Supporting Information

Design, synthesis and evaluation of novel, branched trident small interfering RNA nanostructures for sequence-specific RNAi activity

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Table of Contents

Experimental Section

General. All solvents and reagents were purchased from the suppliers and used without further purification. Reactions were monitored by TLC on silica plates using UV-light or suitable stain for visualization. Evaporation and condensation were carried out in vacuo. $CDCl_3$ (CIL) or DMSO-d_6 (CIL) were used as solvents for obtaining NMR spectra. NMR spectra were recorded with JEOL JNM-ECS 400 and 500 spectrometers with tetramethylsilane as an internal standard (for ^{13}C NMR and ^{1}H NMR). Chemical shifts (δ) and coupling constants (J) are given in ppm (parts per million) and Hz (hertz) respectively. The abbreviations s, d, t, q and m signify singlet, doublet, triplet, quartet, and multiplet respectively.

Synthesis of solid-support analog.

2-(((*tert*-Butyldimethylsilyl)oxy)methyl)-2-(hydroxymethyl)propane-1,3-diol (**D2**)

To a solution of pentaerythritol (1.36 g, 10 mmol) and Et_3N (1.54 mL, 11 mmol) in DMF (80 mL) was added TBDMSCl (1.21 g, 8 mmol) and stirred at room temperature. The reaction mixture was stirred for 16 h at room temperature and partitioned between EtOAc and aqueous $NaHCO_3$ (saturated). The organic layer was washed with brine, dried (Na_2SO_4) and evaporated under reduced pressure. The crude was purified by silica gel chromatography using chloroform/methanol (20:1) to yield D2 (1.30 g, 5.2 mmol, 65%). ^{1}H-NMR (500 MHz, $CDCl_3$), δ 3.72 (d, J = 5.7 Hz, 6H), 3.66 (s, 2H), 2.53 (t, J = 5.7 Hz, 3H), 0.89 (d, J = 11.5 Hz, 9H), 0.08 (d, J = 2.9 Hz, 6H); ^{13}C-NMR (126 MHz, $CDCl_3$) δ 77.42, 76.90, 65.89, 64.73, 45.20, 25.93, 18.23, -5.59, HRMS: 273.149 $[M+Na]^+$.

(3-(Allyloxy)-2,2-bis((allyloxy)methyl)propoxy)(*tert*-butyl)dimethylsilane (**D3**)

To a stirred solution of NaH, in 60% oil immersion (0.78 g,19.4 mmol) in THF (50 mL) was added D2 (1.515 g, 4.85 mmol) dissolved in THF (10 mL) at 0°C. The reaction was stirred at 0°C for 30 minutes before adding the allyl bromide (2.46 mL, 29.1 mmol). The reaction mixture was then stirred for 14 h at room temperature and traced with TLC using $KMnO_4$ as

the stain for the double bond. Excess of NaH was quenched with MeOH and then partitioned between EtOAc and aqueous $NaHCO_3$ (saturated). The organic layer was washed with brine, dried (Na_2SO_4) and evaporated under reduced pressure. The crude was purified by silica gel chromatography using hexane/ethyl acetate (20:1) to yield D3 (1.45 g, 3.92 mmol, 81%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 5.84-5.92 (m, 3H), 5.25 (dq, *J* = 17.2, 1.7 Hz, 3H), 5.11-5.14 (m, 3H), 3.94 (td, *J* = 3.4, 1.5 Hz, 6H), 3.59 (s, 2H), 3.42 (s, 6H), 0.88 (s, 9H), 0.02 (t, *J* = 3.2 Hz, 6H); ^{13}C-NMR (126 MHz, $CDCl_3$) δ 135.47, 116.18, 77.41, 76.90, 72.42, 69.13, 61.74, 46.20, 29.86, 26.02, 18.38, -5.49, HRMS: 393.240 $[M+Na]^+$.

3,3'-((2-(((*tert*-Butyldimethylsilyl)oxy)methyl)-2-((3-hydroxypropoxy)methyl)propane-1,3-diyl)bis(oxy))bis(propan-1-ol) (**D4**)

9- Borabicyclo [3.3.1] nonane (9-BBN, 0.5 M in THF, 33.75 mL) was added dropwise to a solution of compound D3 (1.39 g, 3.75 mmol) in THF (15 mL) and stirred for 17 h at room temperature. Water was added to the reaction mixture until effervescence ceased. 3 N NaOH solution (12.6 mL) was added, and then, slowly 30% aqueous hydrogen peroxide solution (6.4 mL) was added while keeping the temperature between 30 and 50 °C. The mixture was stirred and extracted with water and ethyl acetate. The organic layer was washed with neutral phosphate buffer solution and brine, dried over Na_2SO_4, filtered, and concentrated. The crude material was purified by column chromatography (4% methanol in chloroform) to afford the desired product D4 (1.32 g, 3.11 mmol, 83%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 3.74 (q, *J* = 5.3 Hz, 6H), 3.58 (t, *J* = 5.4 Hz, 6H), 3.50 (d, *J* = 6.3 Hz, 2H), 3.37 (s, 6H), 3.22 (t, *J* = 5.4 Hz, 3H), 1.77-1.82 (m, 6H), 0.88 (s, 10H), 0.03 (s, 6H); ^{13}C-NMR (126 MHz, $CDCl_3$) δ 77.41, 76.90, 70.69, 70.62, 61.92, 61.79, 45.60, 31.86, 25.98, 18.38, 14.33, -5.56, HRMS: 447.273 $[M+Na]^+$.

6,6-bis((3-(Allyloxy)propoxy)methyl)-2,2,3,3-tetramethyl-4,8,12-trioxa-3-silapentadec-14-ene (**D5**)

To a stirred solution of NaH, in 60% oil immersion (0.46g, 11.48 mmol) in THF (30 mL) was added D4 (1.22 g, 2.87 mmol) dissolved in THF (6 mL) at 0°C. The reaction was stirred at 0°C for 30 minutes before adding the allyl bromide (1.46 mL, 17.22 mmol). The reaction mixture was then stirred overnight at room temperature and traced with TLC using $KMnO_4$ as the stain for the double bond. Excess of NaH was quenched with MeOH and then partitioned between EtOAc and aqueous $NaHCO_3$ (saturated). The organic layer was washed with brine, dried (Na_2SO_4) and evaporated under reduced pressure. The crude was purified by silica gel chromatography using hexane/ethyl acetate (10:1) to yield D5 (1.00 g, 1.85 mmol, 64%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 5.91 (qd, *J* = 11.1, 5.4 Hz, 3H), 5.26 (dq, *J* = 17.2, 1.7 Hz, 3H), 5.16 (dd, *J* = 10.3, 1.1 Hz, 3H), 3.96 (td, *J* = 3.4, 2.1 Hz, 6H), 3.53 (s, 2H), 3.50 (t, *J* = 6.6 Hz, 6H), 3.45 (t, *J* = 6.3 Hz, 6H), 3.34 (s, 6H), 1.80-1.85 (m, 6H), 0.88 (s, 9H), 0.01 (d, *J* = 2.9 Hz, 6H); ^{13}C-NMR (126 MHz, $CDCl_3$) δ 135.17, 116.82, 77.42, 76.90, 72.02, 69.51, 68.38, 67.71, 61.82, 46.18, 30.25, 26.03, 18.40, -5.47, HRMS: 567.372 $[M+Na]^+$.

10-(((*tert*-Butyldimethylsilyl)oxy)methyl)-10-((3-(3-hydroxypropoxy)propoxy)methyl)-4,8,12,16-tetraoxanonadecane-1,19-diol (**D6**)

9-Borabicyclo [3.3.1] nonane (9-BBN, 0.5 M in THF, 14.85 mL) was added dropwise to a solution of compound D5 (0.9 g, 1.65 mmol) in THF (10 mL) and stirred for 18 h at room temperature. Water was added to the reaction mixture until effervescence ceased. 3 N NaOH solution (5.6 mL) was added, and then, 30% aqueous hydrogen peroxide solution (6.4 mL) was added carefully, while keeping the temperature between 30 and 50 °C. The mixture was stirred and extracted with water and ethyl acetate. The organic layer was washed with neutral phosphate buffer solution and brine, dried over Na_2SO_4, filtered, and concentrated. The crude was purified by column chromatography (4% methanol in chloroform) to afford the desired product D6 (0.92 g, 1.53 mmol, 93%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 3.76 (q, *J* = 5.5 Hz, 6H),

3.60 (t, *J* = 5.7 Hz, 6H), 3.49-3.52 (m, 8H), 3.44 (t, *J* = 6.3 Hz, 6H), 3.33 (s, 6H), 2.61 (t, *J* = 5.4 Hz, 3H), 1.81 (td, *J* = 12.7, 6.3 Hz, 12H), 0.88 (s, 9H), 0.02 (d, *J* = 2.9 Hz, 6H); ^{13}C-NMR (126 MHz, $CDCl_3$) δ 77.42, 76.90, 70.24, 69.47, 68.53, 68.25, 62.06, 61.74, 46.17, 32.18, 30.14, 26.02, 18.40, -5.48, HRMS: 621.404 $[M+Na]^+$.

12,12-bis((3-(3-(bis(4-methoxyphenyl)(phenyl)methoxy)propoxy)propoxy)methyl)-1,1-bis(4-methoxyphenyl)-15,15,16,16-tetramethyl-1-phenyl-2,6,10,14-tetraoxa-15-silahepta- decane (**D7**)

To a solution of D6 (0.84 g, 1.40 mmol) in pyridine (30 mL) stirred at room temperature, DMTrCl (1.71 g, 5.04 mmol) was added. The mixture was stirred for 12 h and extracted with EtOAc and aqueous $NaHCO_3$ (saturated). The organic layer was washed with brine, dried (Na_2SO_4) and concentrated under vacuum. The residue was purified by silica gel chromatography using hexane/ethyl acetate (5:1) to give D7 (1.90 g, 1.26 mmol, 90%):^{1}H-NMR (500 MHz, $CDCl_3$) 7.42 (d, *J* = 7.4 Hz, 6H), 7.29-7.32 (m, 13H), 7.28 (s, 2H), 7.25 (s, 2H), 7.18 (t, *J* = 7.2 Hz, 3H), 6.79-6.81 (m, 13H), 3.77 (s, 18H), 3.53 (t, *J* = 6.0 Hz, 8H), 3.43 (t, J = 6.6 Hz, 6H), 3.38 (t, *J* = 6.3 Hz, 6H), 3.30 (s, 6H), 3.12 (t, *J* = 6.3 Hz, 6H), 1.73-1.87 (m, 12H), 0.86 (s, 9H), -0.01 (s, 6H); ^{13}C-NMR (126 MHz, $CDCl_3$) δ 158.46, 145.44, 136.76, 130.15, 128.34, 127.82, 126.71, 113.10, 85.86, 77.42, 76.91, 69.45, 68.53, 68.30, 60.53, 60.47, 55.31, 30.60, 30.25, 26.03, 21.20, 14.35, -5.46, HRMS: 1527.807 $[M+Na]^+$.

3-(3-(3-(bis(4-Methoxyphenyl)(phenyl)methoxy)propoxy)propoxy)-2,2-bis((3-(3-(bis(4-methoxyphenyl)(phenyl)methoxy)propoxy)propoxy)methyl)propan-1-ol (**D8**)

To a stirred solution of D7 (1.81 g, 1.19 mmol) in THF (12 mL) was added TBAF (1 M in THF, 2.38 mL) at room temperature. The reaction mixture was stirred for 18 h at room temperature and partitioned between EtOAc and aqueous $NaHCO_3$ (saturated). The organic layer was washed with brine, dried (Na_2SO_4) and evaporated under reduced pressure. The crude

was purified by silica gel chromatography using hexane/ethyl acetate (2:1) to yield D8 (1.50 g, 1.08 mmol, 91%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.41-7.43 (m, 6H), 7.29-7.32 (m, 13H), 7.28 (s, 2H), 7.25 (s, 2H), 7.18 (t, *J* = 7.2 Hz, 3H), 6.79-6.82 (m, 13H), 3.77 (s, 18H), 3.65 (d, J = 6.3 Hz, 2H), 3.53 (t, *J* = 6.6 Hz, 6H), 3.44 (s, 1H), 3.42 (t, *J* = 5.7 Hz, 9H), 3.39 (s, 2H), 3.39 (s, 6H), 3.12 (t, *J* = 6.3 Hz, 6H), 3.00 (t, *J* = 6.3 Hz, 1H), 1.82-1.87 (m, 6H), 1.74-1.79 (m, 6H); ^{13}C-NMR (126 MHz, $CDCl_3$) δ 158.46, 145.43, 136.74, 130.15, 128.32, 127.82, 126.72, 113.10, 85.86, 77.41, 76.90, 71.59, 68.84, 68.34, 68.04, 60.43, 55.32, 44.91, 30.55, 30.11, HRMS: 1413.743 $[M+Na]^+$.

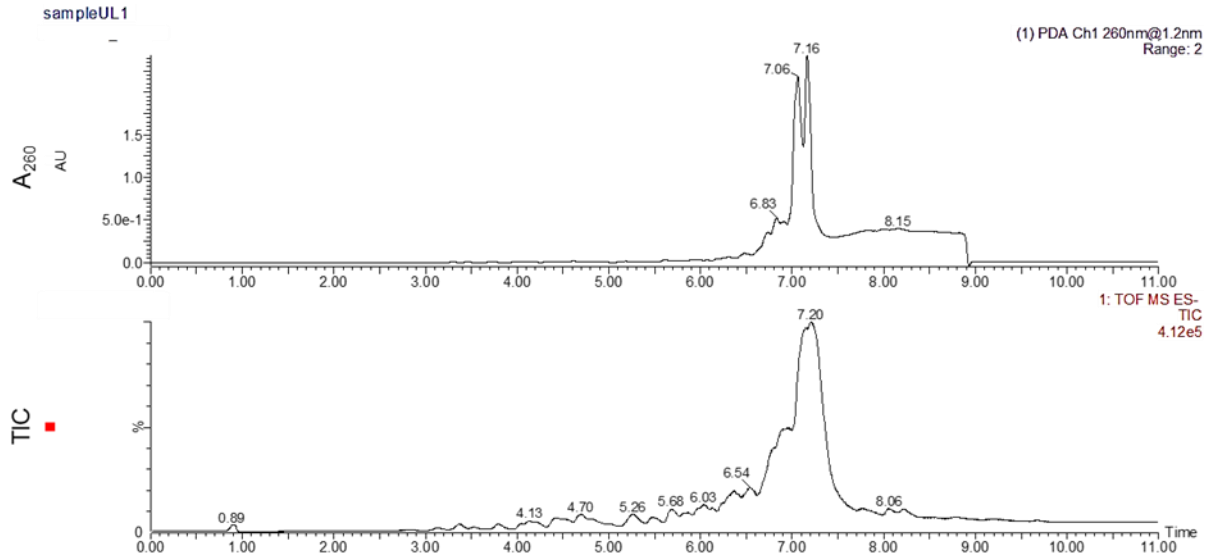


Fig. S2. UPLC chromatogram of branched RNA (crude).

Cellular Uptake Test. HeLa cells (4.0×10^4 cell/mL) were cultured on the 48-micro well plate (400 μL/well) and were grown for 24 h before transfection. Cells were transfected with 50 nM of fluorescein labelled siRNA and equimolar td siRNA in serum-free OptiMEM medium. Transfection of these siRNAs was accomplished using the RNAimax lipofectamine. After 3 h of transfection, cells were washed by PBS, and the accumulations of fluorescein-labelled siRNAs in the transfected cells were visualized by confocal microscope (Zeiss LSM710).

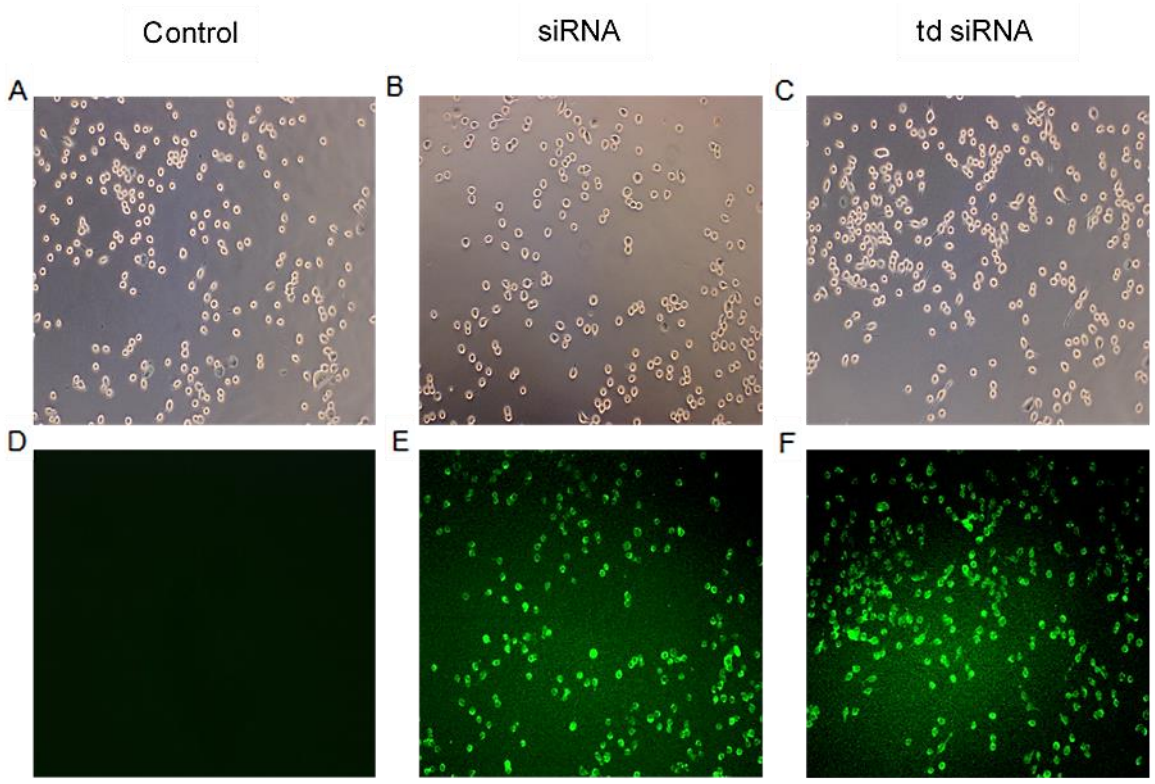


Fig. S3. Microscopy images for cellular uptake of treated cells. The fluorescein-labelled siRNA appears as green.

Table S1.

List of td RNA sequences.

RNA	Sequence	Calculated	Observed
td RNA 1	5'-GGCCUUUCACUACUCCUACUU-C_{18}- D'9-3'	21226.97	10611.88 $[M-2H]^{2-}$
td RNA 2	5'-GUAGGAGUAGUGAAAGGCCUU-C_{18}-D'9-3'	22154.76	11077.62 $[M-2H]^{2-}$
td RNA 3	5'-**F**GGCCUUUCACUACUCCUACUU-C_{18}-D'9-3'	22842.35	11406.64 $[M-2H]^{2-}$

***F** is fluorescein.

Table S2.

Sequences for DLS characterization.

siRNA	ON	Sequence	D_H (nm)
		Sense strand	
1	1	5'-GGCCUUUCACUACUCCUAC**UU**-3'	
	2	3'-**UU**CCGGAAAGUGAUGAGGAUG-5'	2.8 ± 0.6
		Antisense strand	
td siRNA 1	td RNA 1	5'-GGCCUUUCACUACUCCUAC**UU**-C_{18}-**D'9**-3'	6.9 ± 0.5
	2	3'-**UU**CCGGAAAGUGAUGAGGAUG-5'	

D_H – Hydrodynamic diameter

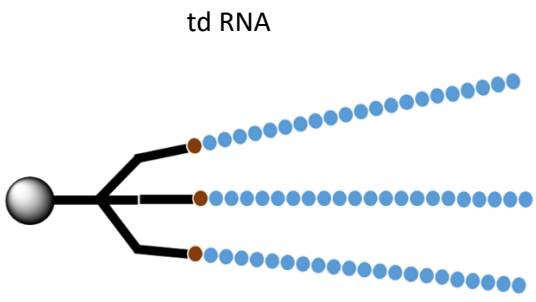


Table S3

Sequences of siRNAs and td siRNAs with melting temperature values.

siRNA	ON	Sequence	T_m (˚C)	ΔT_m (˚C)
Control	Buffer	-	-	-
1		Sense strand		
	1	5'-GGCCUUUCACUACUCCUAC**UU**-3'	77.6 ± 0.2	-
	2	3'-**UU**CCGGAAAGUGAUGAGGAUG-5'		
		Antisense strand		
2	1	5'-GGCCUUUCACUACUCCUAC**UU**-3'	77.0 ± 0.4	-0.7
	3	3'-**11**CCGGAAAGUGAUGAGGAUG-5'		
3	1	5'-GGCCUUUCACUACUCCUAC**UU**-3'	77.1 ± 0.3	-0.5
	4	3'-**22**CCGGAAAGUGAUGAGGAUG-5'		
4	1	5'-GGCCUUUCACUACUCCUAC**UU**-3'	77.2 ± 0.4	-0.4
	5	3'-**33**CCGGAAAGUGAUGAGGAUG-5'		
td siRNA 2	td RNA 1	5'-GGCCUUUCACUACUCCUAC**UU**-C_{18}-**D'9**-3'	77.0 ± 0.2	-0.7
	3	3'-**11**CCGGAAAGUGAUGAGGAUG-5'		
td siRNA 3	td RNA 1	5'-GGCCUUUCACUACUCCUAC**UU**-C_{18}-**D'9**-3'	77.5 ± 0.3	-0.2
	4	3'-**22**CCGGAAAGUGAUGAGGAUG-5'		
td siRNA 4	td RNA 1	5'-GGCCUUUCACUACUCCUAC**UU**-C_{18}-**D'9**-3'	77.5 ± 0.3	-0.1
	5	3'-**33**CCGGAAAGUGAUGAGGAUG-5'		

Table S4

RNAi activity for modified td siRNAs with sense strand as branching unit.

siRNA	**Sequence**	**Upper: 10 nM** **Lower: 1 nM**
Control	(buffer)	100 ± 9.3
siRNA 1	Sense strand 5'-GGCCUUUCACUACUCCUAC**UU**-3' 3'-**UU**CCGGAAAGUGAUGAGGAUG-5' Antisense strand	 21.9 ± 1.4 31.5 ± 4.5
td siRNA 1	5'-GGCCUUUCACUACUCCUAC**UU**-C_{18}-**D'9**-3' 3'-**UU**CCGGAAAGUGAUGAGGAUG-5'	19.4 ± 1.0 38.7 ± 1.8
td siRNA 2	5'-GGCCUUUCACUACUCCUAC**UU**-C_{18}-**D'9**-3' 3'-**11**CCGGAAAGUGAUGAGGAUG-5'	22.1 ± 1.1 42.9 ± 4.2
td siRNA 3	5'-GGCCUUUCACUACUCCUAC**UU**-C_{18}-**D'9**-3' 3'-**22**CCGGAAAGUGAUGAGGAUG-5'	22.0 ± 2.2 38.9 ± 2.6
td siRNA 4	5'-GGCCUUUCACUACUCCUAC**UU**-C_{18}-**D'9**-3' 3'-**33**CCGGAAAGUGAUGAGGAUG-5'	22.1 ± 2.0 46.0 ± 3.3

Gene expression from cells transfected with each siRNA has been normalized and presented as the percentage from three independent experiments, with three replicate samples per experiment.

Table S5

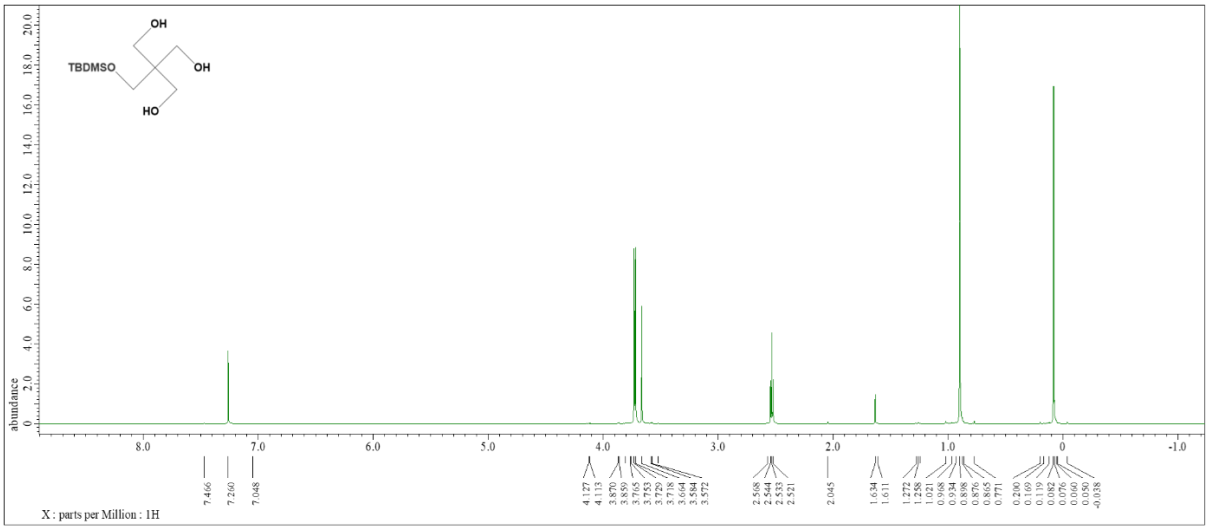
RNAi activity for modified td siRNAs with antisense strand as branching unit.

siRNA	**Sequence**	**Upper: 10 nM** **Lower: 1 nM**
Control	(buffer)	100 ± 9.3
siRNA 1	Sense strand 5'-GGCCUUUCACUACUCCUAC**UU**-3' 3'-**UU**CCGGAAAGUGAUGAGGAUG-5' Antisense strand	 19.9 ± 1.4 31.5 ± 4.5
td siRNA 5	5'-GGCCUUUCACUACUCCUAC**UU**-3' 3'-**D'9-**C_{18}**-UU**CCGGAAAGUGAUGAGGAUG-5'	42.1 ± 4.4 76.3 ± 5.0
td siRNA 6	5'-GGCCUUUCACUACUCCUAC**11**-3' 3'-**D'9-**C_{18}**-UU**CCGGAAAGUGAUGAGGAUG-5'	33.9 ± 1.5 70.1 ± 4.0
td siRNA 7	5'-GGCCUUUCACUACUCCUAC**22**-3' 3'-**D'9-**C_{18}**-UU**CCGGAAAGUGAUGAGGAUG-5'	30.7 ± 4.7 59.1 ± 3.2
td siRNA 8	5'-GGCCUUUCACUACUCCUAC**33**-3' 3'-**D'9-**C_{18}**-UU**CCGGAAAGUGAUGAGGAUG-5'	28.0 ± 3.3 71.0 ± 5.1

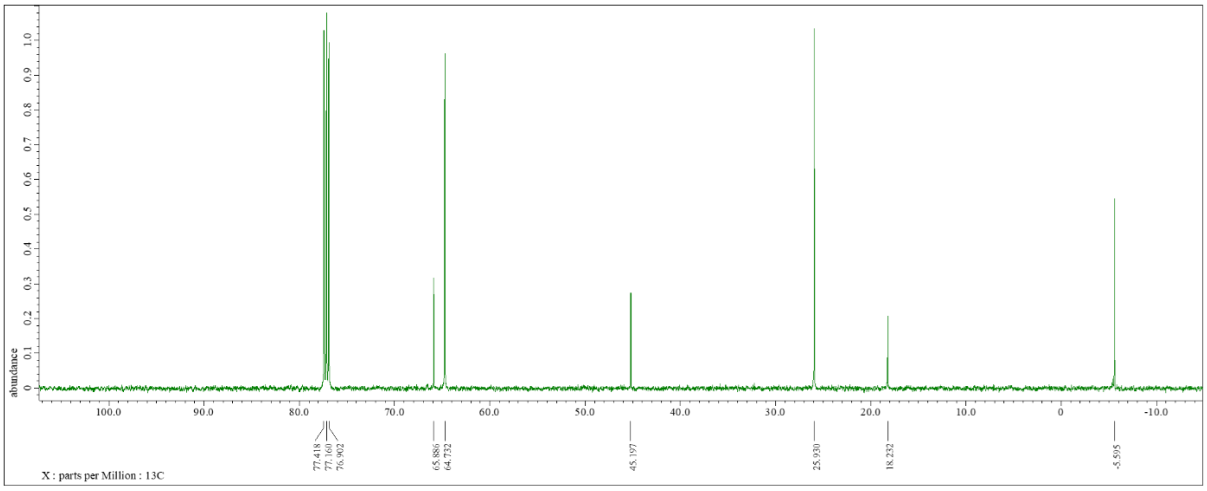
Gene expression from cells transfected with each siRNA has been normalized and presented as the percentage from three independent experiments, with three replicate samples per experiment.

^{1}H and ^{13}C NMR spectra of compounds

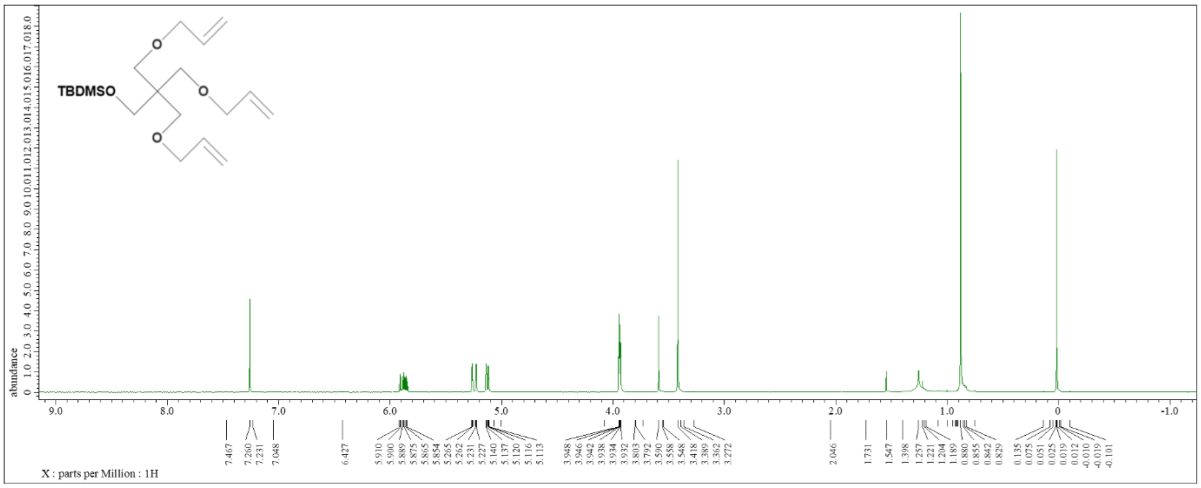
^{1}H NMR spectrum of compound D2



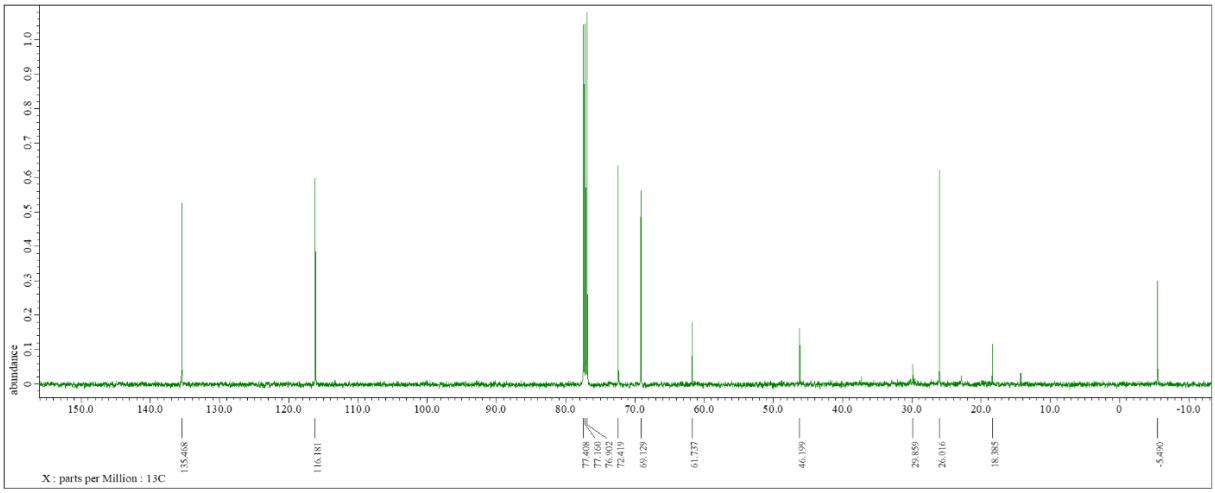
^{13}C NMR spectrum of compound D2



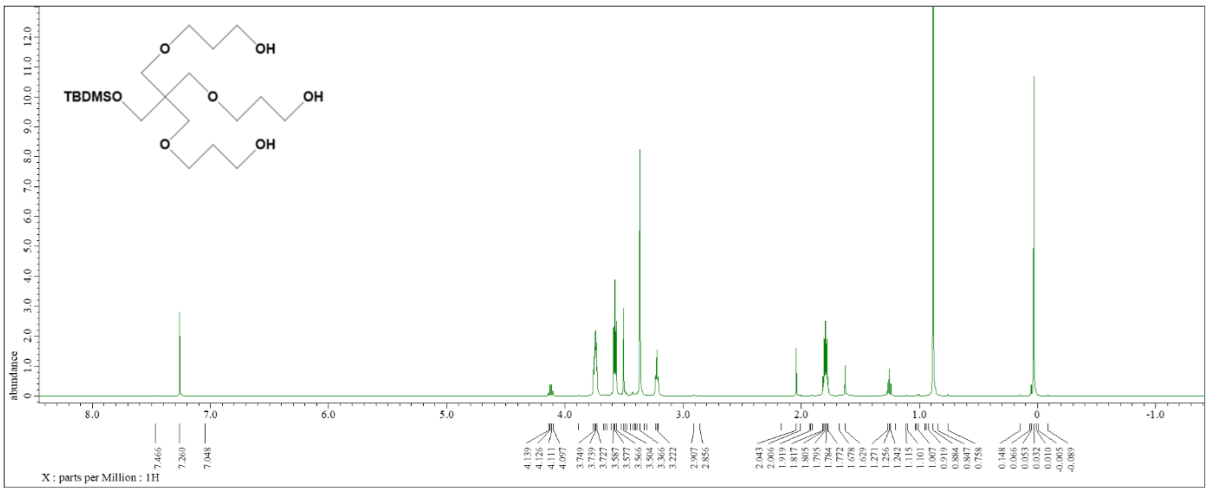
^{1}H NMR spectrum of compound D3



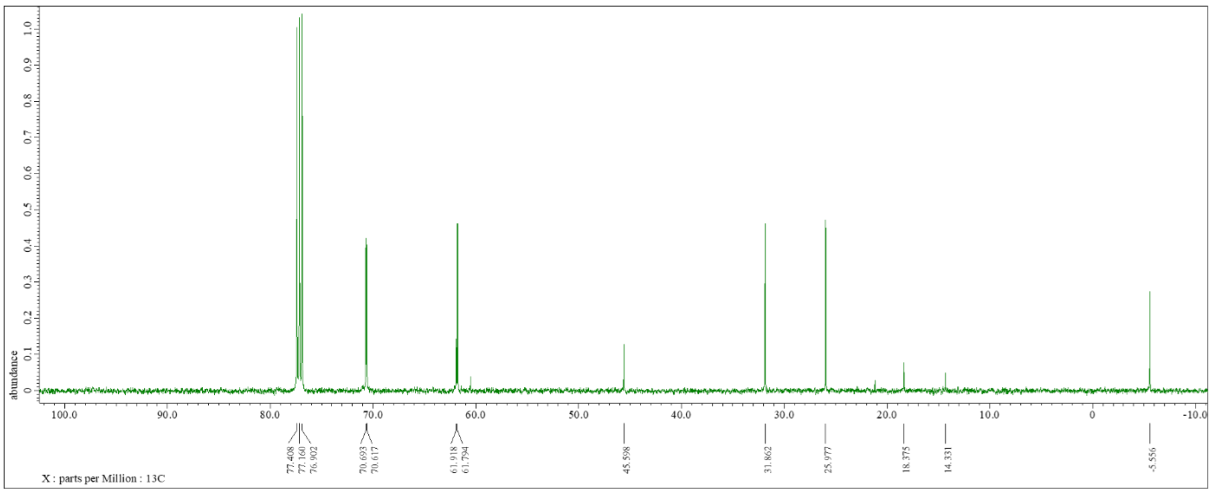
^{13}C NMR spectrum of compound D3



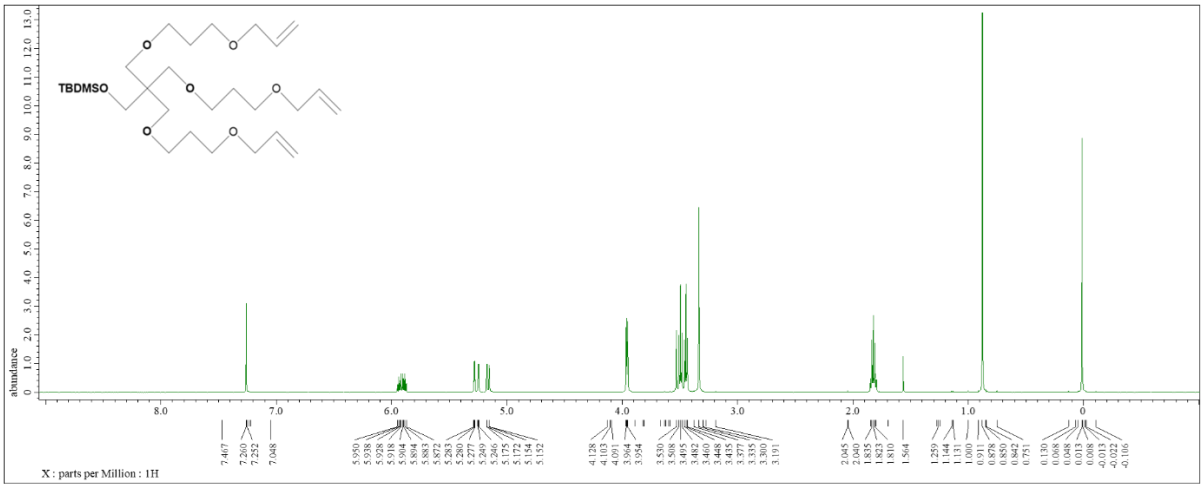
^{1}H NMR spectrum of compound D4



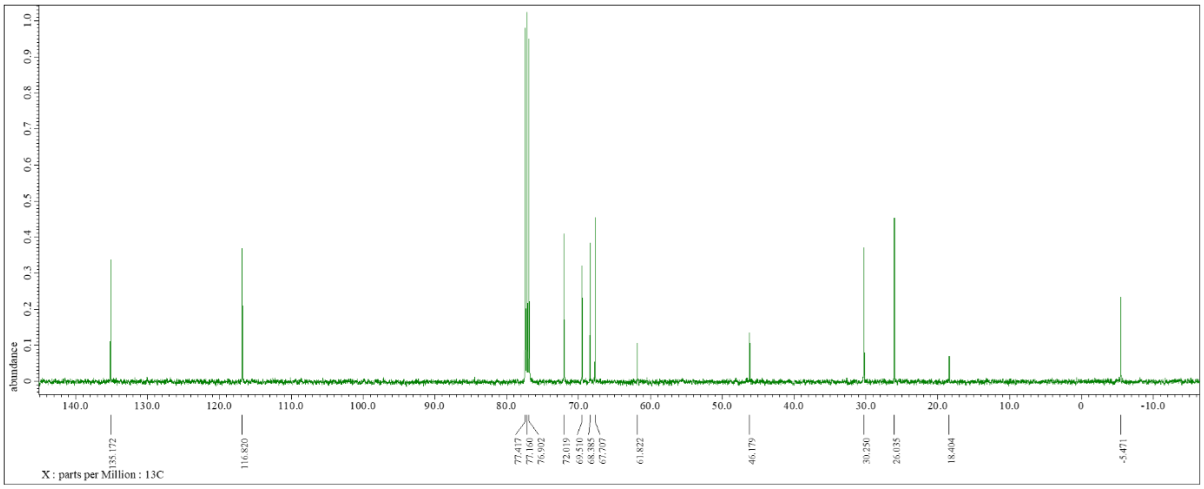
^{13}C NMR spectrum of compound D4



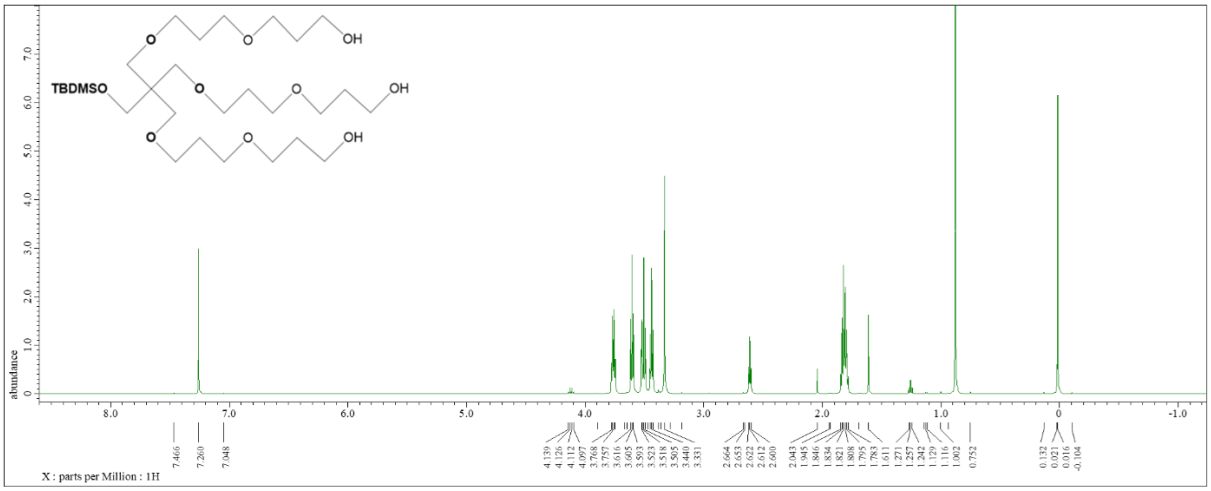
^{1}H NMR spectrum of compound D5



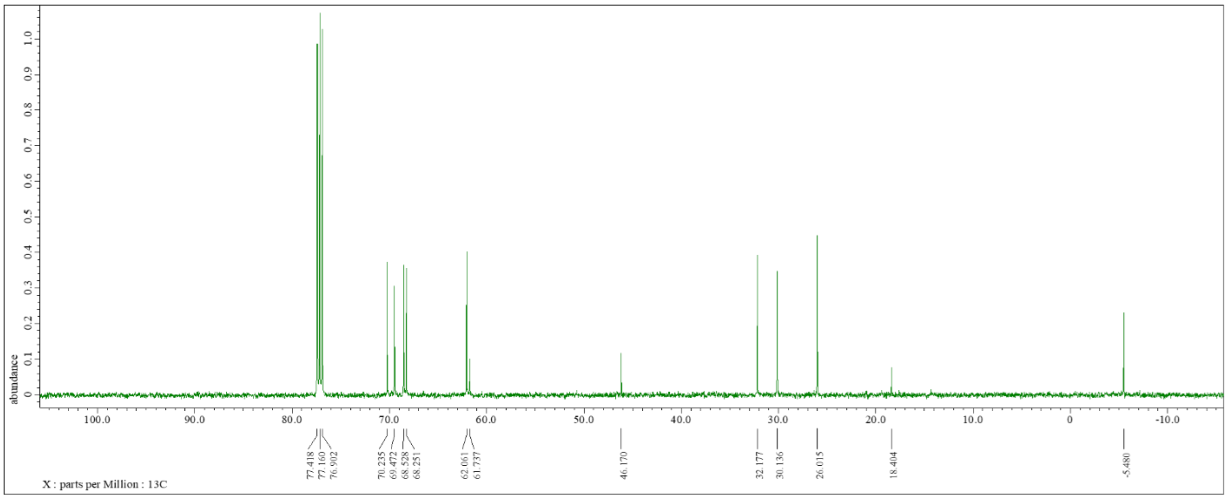
^{13}C NMR spectrum of compound D5



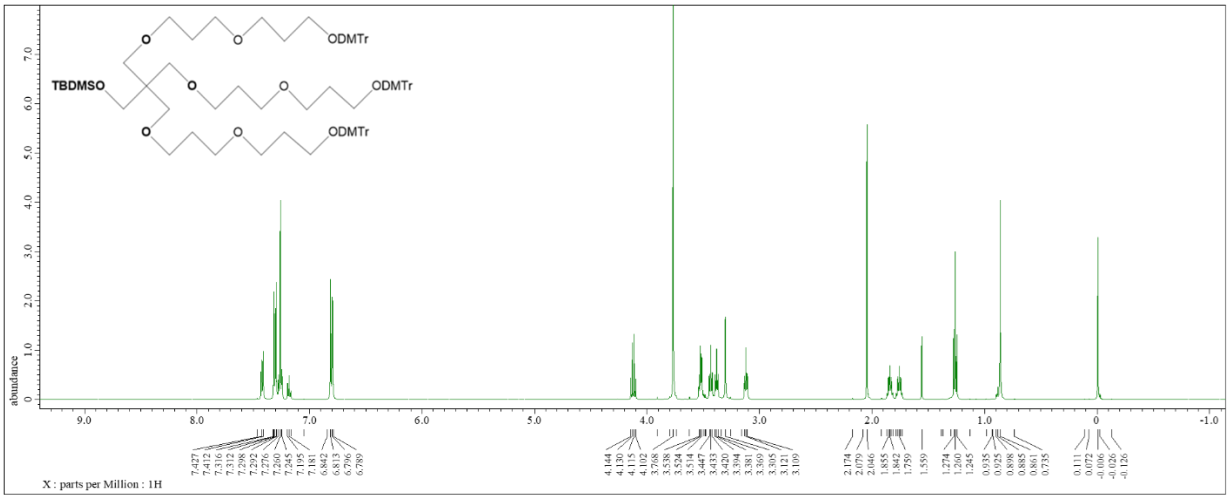
^{1}H NMR spectrum of compound D6



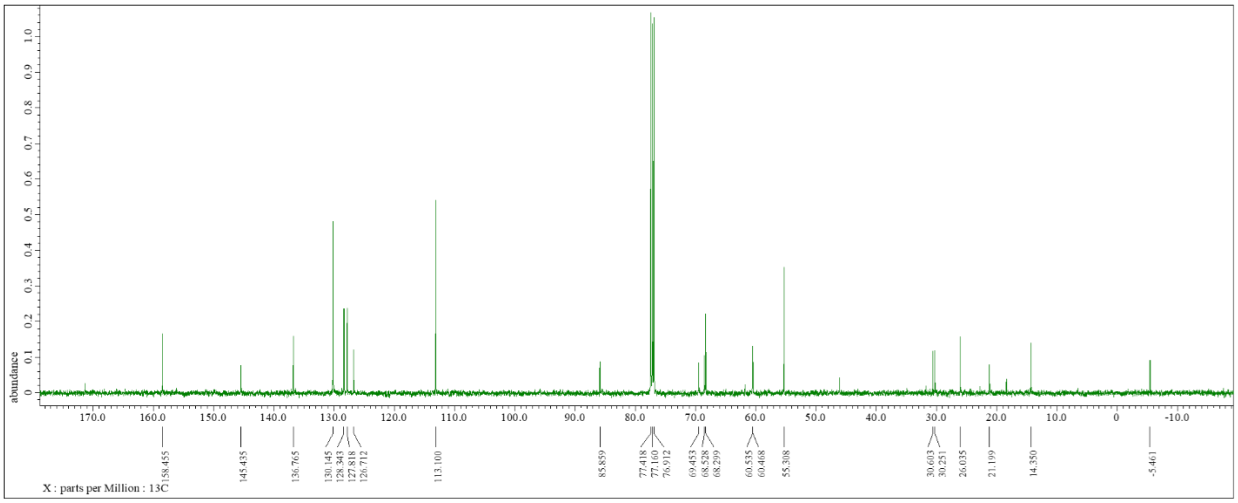
^{13}C NMR spectrum of compound D6



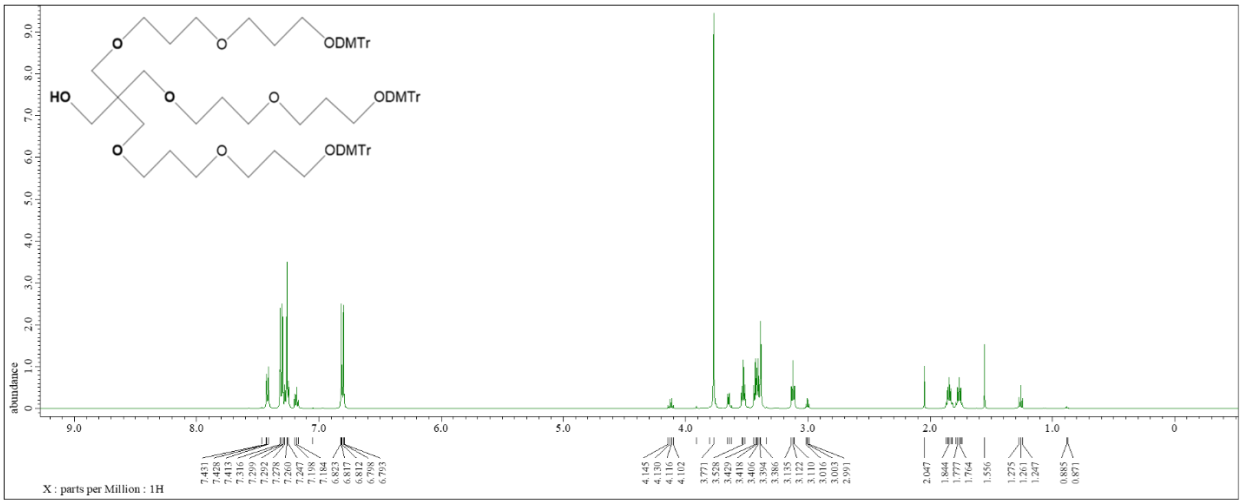
^{1}H NMR spectrum of compound D7



^{13}C NMR spectrum of compound D7



^{1}H NMR spectrum of compound D8



^{13}C NMR spectrum of compound D8