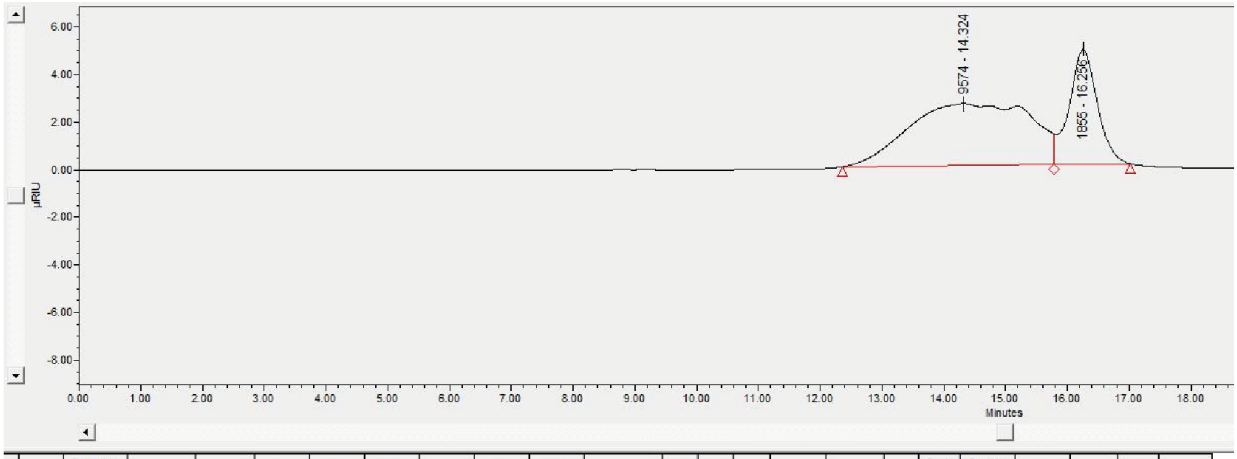


	Name	Retention Time (min)	Peak Codes	Dist Name	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mv (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Polydispersity	[n]P (dl/g)	K (dl/g)	alpha	Mz/Mw	Mz+1/Mw	Id	Peak Sigma (mL)	System Sigma (mL)	Area (μV*sec)	% Area	Height (μV)	Int Type
1	Broad	13.502			26471	31205	19963		38409	47738	1.178862				1.230849	1.529814	5052			62380	17.29	1179	bv
2	Peak2	13.999			12113	12445	12744		12776	13097	1.027423				1.026579	1.052397	5053			55032	15.25	1706	vv
3	Peak3	14.905			5158	5437	5806		5704	5957	1.054088				1.049175	1.095679	5054			243459	67.46	6253	vb

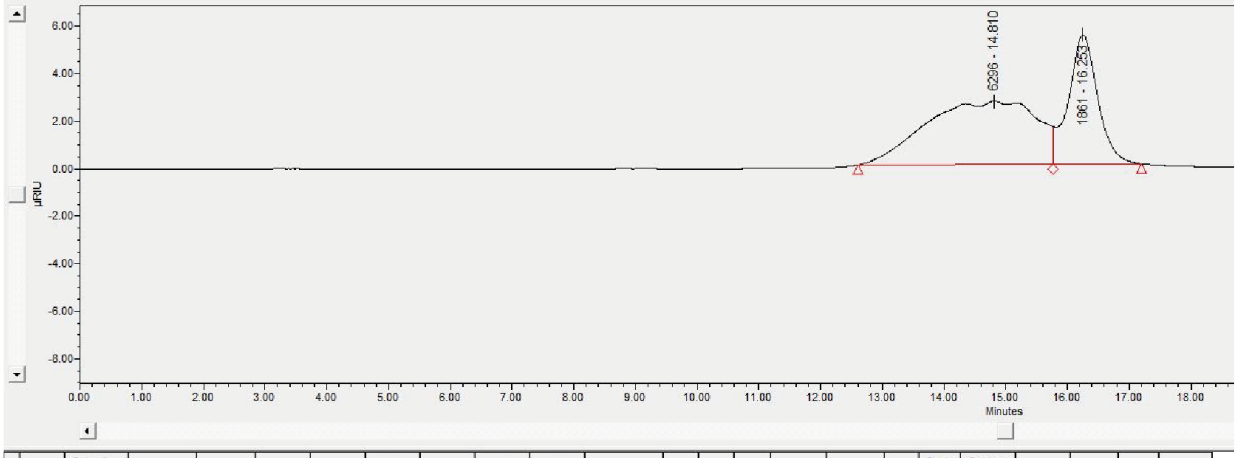


	Name	Retention Time (min)	Peak Codes	Dist Name	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mv (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Polydispersity	[n]P (dl/g)	K (dl/g)	alpha	Mz/Mw	Mz+1/Mw	Id	Peak Sigma (mL)	System Sigma (mL)	Area (μV*sec)	% Area	Height (μV)	Int Type
1	Broad	13.501			27404	33714	19981		43939	57407	1.230238				1.303319	1.702792	5056			72989	19.06	1236	bv
2	Peak2	13.999			12018	12345	12737		12670	12986	1.027205				1.026345	1.051880	5057			52138	13.61	1587	vv
3	Peak3	14.904			5172	5455	5812		5722	5974	1.054653				1.049106	1.095198	5058			257823	67.33	6717	vb

Figure 22: Linear PEG 5000 and EDT in PBS. Three populations are visible.

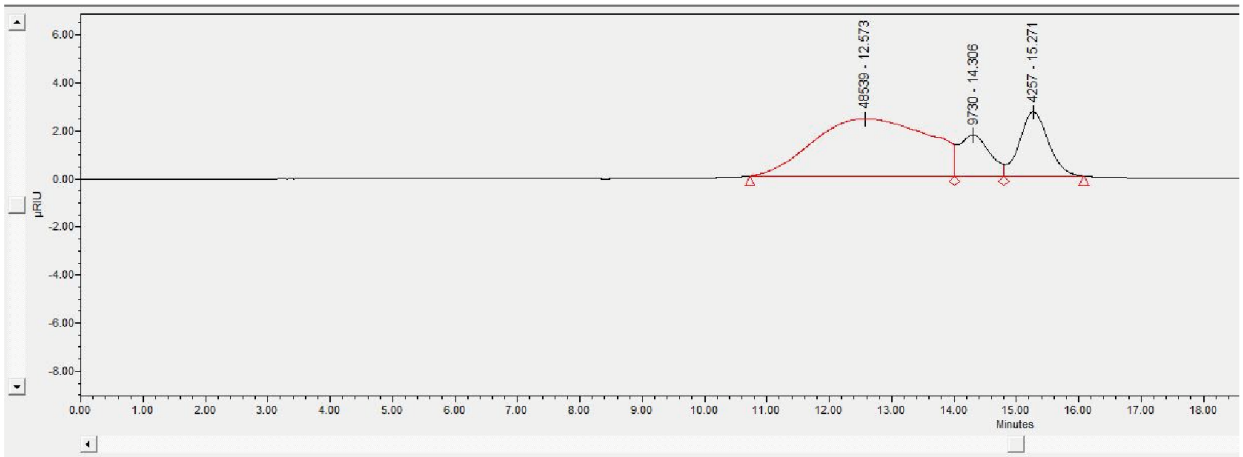


	Name	Retention Time (min)	Peak Codes	Dist Name	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mv (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Polydispersity	[n]P (dl/g)	K (dl/g)	alpha	Mz/Mw	Mz+1/Mw	Id	Peak Sigma (mL)	System Sigma (mL)	Area (µV*sec)	% Area	Height (µV)	Int Type
1	Broad	9.631															0						Missing
2	Peak2	14.324			7321	11237	9574		17102	23533	1.534828				1.521989	2.094275	5063			356738	69.82	2608	bv
3	Peak3	16.256			1812	1877	1855		1943	2008	1.036201				1.035022	1.069818	5064			154201	30.18	4820	vb

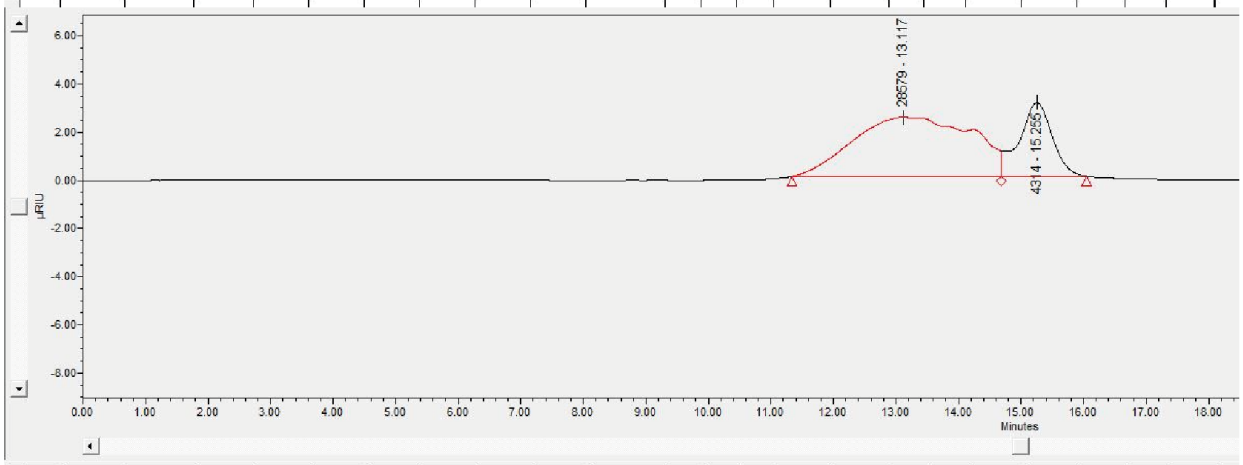


	Name	Retention Time (min)	Peak Codes	Dist Name	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mv (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Polydispersity	[n]P (dl/g)	K (dl/g)	alpha	Mz/Mw	Mz+1/Mw	Id	Peak Sigma (mL)	System Sigma (mL)	Area (µV*sec)	% Area	Height (µV)	Int Type
1	Broad	9.631															0						Missing
2	Peak3	14.810			6762	9775	6296		14247	19206	1.445646				1.457500	1.964739	5066			330377	64.74	2656	bv
3	Peak2	16.253			1808	1883	1861		1957	2029	1.041389				1.039110	1.077444	5067			179905	35.26	5424	vb

Figure 23: Linear PEG 2000 and EDT in DMF. Distinction between two populations was made.



	Name	Retention Time (min)	Peak Codes	Dist Name	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mv (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Polydispersity	[n]P (dl/g)	K (dl/g)	alpha	Mz/Mw	Mz+1/Mw	Id	Peak Sigma (mL)	System Sigma (mL)	Area (µV*sec)	% Area	Height (µV)	Int Type
1	Broad	12.573			35189	58432	48539		96280	139815	1.660538				1.647710	2.392788	5072			304737	67.86	2389	bv
2	Peak2	14.306			9254	9544	9730		9827	10093	1.031392				1.029551	1.057517	5073			58526	13.03	1722	vv
3	Peak3	15.271			4105	4250	4257		4394	4536	1.035315				1.033822	1.067172	5074			85784	19.10	2672	vb



	Name	Retention Time (min)	Peak Codes	Dist Name	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mv (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Polydispersity	[n]P (dl/g)	K (dl/g)	alpha	Mz/Mw	Mz+1/Mw	Id	Peak Sigma (mL)	System Sigma (mL)	Area (µV*sec)	% Area	Height (µV)	Int Type
1	Broad	13.117			19835	32196	28579		51349	72376	1.623164				1.594899	2.247982	5076			323349	74.25	2461	bv
2	Peak3	15.255			4320	4529	4314		4747	4969	1.048307				1.048115	1.097218	5077			112137	25.75	3051	vb

Figure 24: Linear PEG 5000 and EDT in DMF. Distinction between either three or two populations was made.

4 References

1. Klimek, R. *et al.* Visible light-activatable Q-dye molecular beacons for long-term mRNA monitoring in neurons. *Chem. Commun.* **57**, 12683–12686 (2021).
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