Supplementary Information

Synthesis and Biophysical Evaluation of C-5-Triazolyl-Functionalized Morpholino Thymidine Analogs

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Contents

1. General chemical methods:

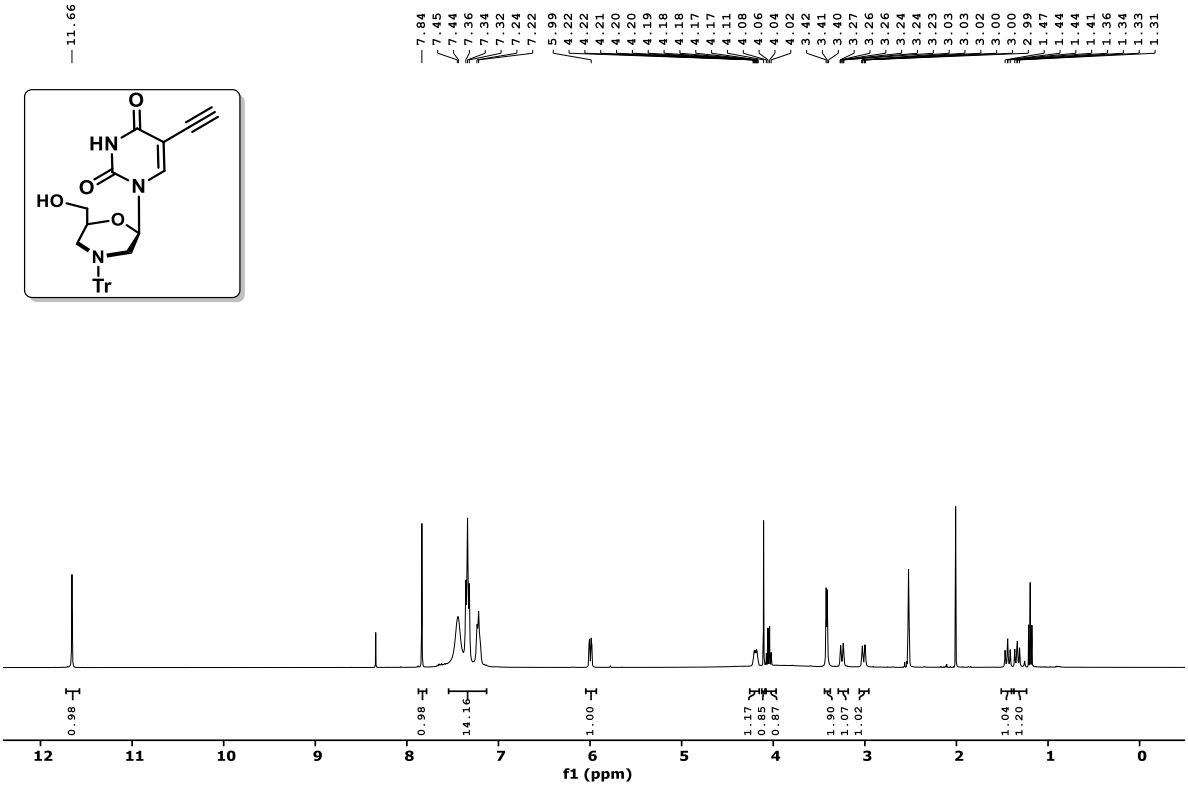
All chemical reagents were purchased from commercially available sources and used without further purification, unless otherwise specified. Reactions were conducted in glassware that had been thoroughly dried in oven and placed under argon atmosphere. Solvents were purified and dried following standard protocols. Thin layer chromatography (TLC) was performed using silica gel 60 F254-coated aluminium sheets (0.25 mm layer thickness, Merck). TLC visualization was accomplished using UV light (254 nm), and staining was carried out using standard staining solutions such as CAM, Ninhydrin etc. For purification, column chromatography has been performed on silica gel columns (mesh sizes: 60-120 and 100-200). Nuclear Magnetic Resonance (NMR) spectra, including ^{1}H, $^{13}C\{^{1}H\}$, and ^{31}P NMR, were recorded using Bruker NMR spectrometers operating at 300 MHz.$^{13}C\{^{1}H\}$ NMR spectra were recorded at 75 MHz.^{31}P NMR spectra were recorded at 121 MHz. Chemical shifts (δ) are reported in parts per million (ppm) relative to the solvent residual peak (δ = 7.26 ppm, for $CDCl_3$) or the TMS standard. Multiplicity of NMR signals is abbreviated as follows: singlet (s), doublet (d), triplet (t), septet (sept), broad signal (brs), or multiplet (m). High-Resolution Mass Spectra (HRMS) were obtained using a QTOF I (Quadrupole hexapole TOF) mass spectrometer equipped with an orthogonal Z spray electrospray interface on a Micro (YA 263) mass spectrometer (Manchester, UK). ESI mass was recorded using Agilent advance Bio 654XT LC/QTOF in linear negative mode. Matrix-Assisted Laser Desorption Ionization (MALDI) mass spectra were recorded using a Bruker UltrafleXtreme MALDI-TOF/TOF system. The matrix used was Cyano-4-hydroxycinnamic acid (CHCA) in 0.1% TFA in ACN/H_2O (1:1).

1.2 HPLC Purification of Synthesized ONs:

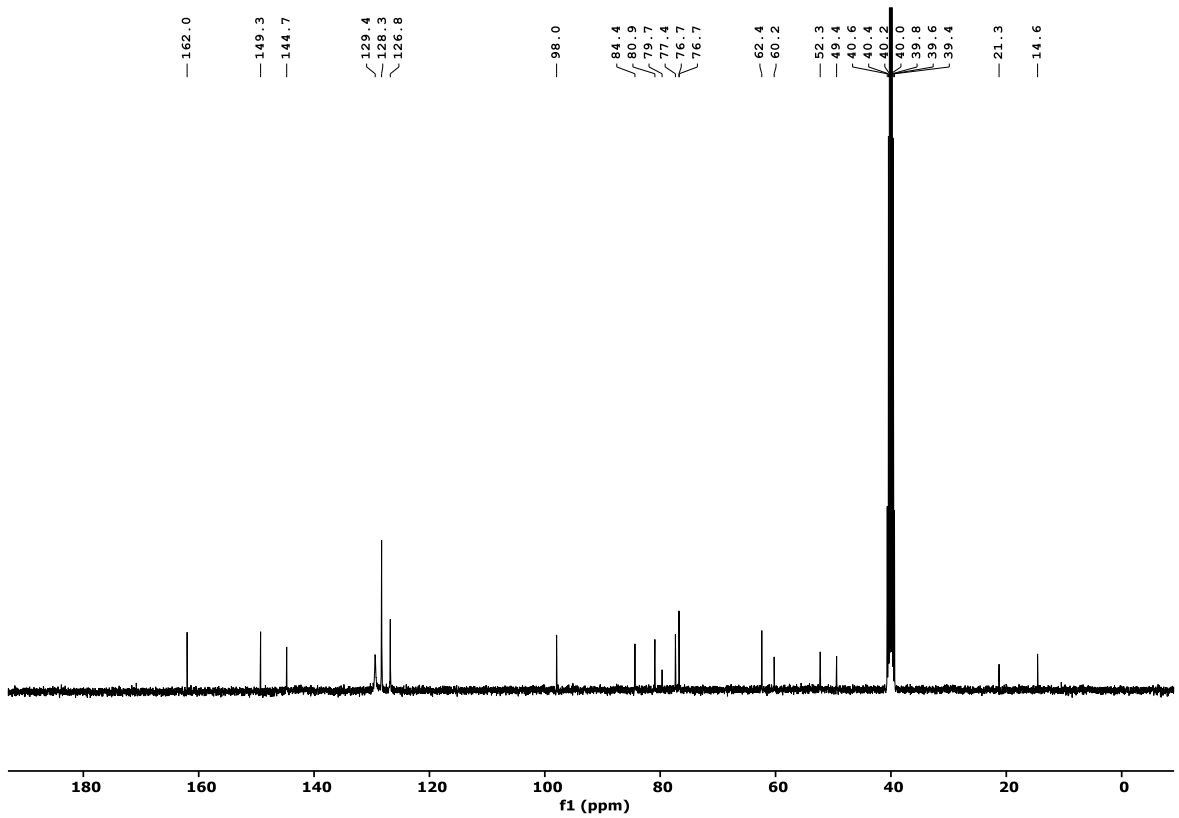
High-Performance Liquid Chromatography (HPLC) purification of all the PMOs, were performed using a Waters HPLC module equipped with Waters 1525 Binary HPLC pump and Waters 2998 PDA detector and C_{18} (Waters XBridge BEH Shield RP18) column in 2 ml/min and Shimadzu SP-20AD system with C18 (CAPCELL PAK) column using 50mM Ammonium acetate buffer (in H_2O)-CH_3CN gradient system (10-50%) flow rate = 1 ml/min.

2. NMR Spectra:

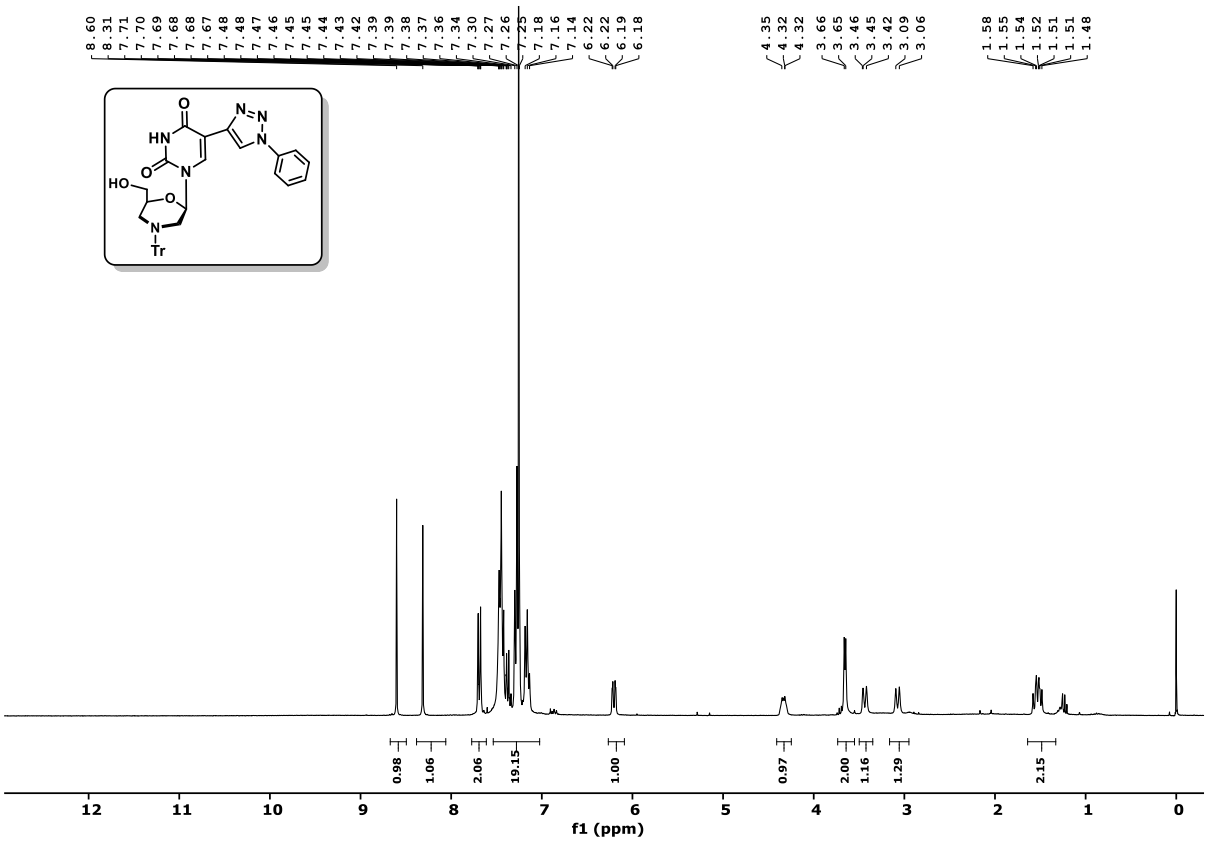
^{1}H-NMR (300 MHz, $CDCl_3$), of **Compound 1.**



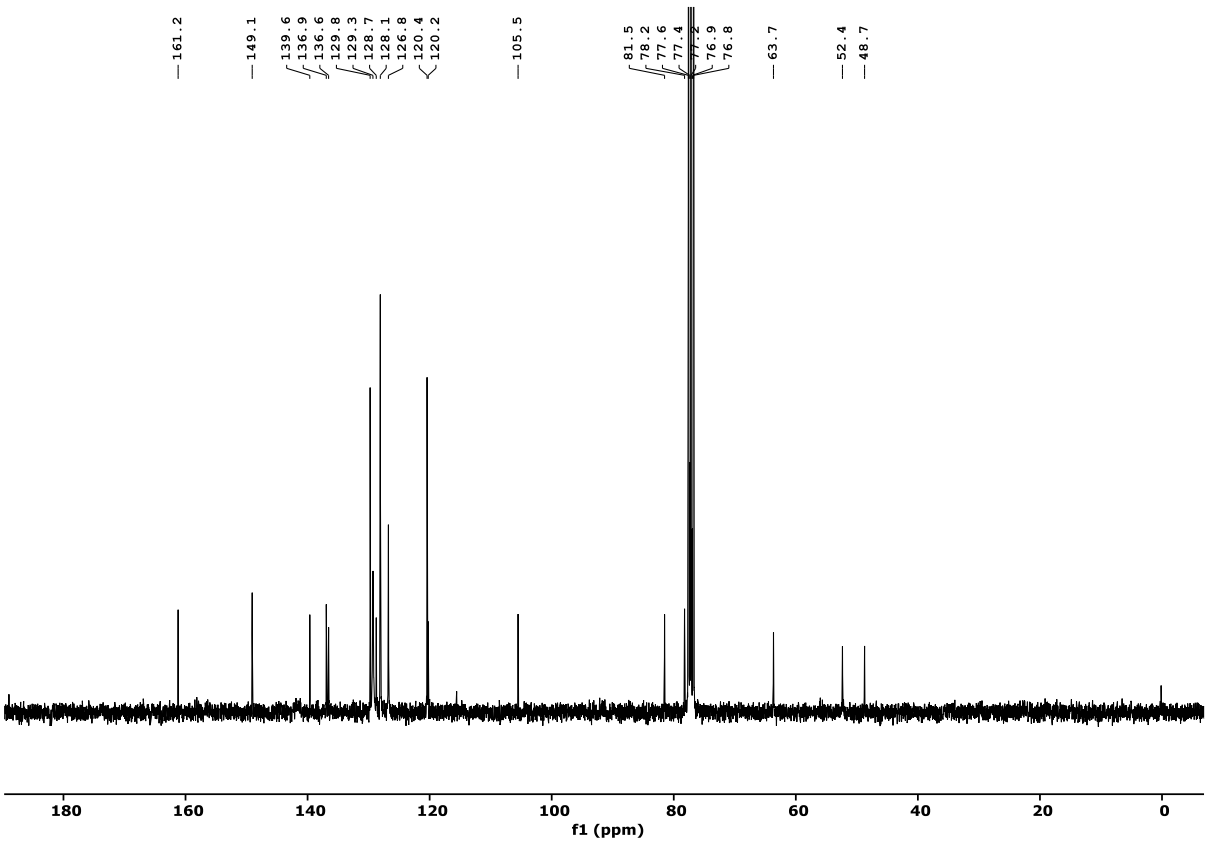
13C{1H} NMR (75 MHz, $CDCl_3$)



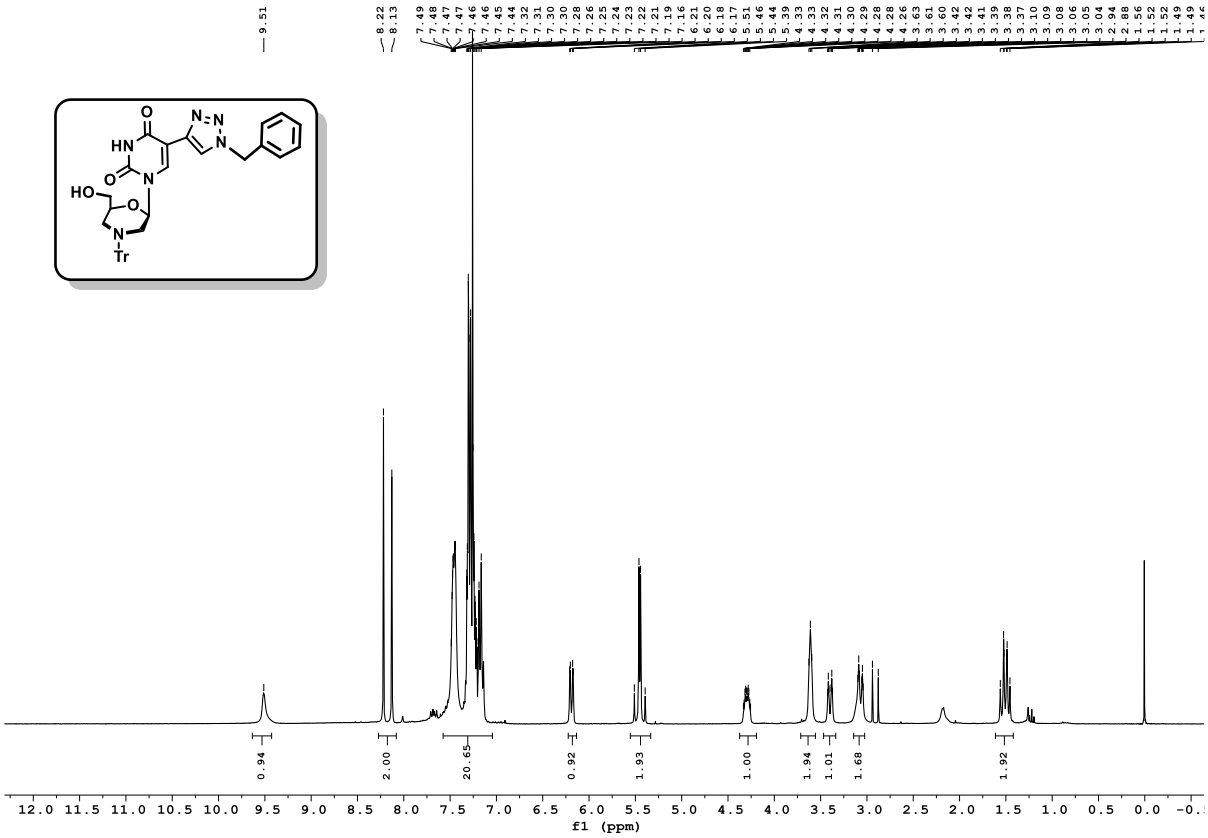
^{1}H-NMR (300 MHz, $CDCl_3$), of **Compound 2.**



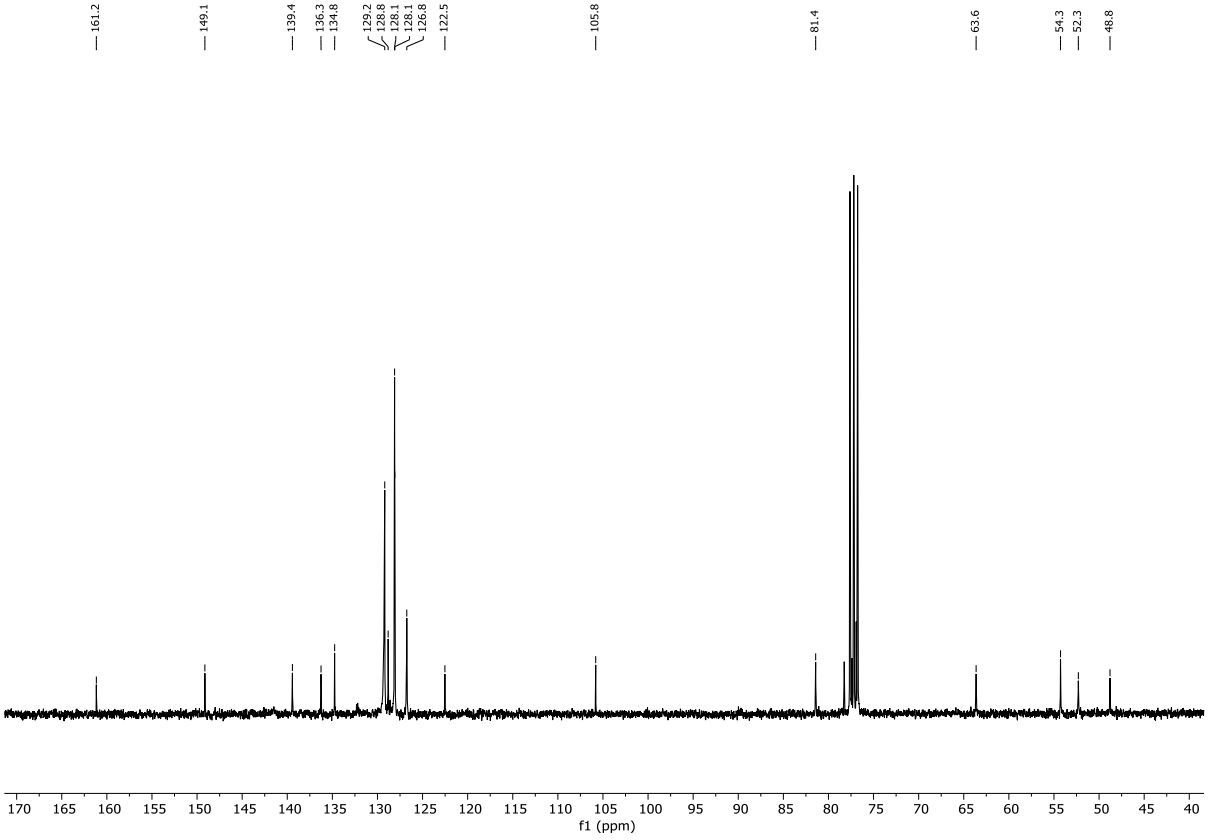
^{13}C{1H} NMR (75 MHz, $CDCl_3$) of **Compound 2.**



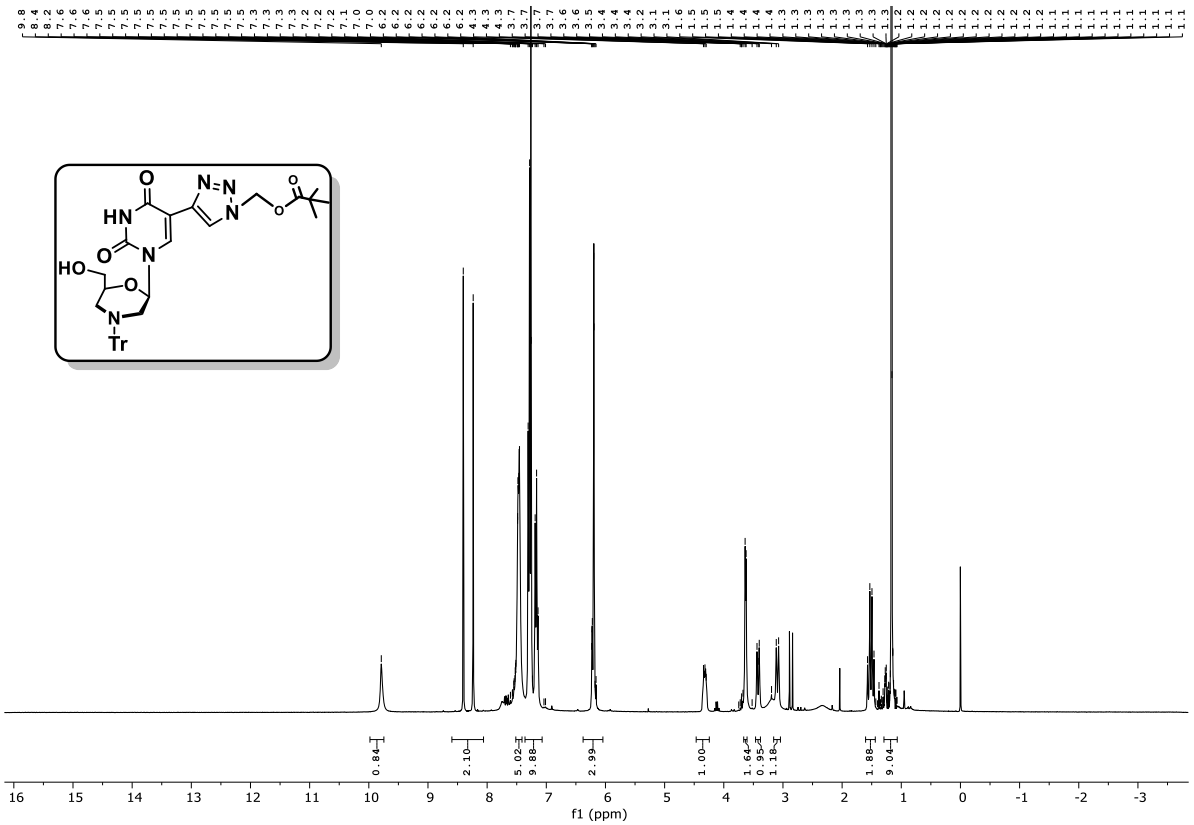
^{1}H-NMR (300 MHz, $CDCl_3$) **compound 3**



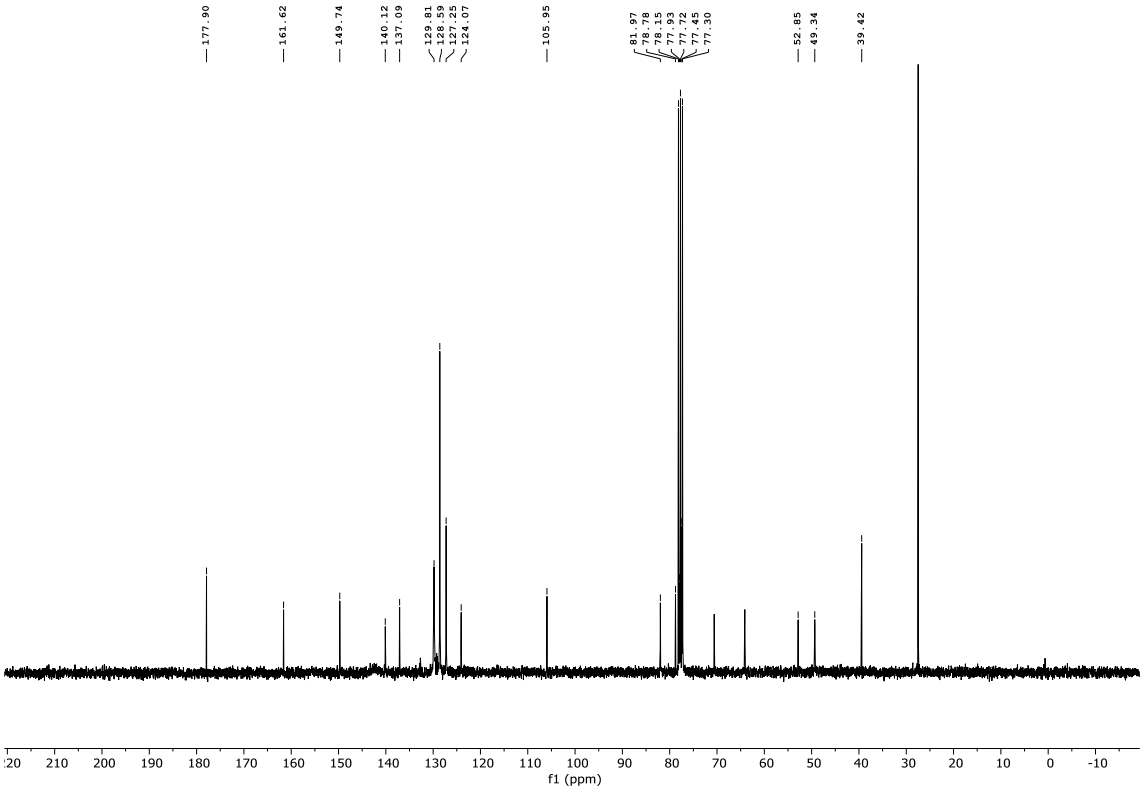
^{13}C{1H} NMR (75 MHz, $CDCl_3$) of **Compound 3**



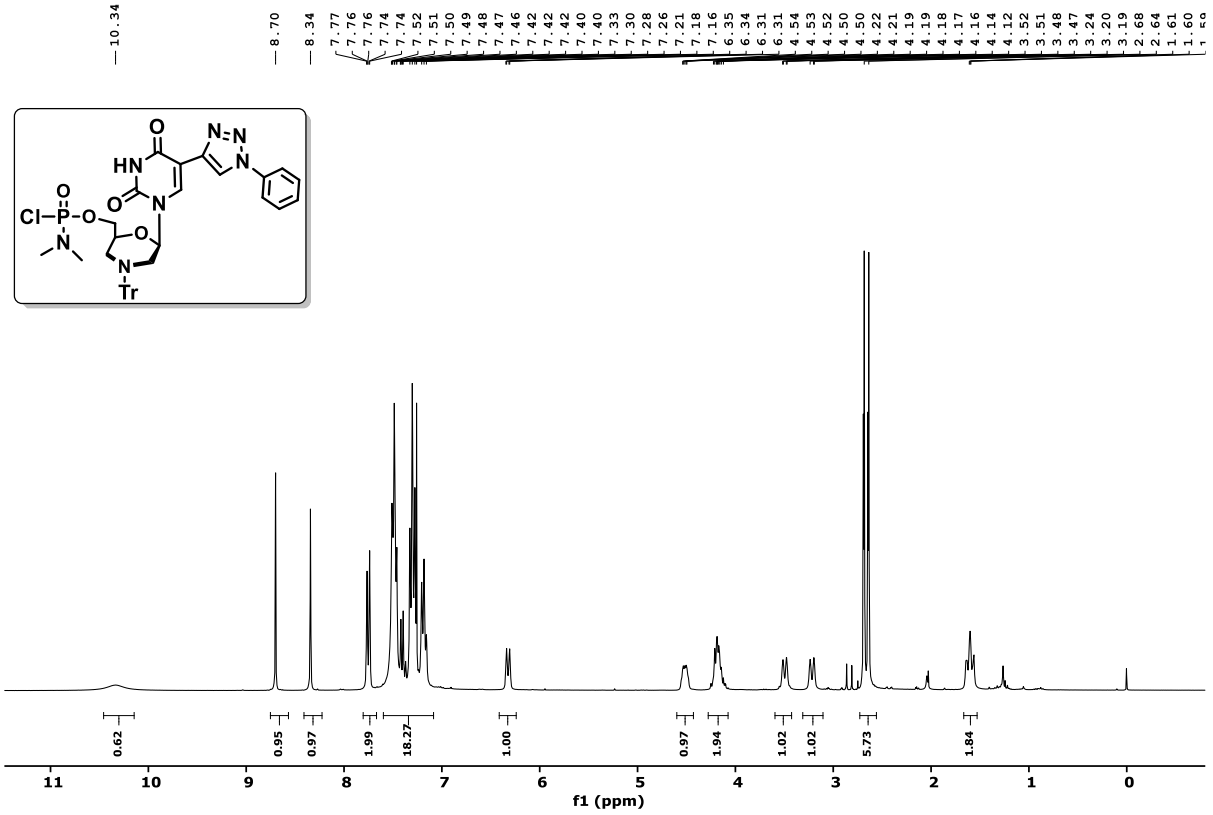
^{1}H-NMR (300 MHz, $CDCl_3$), **Compound 4**



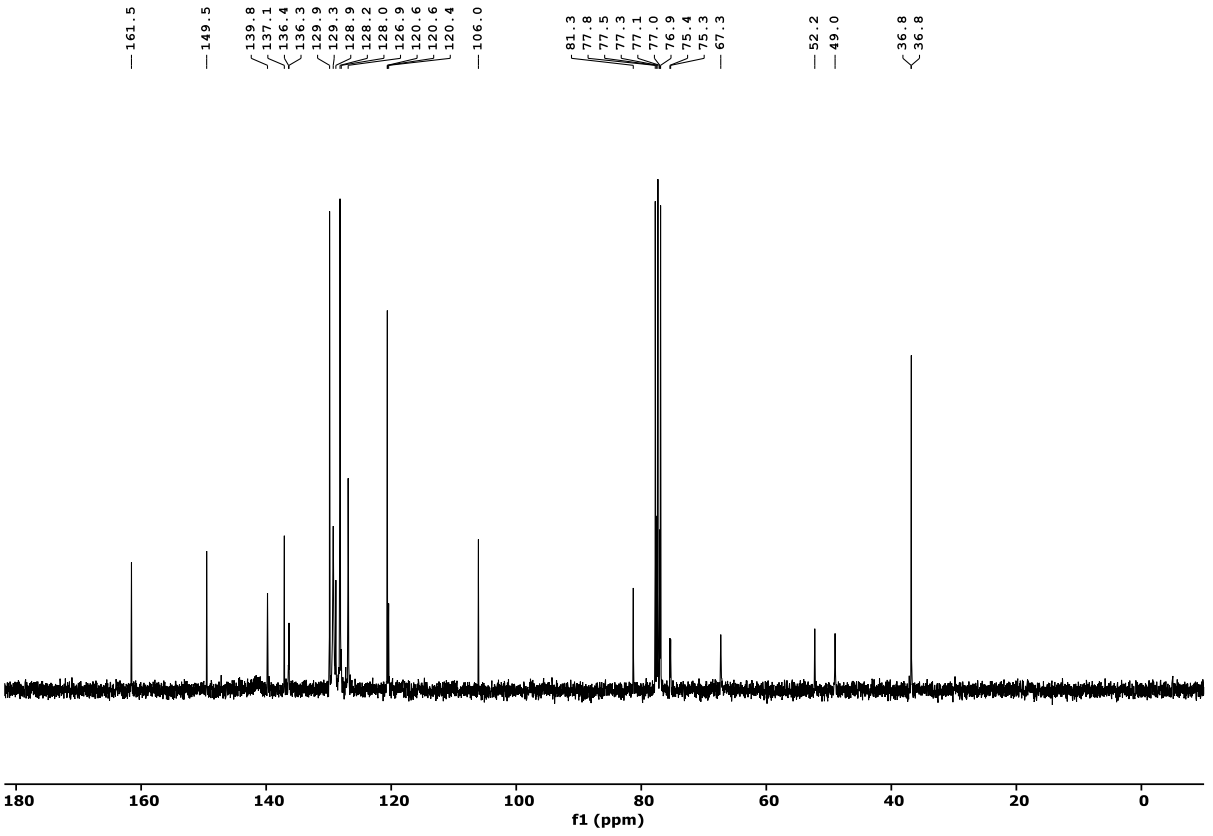
^{13}C{1H} NMR (75 MHz, $CDCl_3$) of **Compound 4**



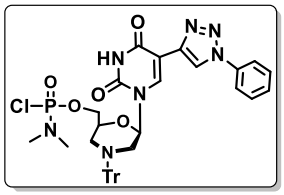
^{1}H-NMR (300 MHz, $CDCl_3$), of **Compound 6.**



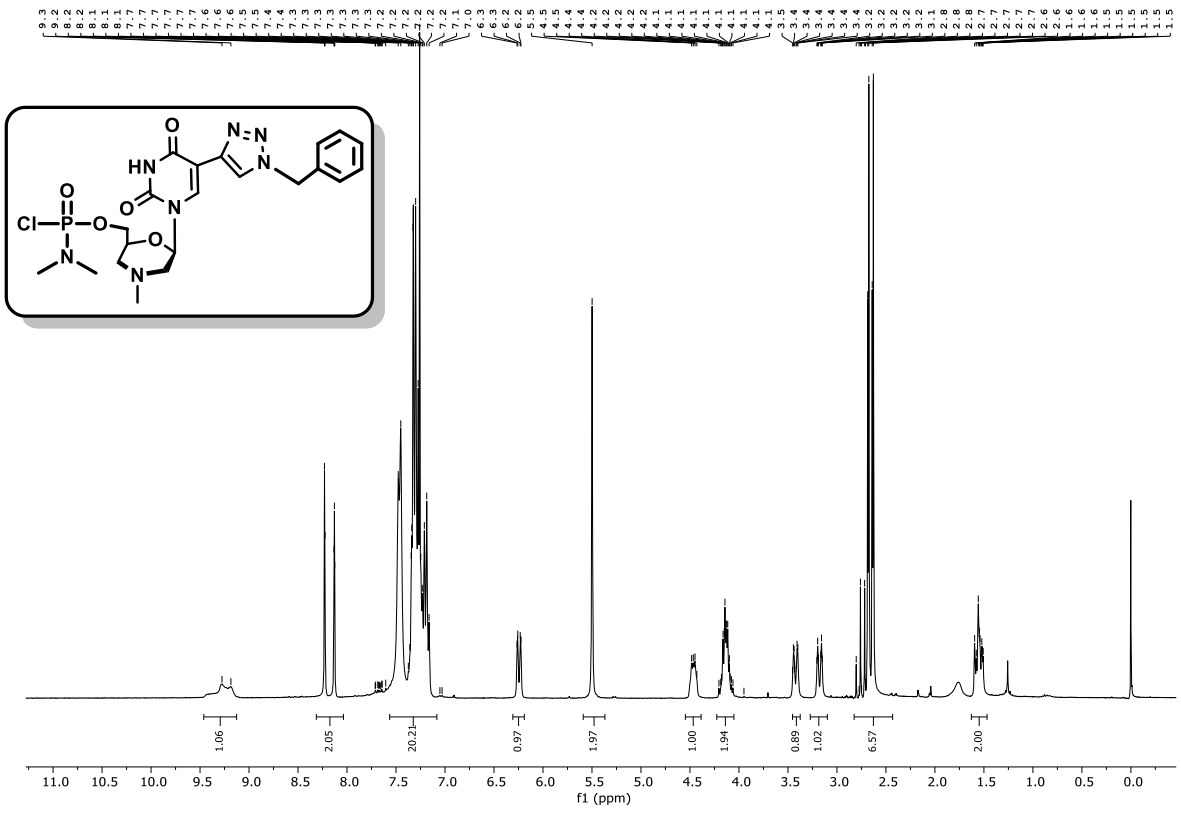
^{13}C{1H} NMR (75 MHz, $CDCl_3$) of **Compound 6**



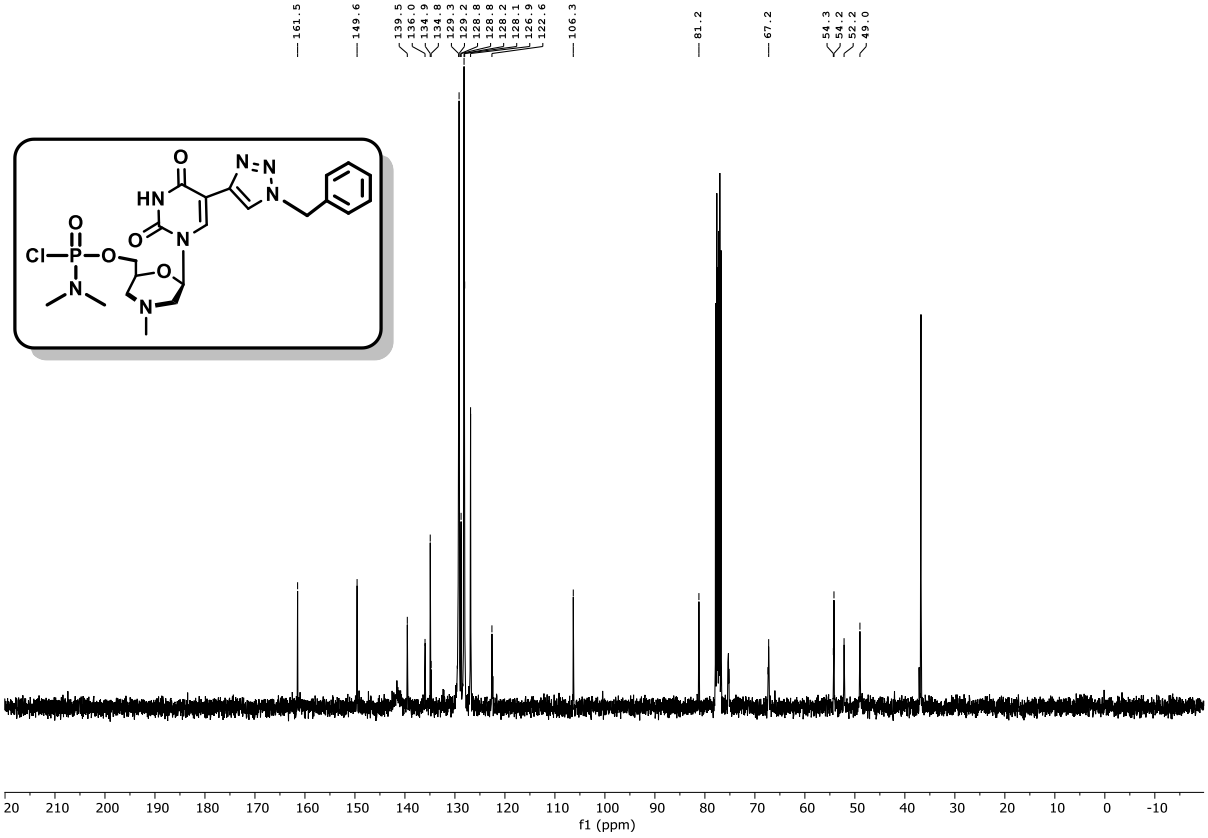
^{31}P-NMR (121 MHz, $CDCl_3$) of **Compound 6.**



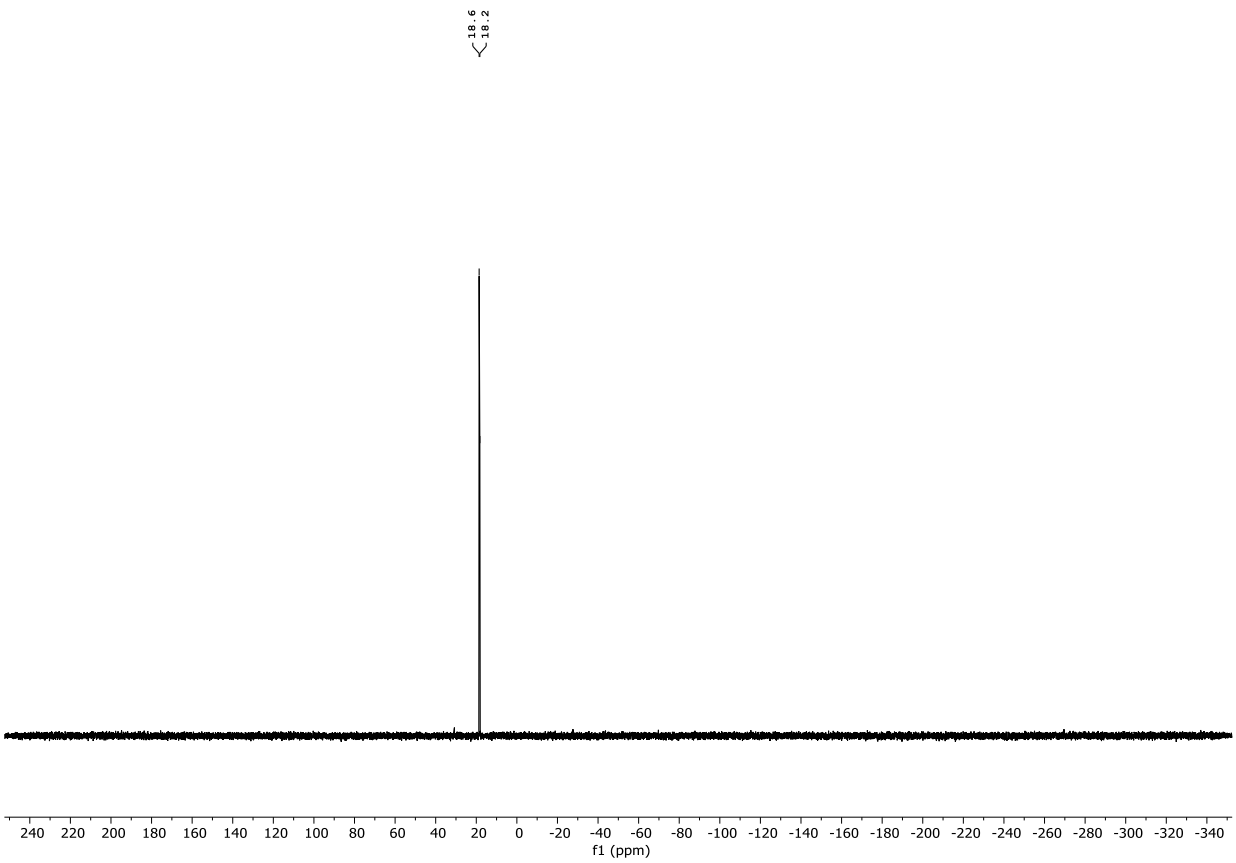
^{1}H-NMR (300 MHz, $CDCl_3$), of **compound 7**



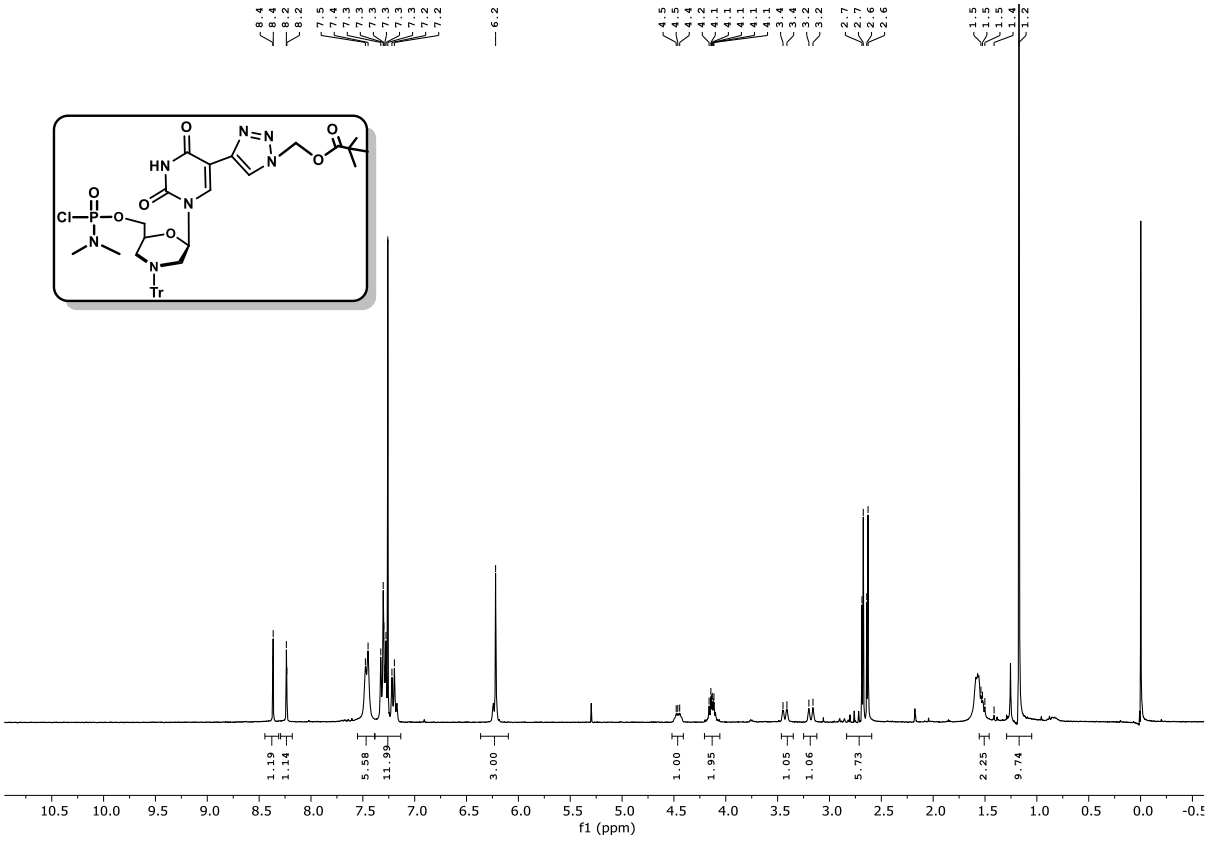
$^{13}C\{1H\}$ NMR (75 MHz, $CDCl_3$), of **Compound 7**



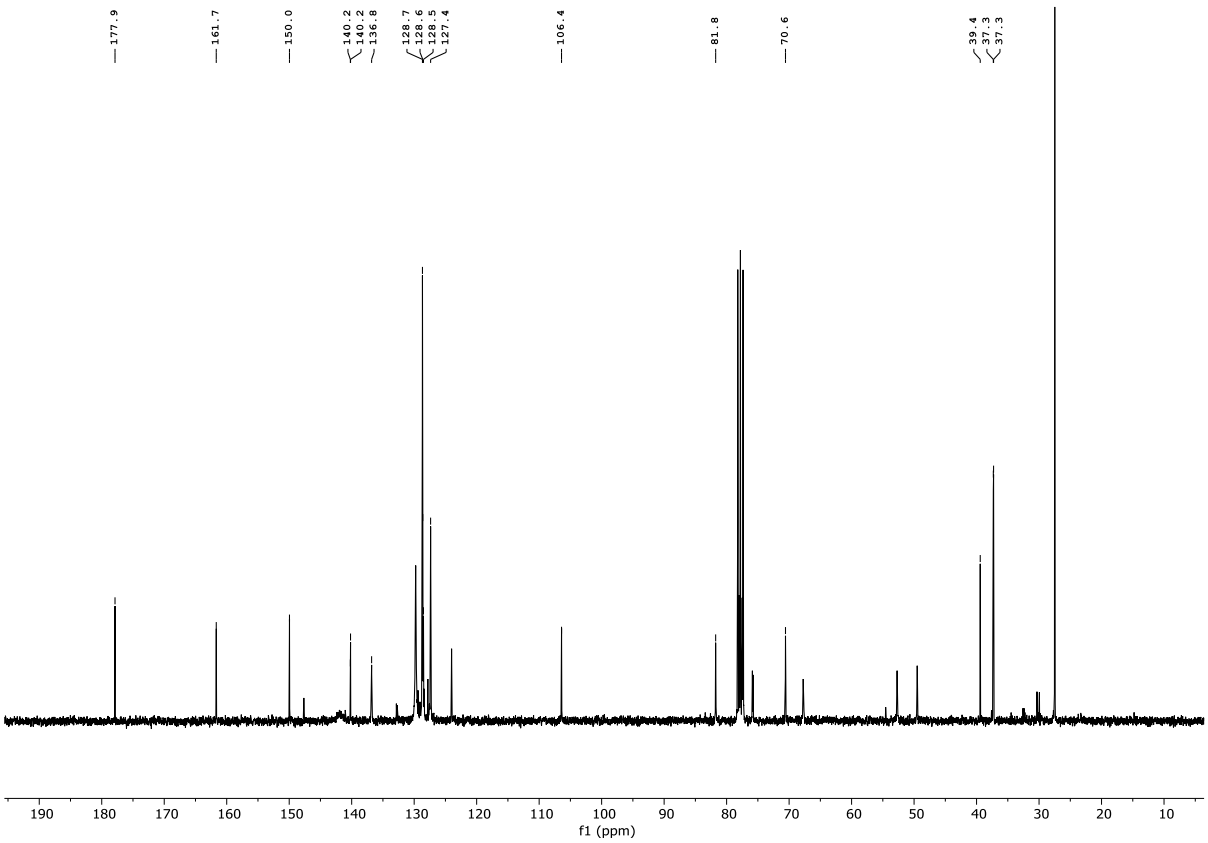
^{31}P-NMR (121 MHz, $CDCl_3$) of **Compound 7**



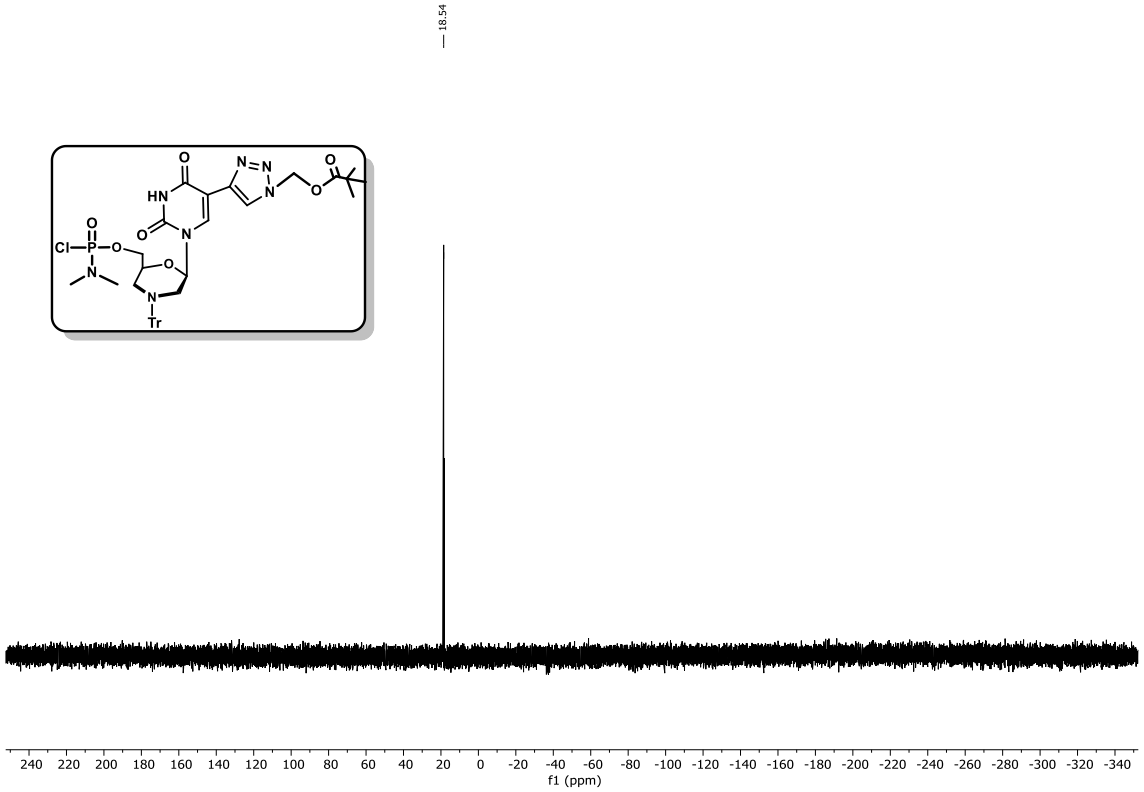
^{1}H-NMR (300 MHz, $CDCl_3$), of **Compound 8**



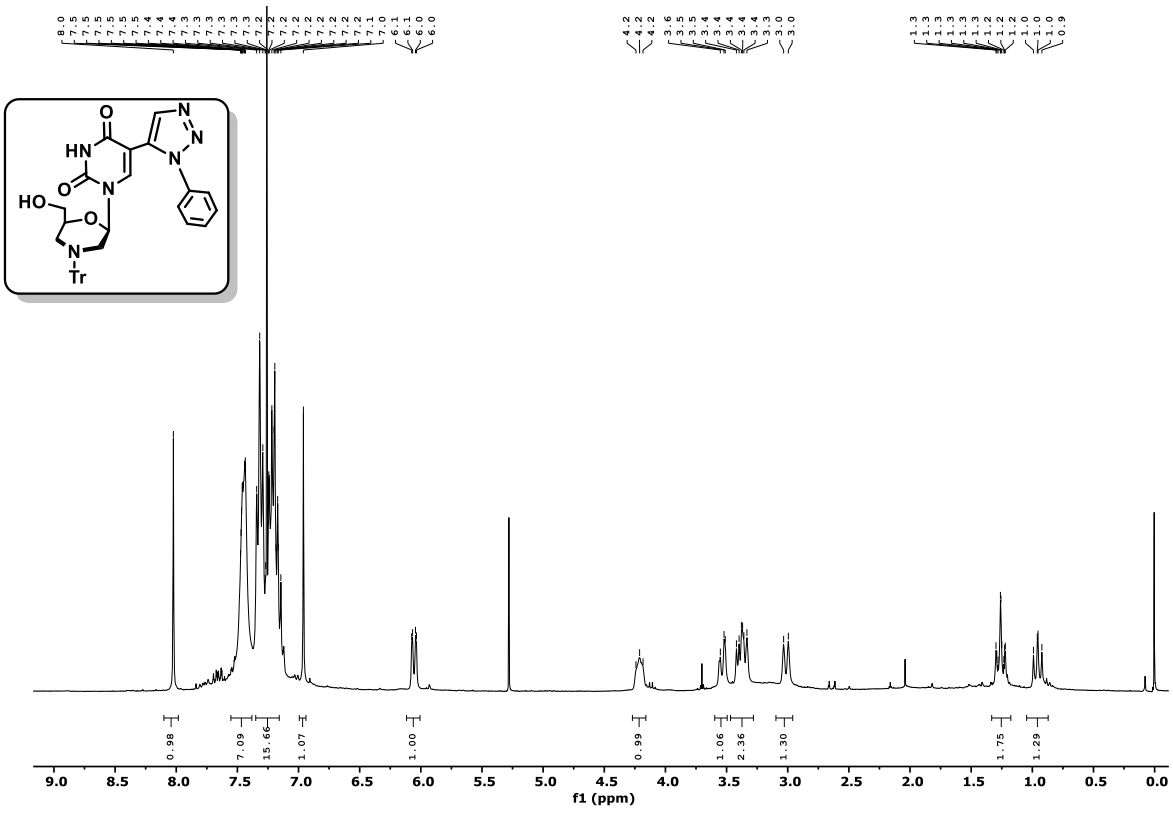
^{13}C{1H} NMR (75 MHz, $CDCl_3$) of **Compound 8**



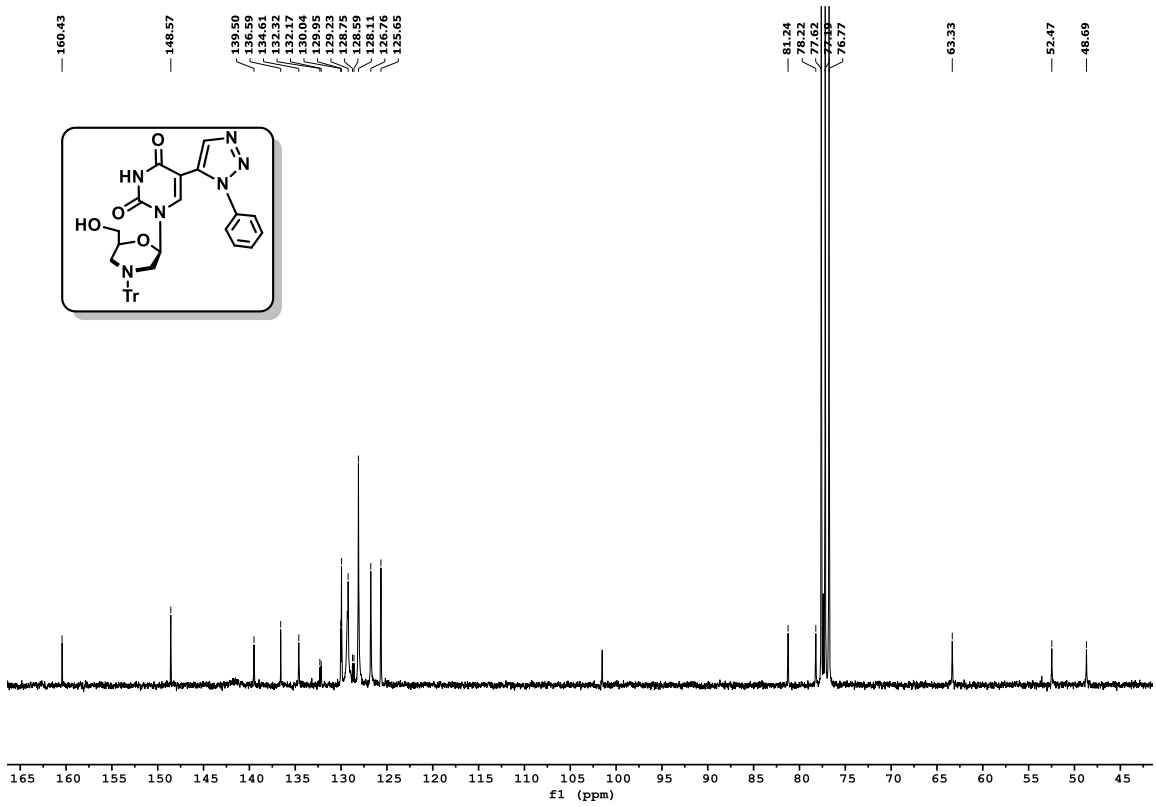
^{31}P-NMR (121 MHz, $CDCl_3$) of **Compound 8**



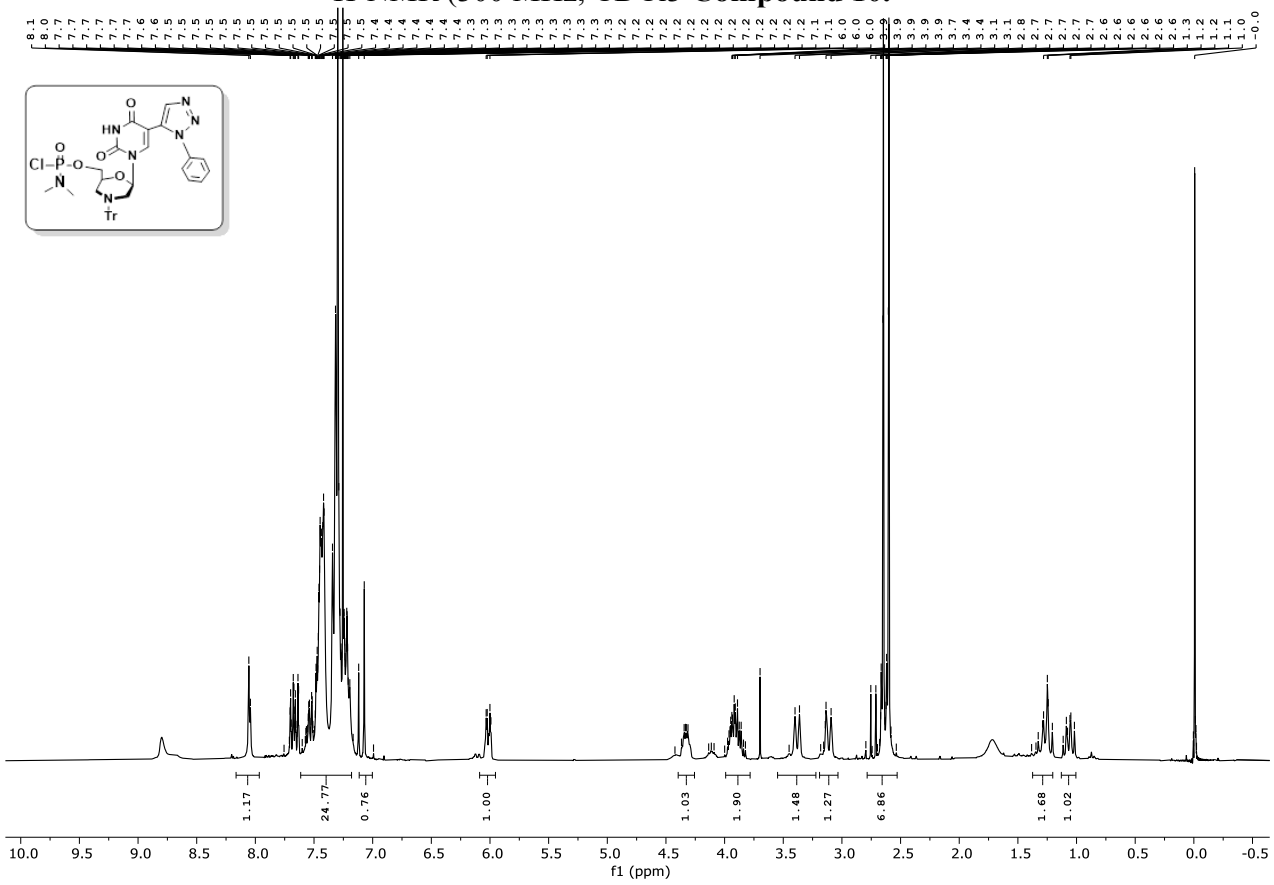
^{1}H-NMR (300 MHz, CDCl3) of **compound 9.**



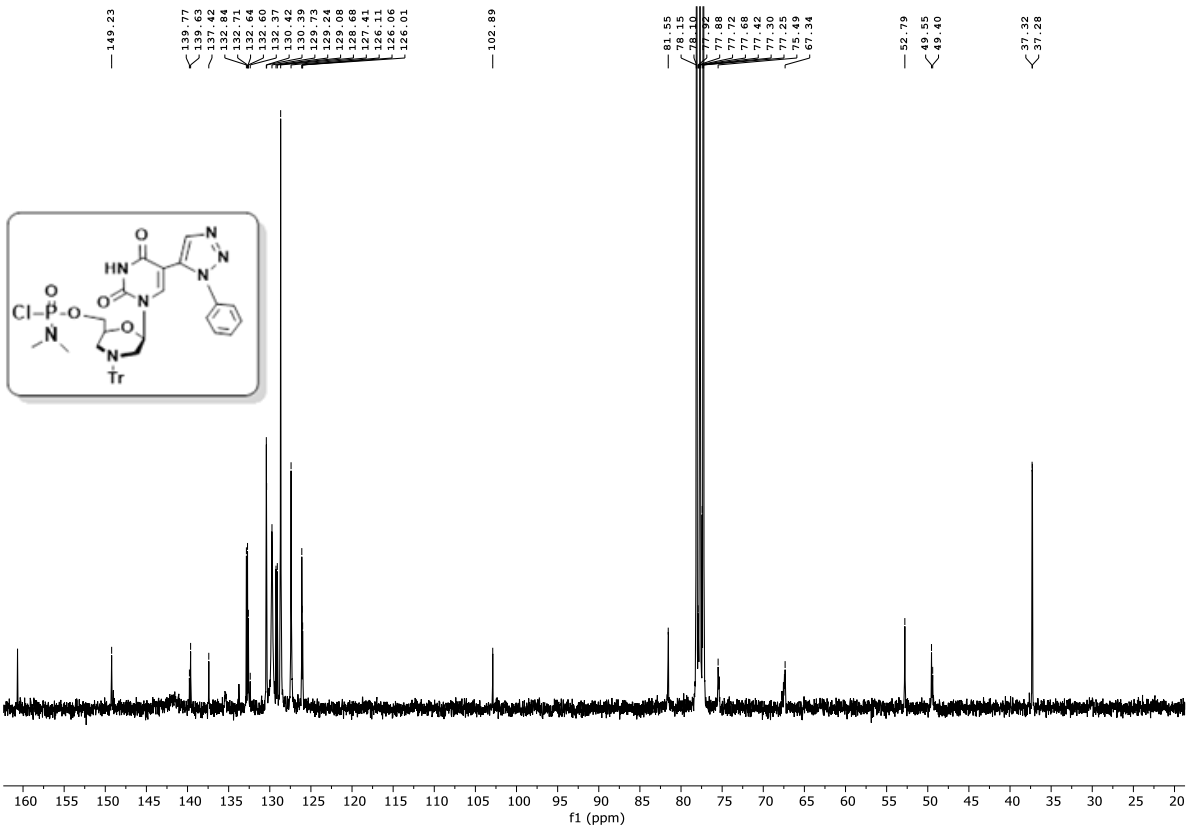
$^{13}C\{1H\}$ NMR (75 MHz, $CDCl_3$) **of compound 9**



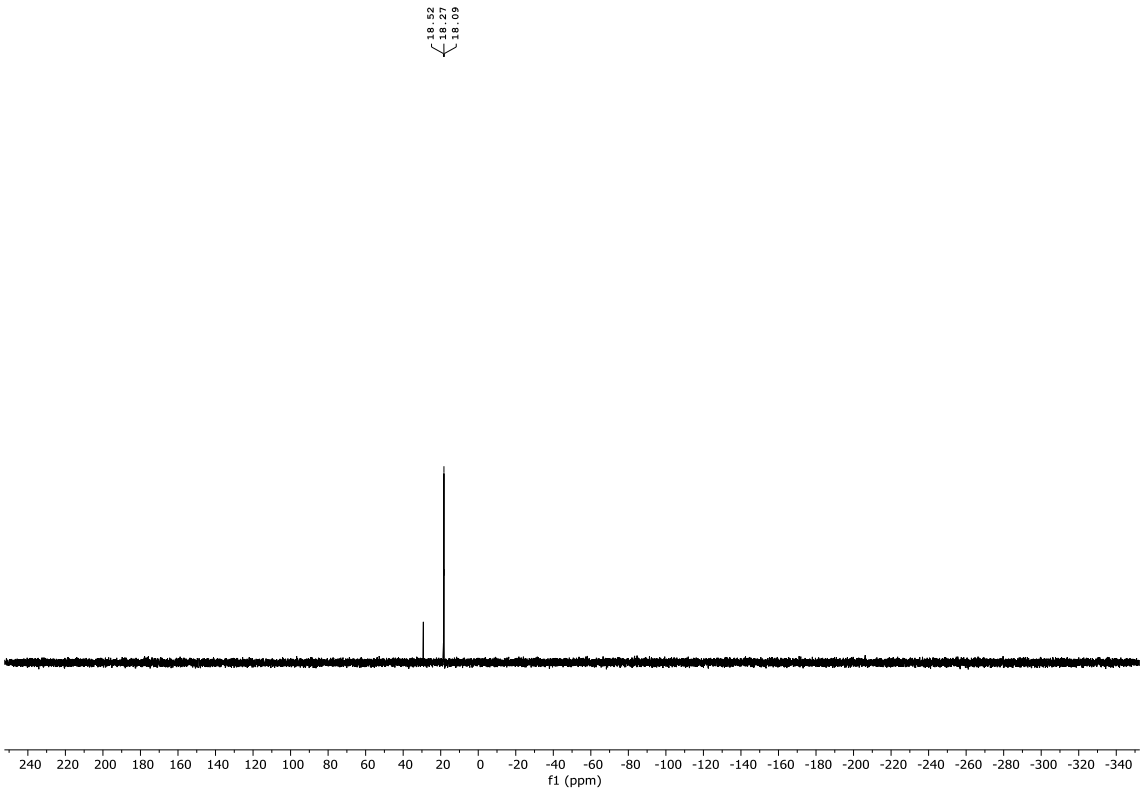
^{1}H-NMR (300 MHz, CDCl3 **Compound 10.**



13C{1H} NMR (75 MHz, CDCl3), of **Compound 10.**



^{31}P-NMR (121 MHz, $CDCl_3$) of **Compound 10.**



3. Solid phase synthesis protocol for PMO synthesis

The solid-supported synthesis of triazole-incorporated PMOs was carried out in Ramage Chem matrix resin. The standard coupling cycle involves a sequence of four fundamental steps: (i) deblocking: CYPMSA (1 g 3-cyano pyridine, 780 mg methanesulfonic acid (MSA), 20 ml MeOH, 60 ml DCM) 3 × 2 min = 6 min; (ii) coupling: chlorophosphoramidate morpholino monomer (0.2 M in DMI), ETT (5-ethylthio tetrazole) (0.3 M in DMI), and NEM (4-ethylmorpholine) 40 min, for functionalized chlorophosphoramidate morpholino monomer (3 × 40 min = 2 h); (iii) capping: (1 : 1)–10% Ac_2O-NMP and 10% DIPEA-NMP (3 × 1 = 3 min); and (iv) cleavage from the solid support: 30% aq. NH_3, 55 °C, 16 h. Throughout the coupling reaction anhydrous condition was maintained given the susceptibility of chlorophosphoramidate monomers. The coupling-capping-deprotection cycle is repeated until the desired sequence is achieved.[1]

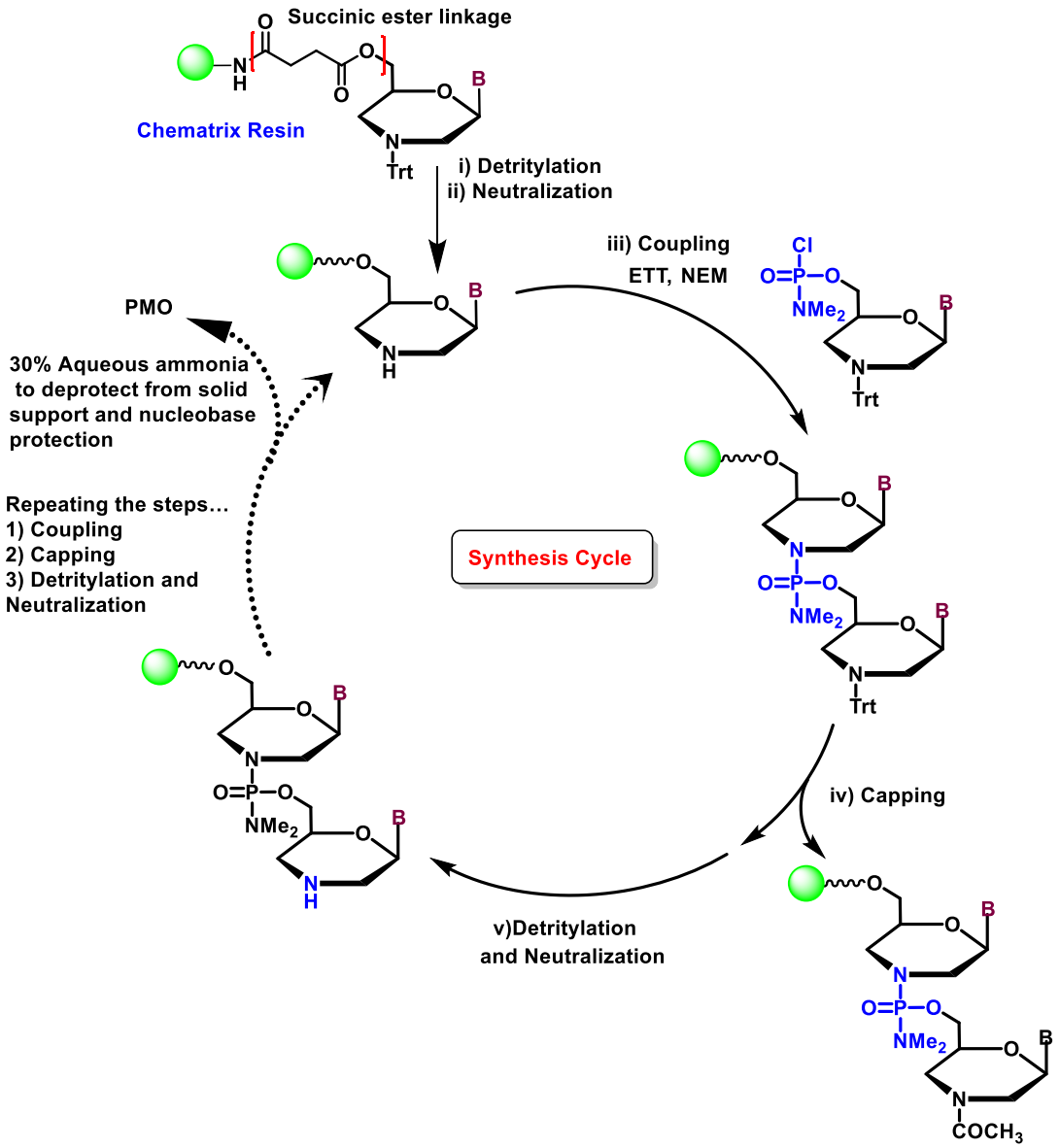
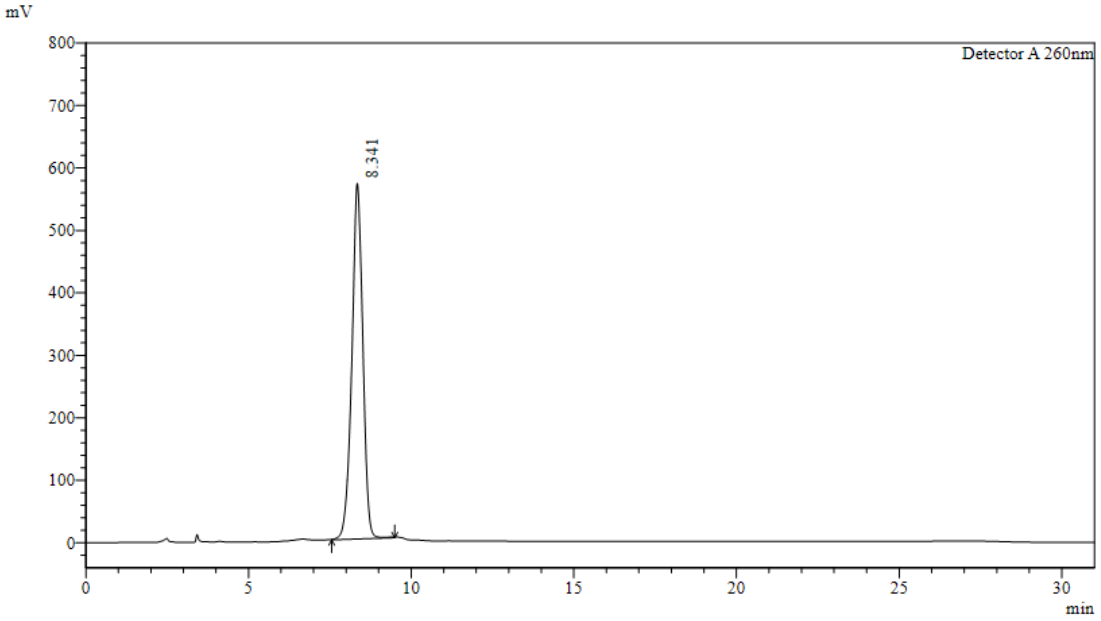


Fig-S1: Solid phase PMO synthesis cycle in Ramge Chemmatrix resin

4. Table-S1: HPLC yields and Mass analyses of Oligonucleotides

ONs	*Sequence* (5'→3')	*Molecular Formula*	*Calculated Mass*	*Found Mass*	*Pure Yield (%)*
PMO-1	GTGTTTTGC	$C_{105}H_{164}N_{45}O_{40}P_8Na$	2967.52	2267.44	40
PMO-2	GTGT**W**TTGC	$C_{112}H_{167}N_{48}O_{40}P_8Na$	3096.64	3096.40	38
PMO-3	GTGT**WW**TGC	$C_{119}H_{171}N_{51}O_{40}P_8Na$	3226.77	3226.27	37
PMO-4	GTG**WWW**TGC	$C_{126}H_{176}N_{54}O_{40}P_8Na$	3357.91	3357.45	32
PMO-5	G**W**GT**W**T**W**GC	$C_{126}H_{174}N_{54}O_{40}P_8K$	3372.00	3372.24	31
PMO-6	GTGT**YY**TGC	$C_{107}H_{164}N_{51}O_{40}P_8Na$	3075.59	3075.98	36
PMO-7	GTG**YYY**TGC	$C_{108}H_{163}N_{54}O_{40}P_8Na$	3128.61	3128.15	31
PMO-8	GTGT**XX**TGC	$C_{121}H_{173}N_{54}O_{40}P_8K$	3267.06	3266.03	37
PMO-9	GTG**XXX**TGC	$C_{129}H_{178}N_{54}O_{40}P_8K$	3410.10	3409.79	32
PMO-10	GTGT**ZZ**TTGC	$C_{119}H_{171}N_{51}O_{40}P_8K$	3242.88	3242.33	35
PMO-11	GTGT**ZZZ**TGC	$C_{126}H_{172}N_{54}O_{40}P_8K$	3368.06	3367.16	30

5. HPLC chromatograms and MALDI-TOF MS of Synthesized PMOs



Peak Table

Detector A 260nm

Peak#	Ret. Time	Area	Height	Area%
1	8.341	13420742	569375	100.000
Total		13420742	569375	100.000

Fig S2: Pure HPLC Chromatogram of 9 mer **PMO 1 (**5'-GTGTTTTGC-3') was obtained on Shimadzu SP-20AD system with C18 (CAPCELL PAK) column using 50 mM Ammonium acetate buffer (in H_2O)-CH_3CN gradient system (10-50%) flow rate = 1 ml/min.

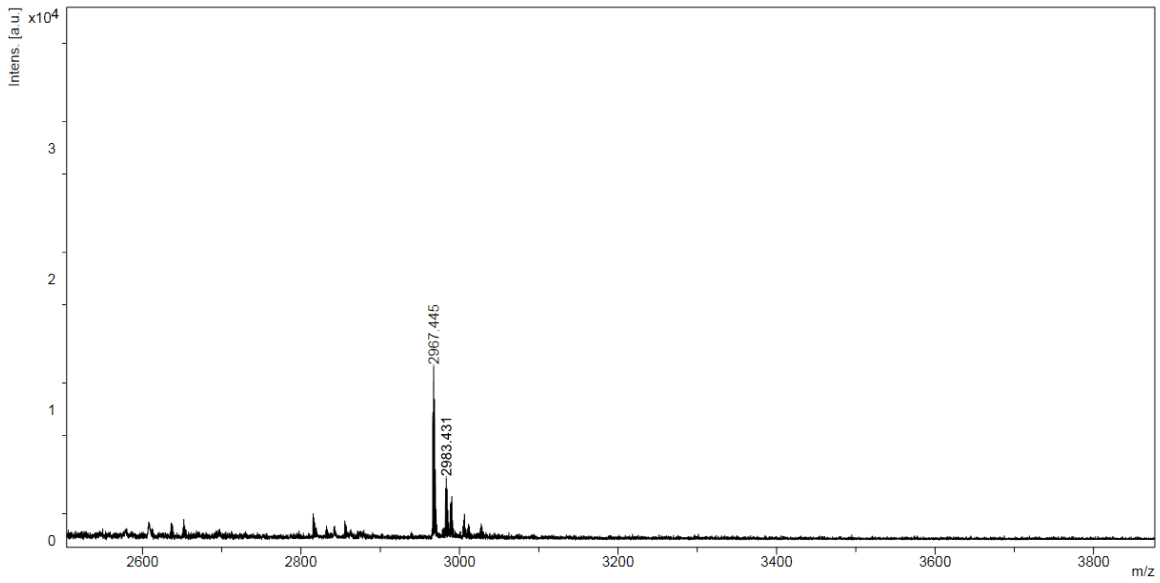
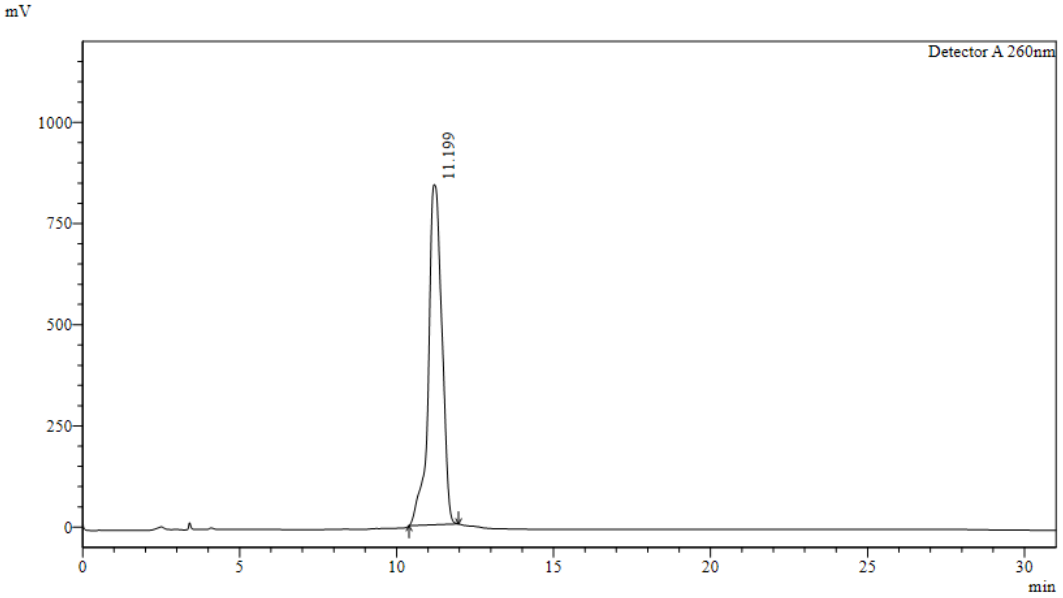


Fig S3: MALDI-TOF mass of PMO1. Calculated mass of molecular formula $[C_{105}H_{164}N_{45}O_{40}P_8Na]^+$ = 2964.52, found = 2967.44



Peak Table

Detector A 260nm

Peak#	Ret. Time	Area	Height	Area%
1	11.199	25135208	840294	100.000
Total		25135208	840294	100.000

Fig S4: Pure HPLC Chromatogram of 9 mer **PMO 2 (**5'- GTGT**W**TTGC-3') was obtained on Shimadzu SP-20AD system with C18 (CAPCELL PAK) column using 0.05M Ammonium acetate buffer (in H_2O)-CH_3CN gradient system (10-50%) flow rate = 1 ml/min.

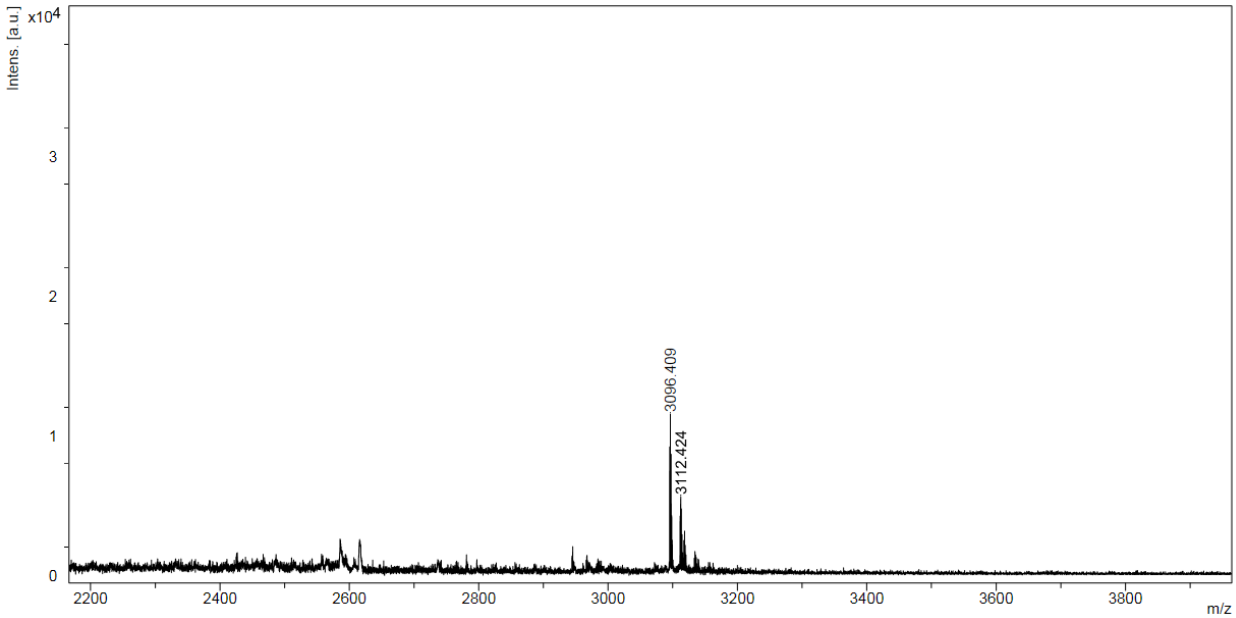
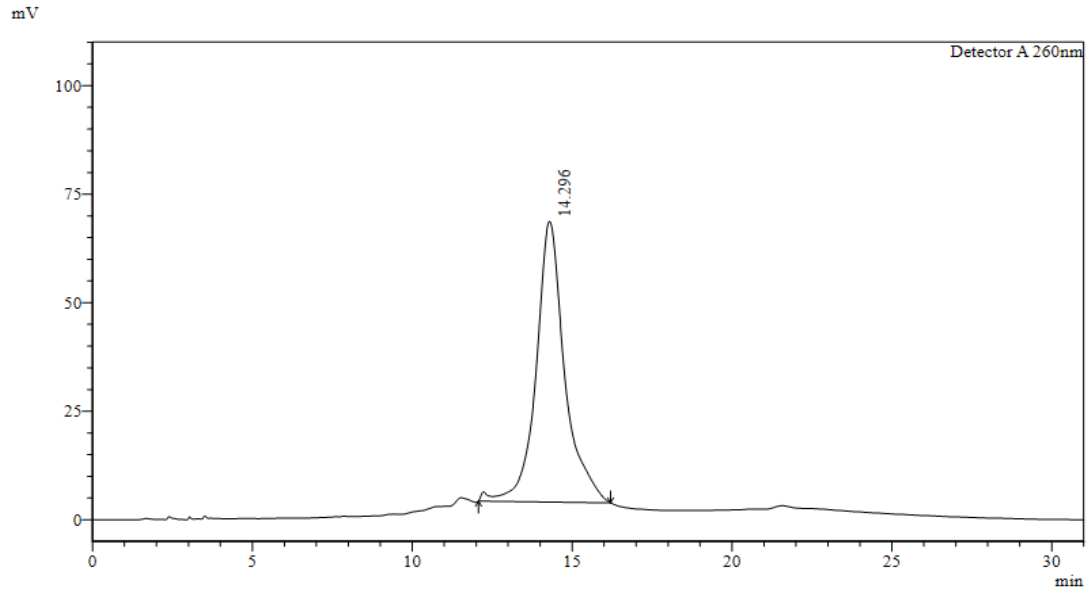


Fig S5: MALDI-TOF mass of PMO2. Calculated mass of molecular formula $[C_{112}H_{167}N_{48}O_{40}P_8Na]^+$ = 3096.64, found = 3096.40



Peak Table

Detector A 260nm

Peak#	Ret. Time	Area	Height	Area%
1	14.296	4024495	64630	100.000
Total		4024495	64630	100.000

Fig S6: Pure HPLC Chromatogram of 9 mer **PMO 3 (**5'- GTGT**WW**TGC-3') was obtained on Shimadzu SP-20AD system with C18 (CAPCELL PAK) column using 50mM Ammonium acetate buffer (in H_2O)-CH_3CN gradient system (10-60%) flow rate = 1 ml/min.

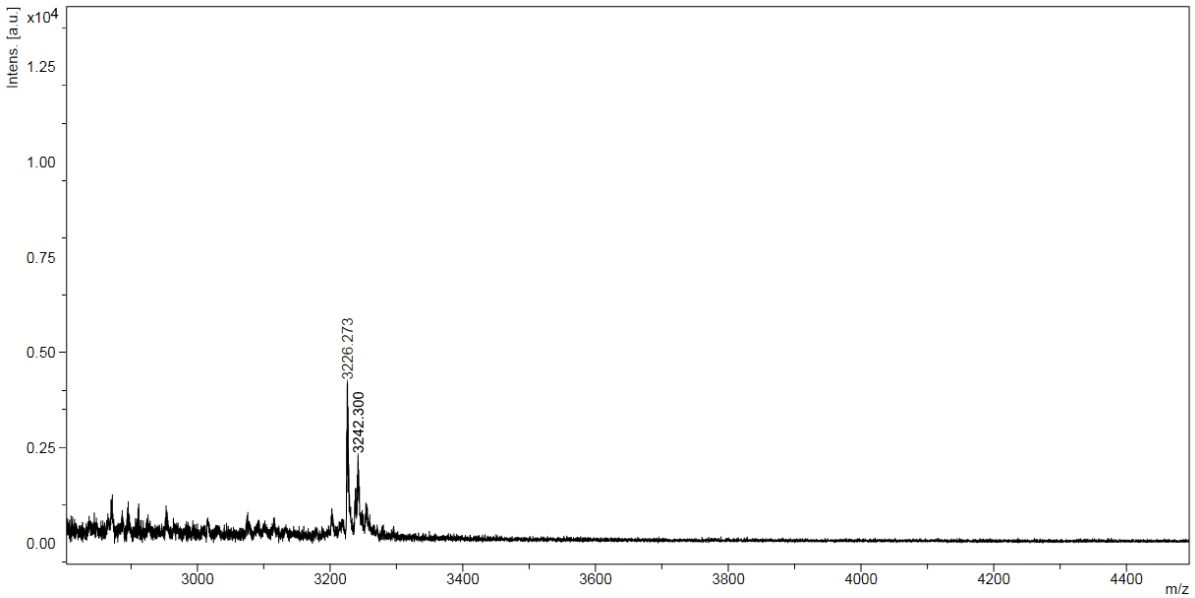
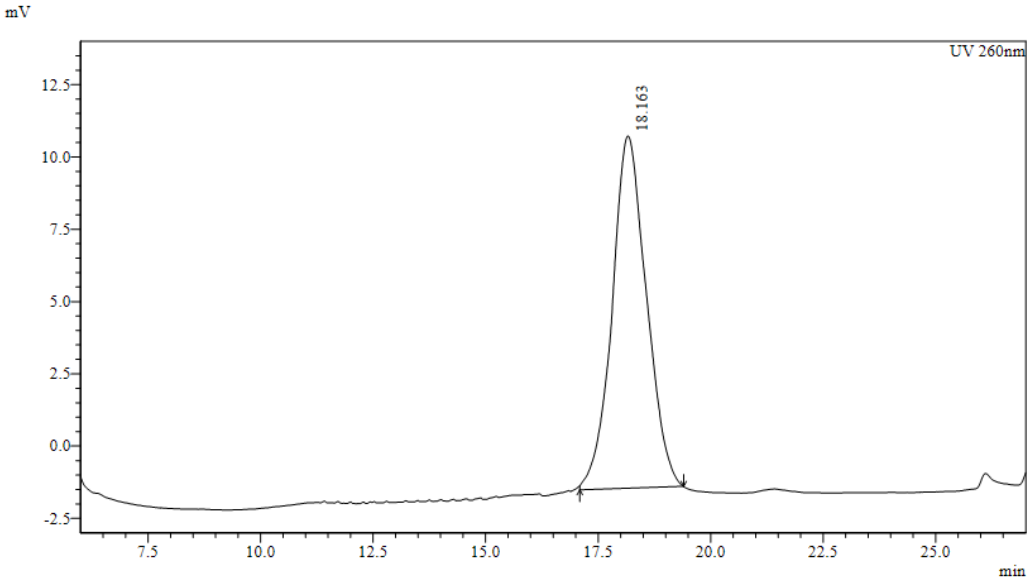


Fig S7: MALDI-TOF mass of PMO3. Calculated mass of molecular formula $[C_{119}H_{171}N_{51}O_{40}P_8Na]^+$ = 3226.77, found 3226.27



Peak Table

UV 260nm

Peak#	Ret. Time	Area	Height	Area%
1	18.163	646186	12178	100.000
Total		646186	12178	100.000

Fig S8: Pure HPLC Chromatogram of 9 mer **PMO 4** (5'- GTG**WWW**TGC-3') was obtained on Shimadzu SP-20AD system with C18 (CAPCELL PAK) column using 50 mM Ammonium acetate buffer (in H_2O)-CH_3CN gradient system (10-70%) flow rate = 1 ml/min.

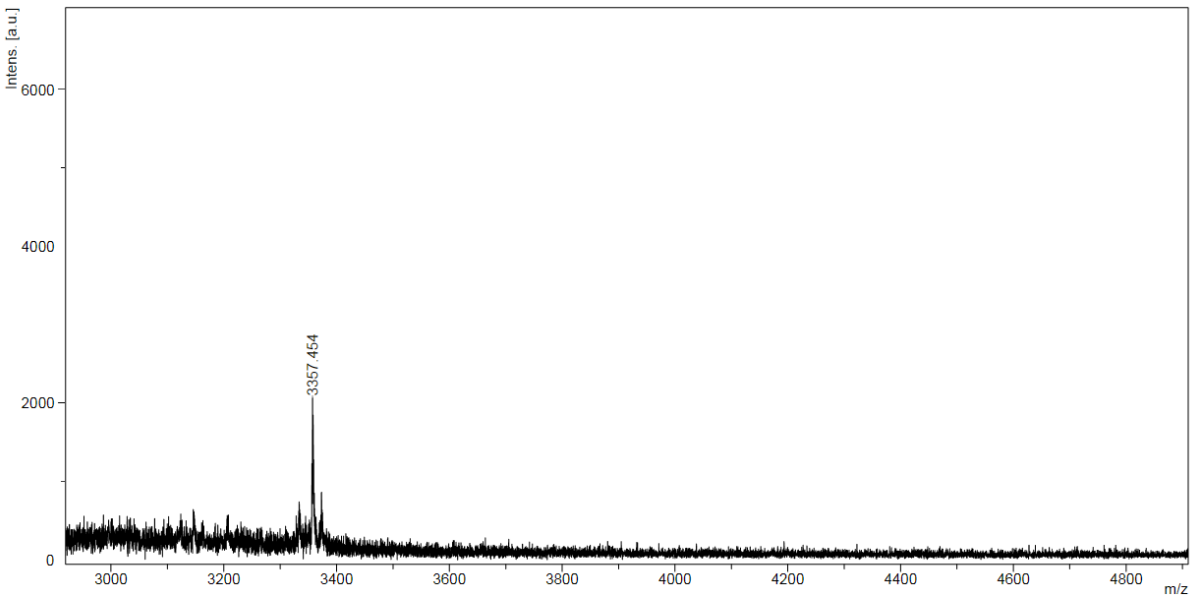
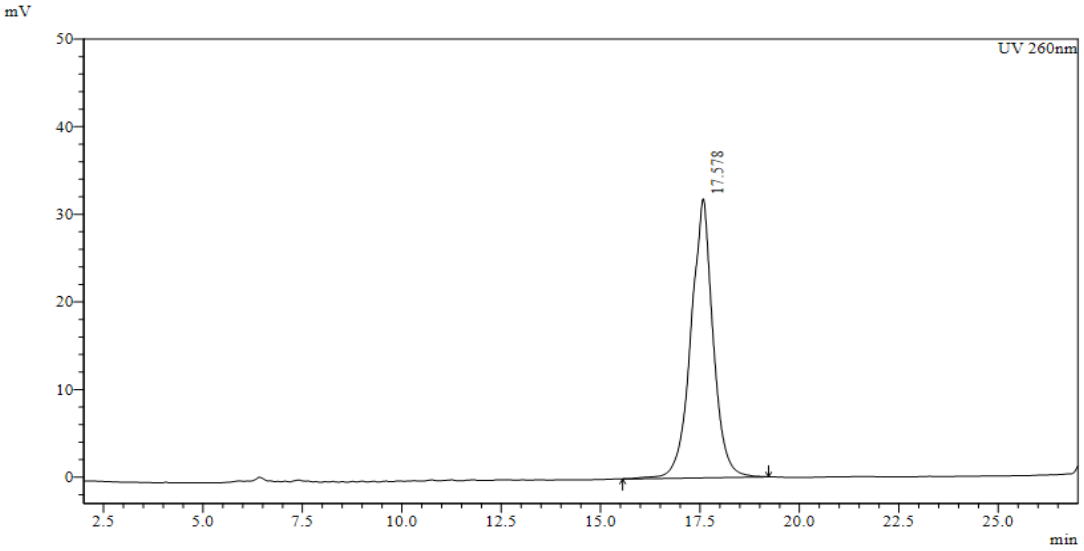


Fig S9: MALDI-TOF mass of PMO4. Calculated mass of molecular formula $[C_{126}H_{176}N_{54}O_{40}P_8Na]^+$ = 3357.91, found = 3357.45



Peak Table

UV 260nm

Peak#	Ret. Time	Area	Height	Area%
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Total		1234184	31833	100.000

Fig S10: Pure HPLC Chromatogram of 9 mer **PMO 5** (5'- G**W**GT**W**T**W**GC -3') was obtained on Shimadzu SP-20AD system with C18 (CAPCELL PAK) column using 50mM Ammonium acetate buffer (in H2O)-CH3CN gradient system (10-70%) flow rate = 1 ml/min.

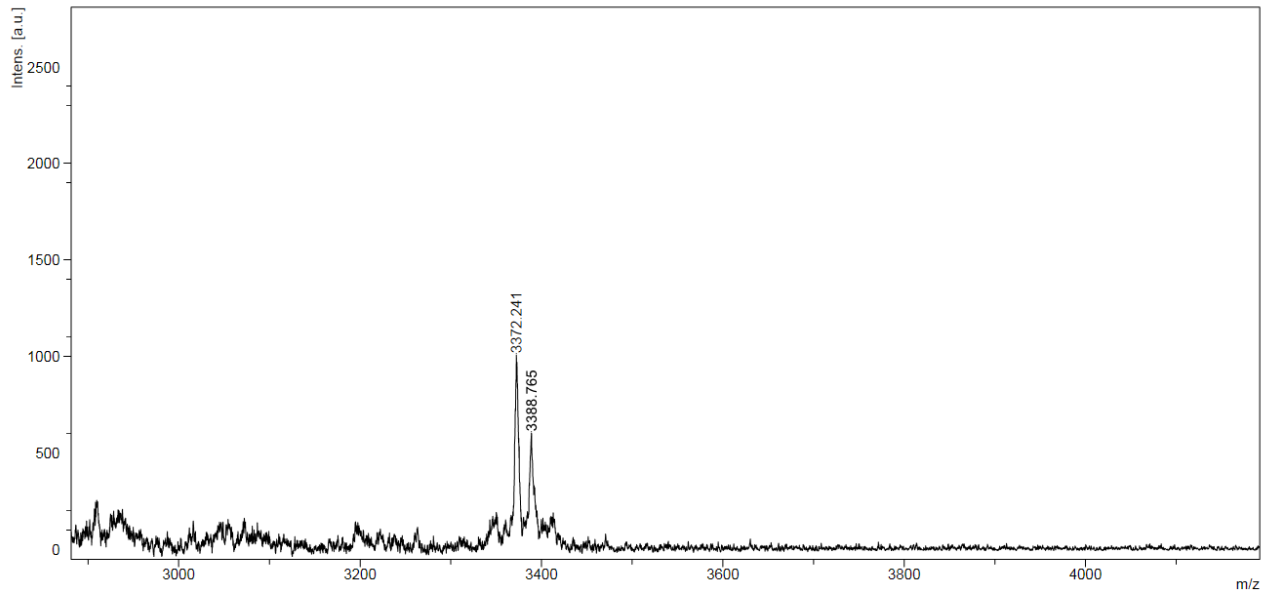
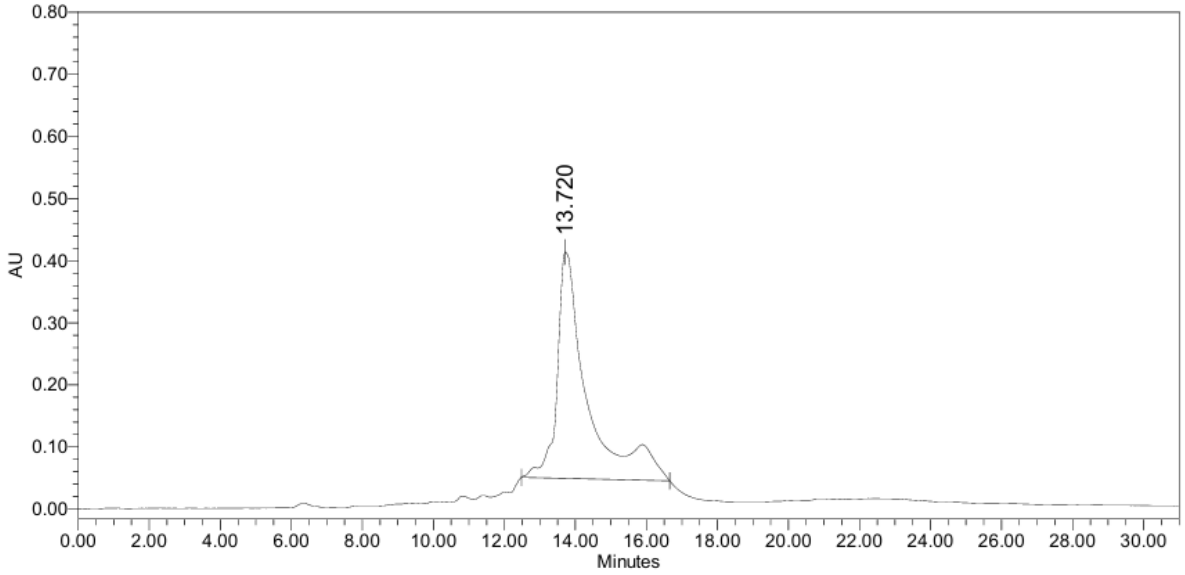


Fig S11: MALDI-TOF mass of PMO5. Calculated mass of molecular formula $[C_{126}H_{174}N_{54}O_{40}P_8K]^+$ = 3372.00, found = 3372.24



Peak table

	RT	Area	Height	% Area
1	13.720	21465078	364889	100.00

Fig S12: Pure HPLC Chromatogram of 9 mer **PMO 6** (5'- GTGT**YY**TGC -3') was obtained on Waters 1525 Binary HPLC pump, C18 (Waters XBridge BEH Shield RP18) column. Using 50mM Ammonium acetate buffer (in H_2O)-CH3CN gradient system (10-50%) flow rate = 2 ml/min.

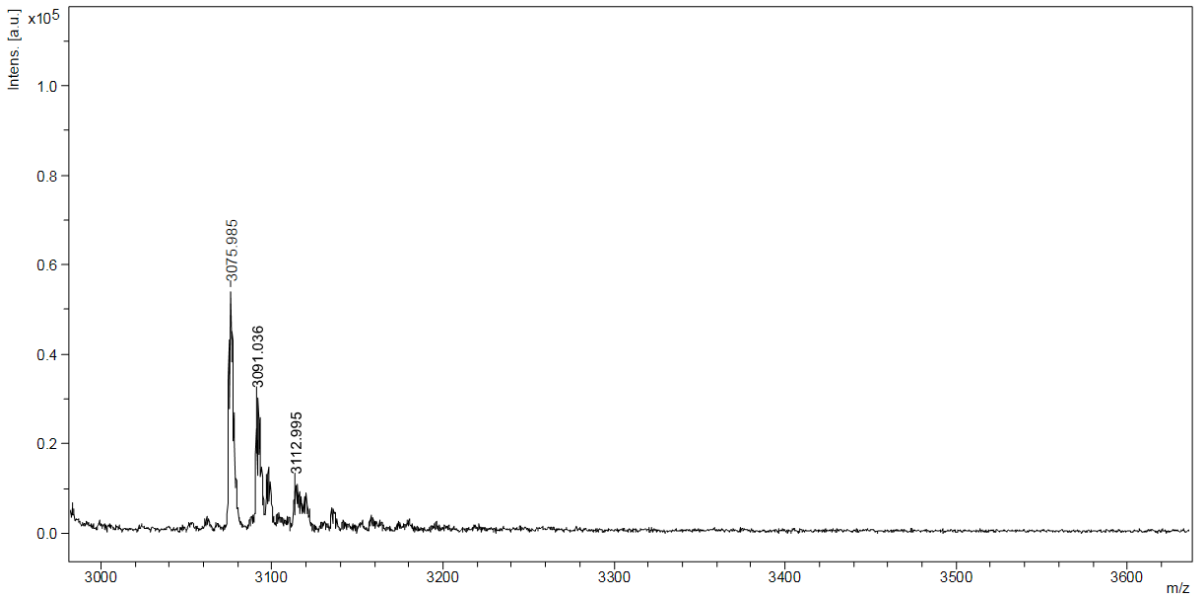
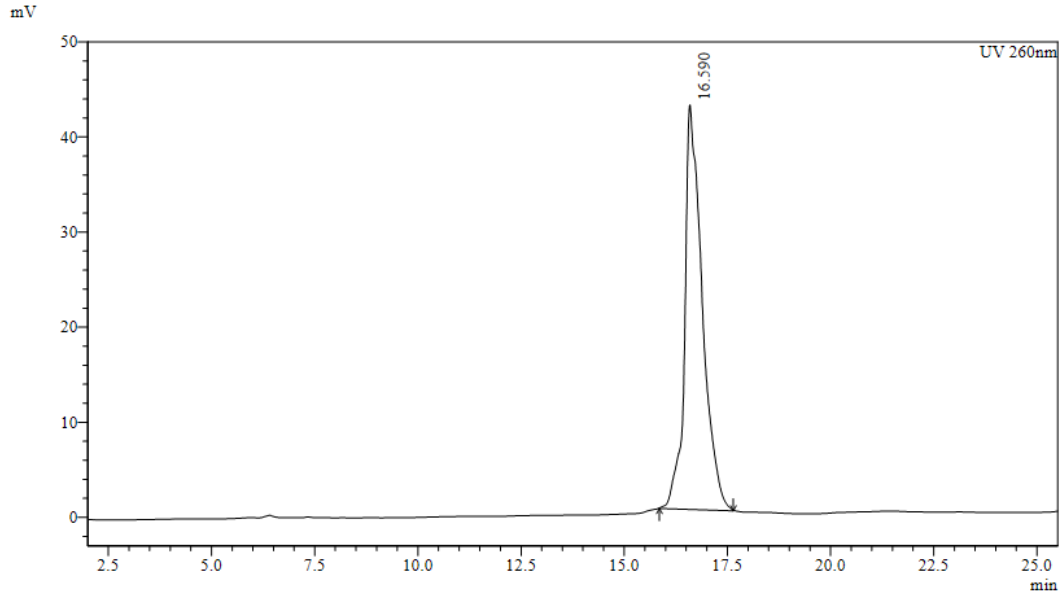


Fig S13: MALDI-TOF mass of PMO6. Calculated mass of molecular formula $[C_{107}H_{164}N_{51}O_{40}P_8Na]^+$ = 3075.59, found = 3075.98.



Peak Table

UV 260nm

Peak#	Ret. Time	Area	Height	Area%
1	16.590	1263815	42553	100.000
Total		1263815	42553	100.000

Fig S14: Pure HPLC Chromatogram of 9 mer **PMO 7** (5'- GTG**YYY**TGC-3') was obtained on Shimadzu SP-20AD system with C18 (CAPCELL PAK) column using 50mM Ammonium acetate buffer (in H_2O)-CH_3CN gradient system (10-70%) flow rate = 1 ml/min.

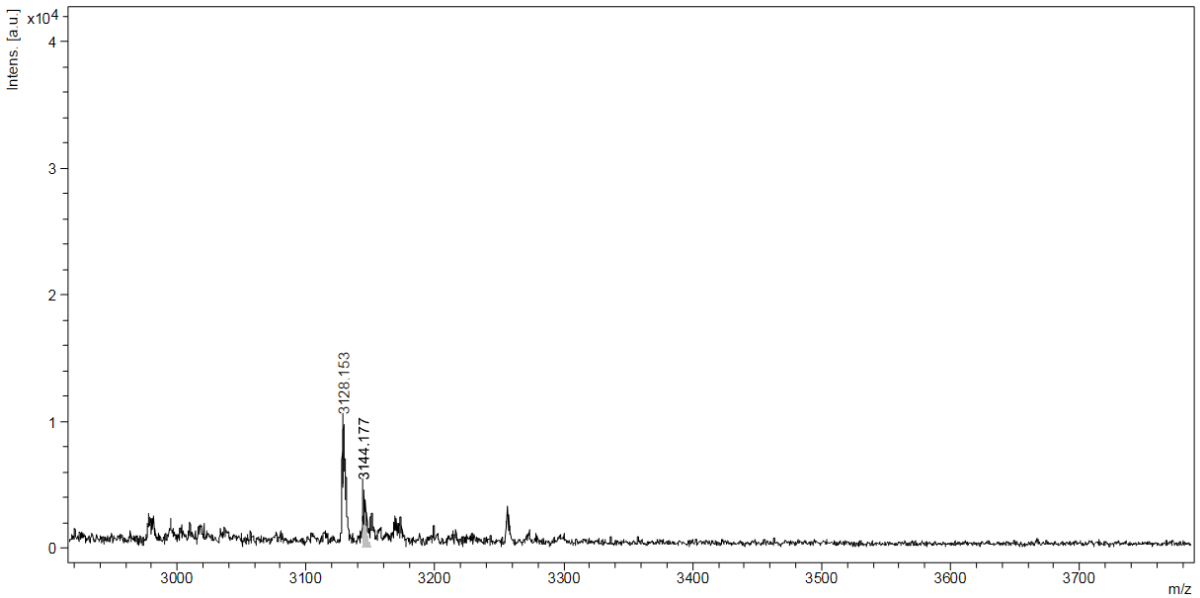
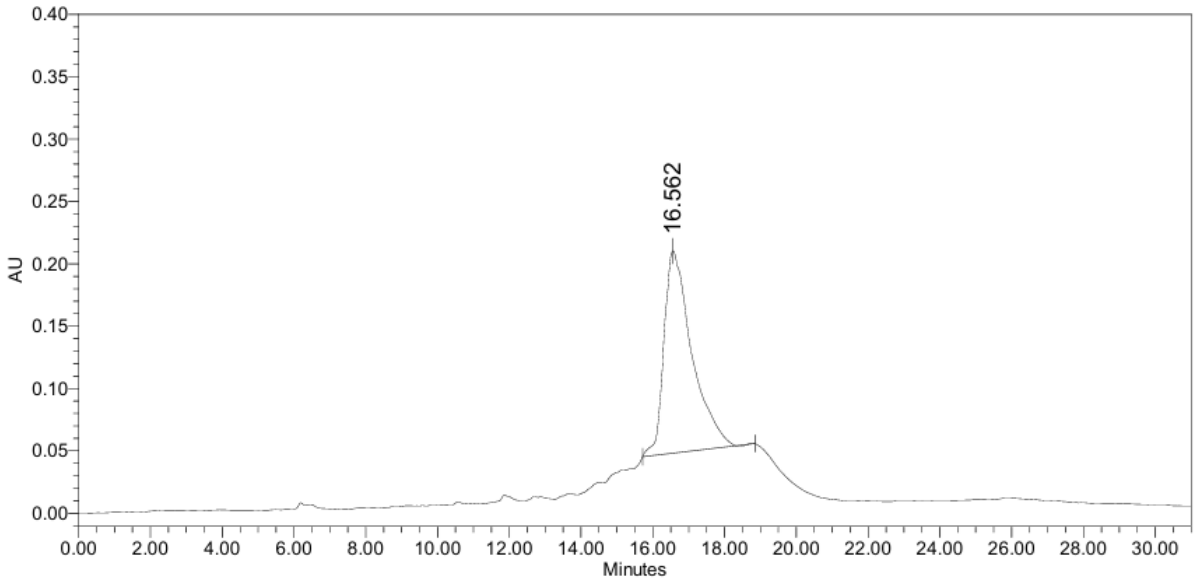


Fig S15: MALDI-TOF mass of PMO7. Calculated mass of molecular formula $[C_{108}H_{163}N_{54}O_{40}P_8Na]^+$ = 3128.61, found = 3128.15



Peak table

	RT	Area	Height	% Area
1	16.562	8776867	162147	100.00

Fig S16: Pure HPLC Chromatogram of 9 mer **PMO 8** (5'- GTGT**XX**TGC-3') was obtained on Waters 1525 Binary HPLC pump, C18 (Waters XBridge BEH Shield RP18) column. Using 50mM Ammonium acetate buffer (in H_2O)-CH_3CN gradient system (10-50%) flow rate = 2 ml/min.

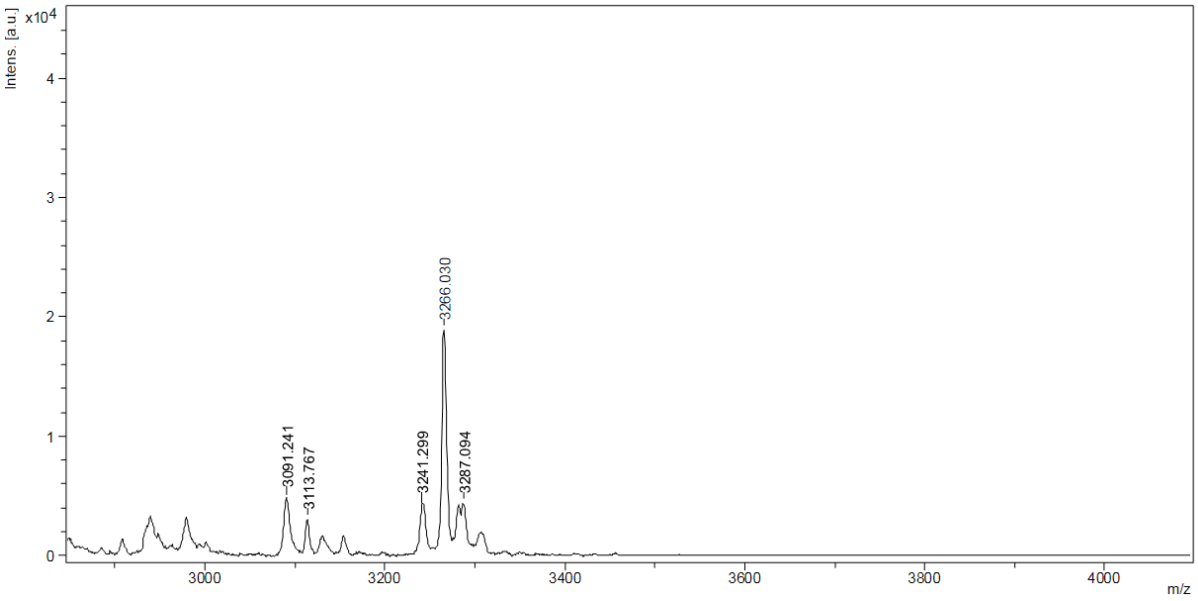
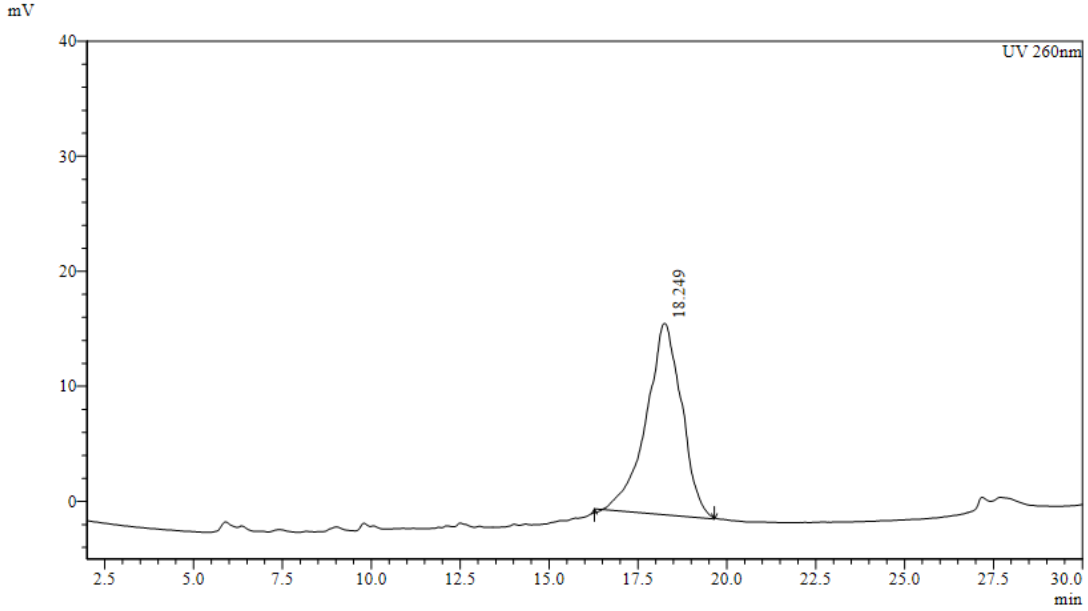


Fig S17: MALDI-TOF mass of PMO8. Calculated mass of molecular formula $[C_{121}H_{173}N_{54}O_{40}P_8K]^+$ = 3267.06, found = 3266.03



Peak Table

UV 260nm

Peak#	Ret. Time	Area	Height	Area%
1	18.249	1154392	16638	100.000
Total		1154392	16638	100.000

Fig S18: Pure HPLC Chromatogram of 9 mer **PMO 9** (5'- GTG**XXX**TGC-3') was obtained on Shimadzu SP-20AD system with C18 (CAPCELL PAK) column using 50mM Ammonium acetate buffer (in H_2O)-CH_3CN gradient system (10-70%) flow rate = 1 ml/min.

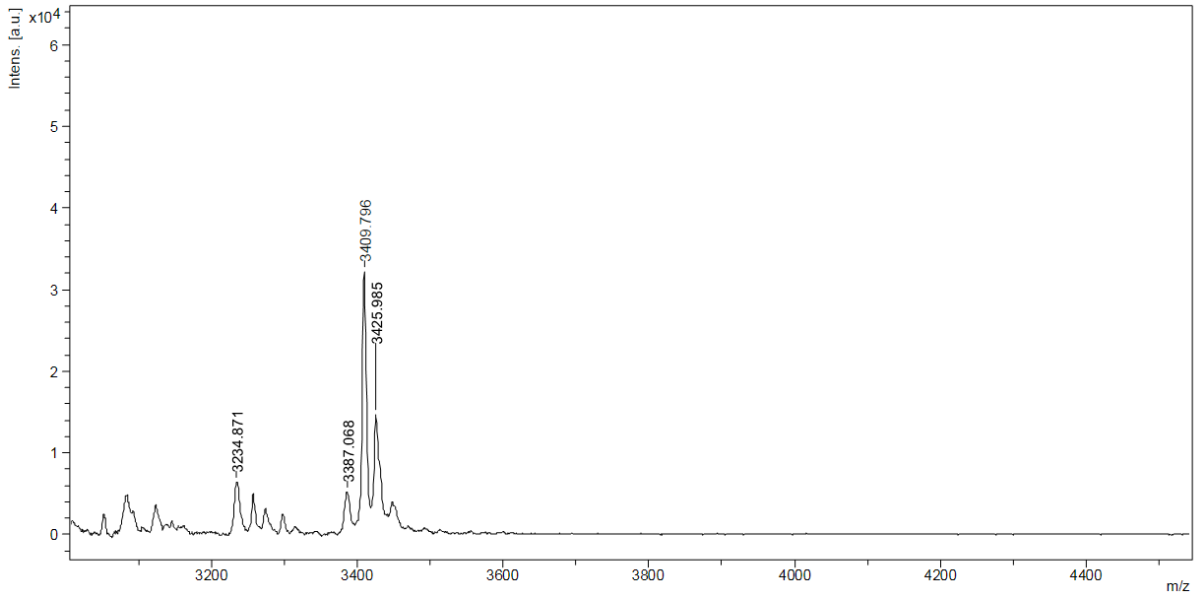
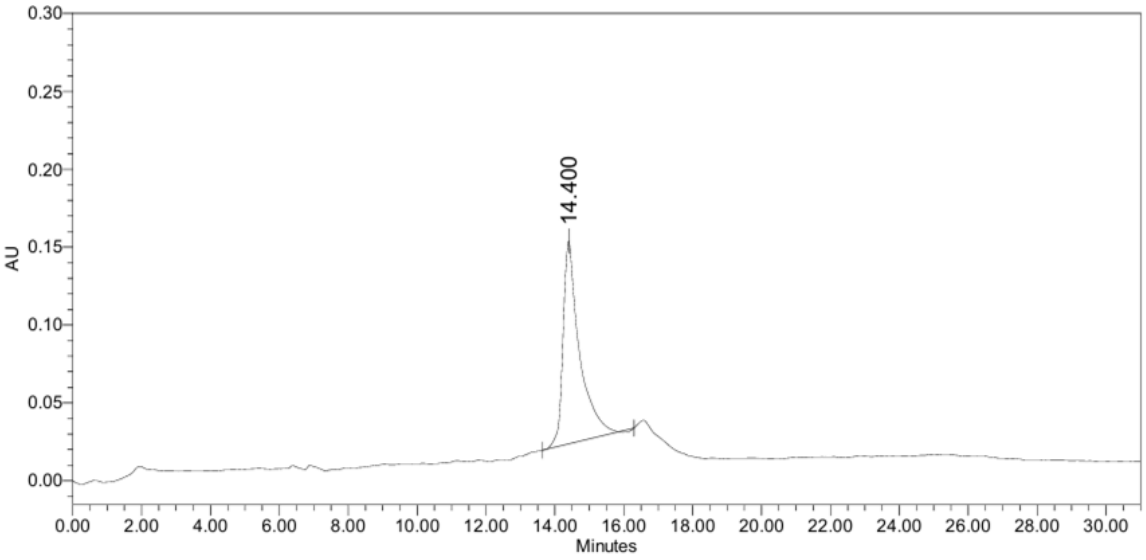


Fig S19: MALDI-TOF mass of PMO9. Calculated mass of molecular formula $[C_{119}H_{171}N_{51}O_{40}P_8K]^+$ = 3410.109, found = 3409.796



Peak table

	RT	Area	Height	% Area
1	14.400	4343832	130246	100.00

Fig S20: Pure HPLC Chromatogram of 9 mer **PMO 10** (5'- GTGT**ZZ**TGC-3') was obtained on Waters 1525 Binary HPLC pump, C18 (Waters XBridge BEH Shield RP18) column. Using 50mM Ammonium acetate buffer (in H_2O)-CH_3CN gradient system (10-50%) flow rate = 2 ml/min.

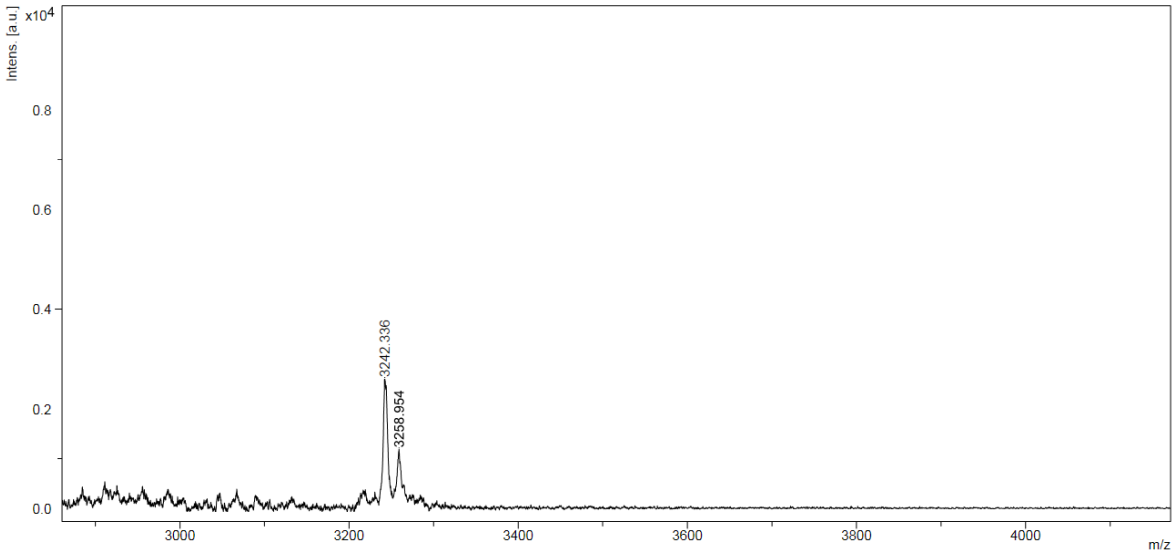
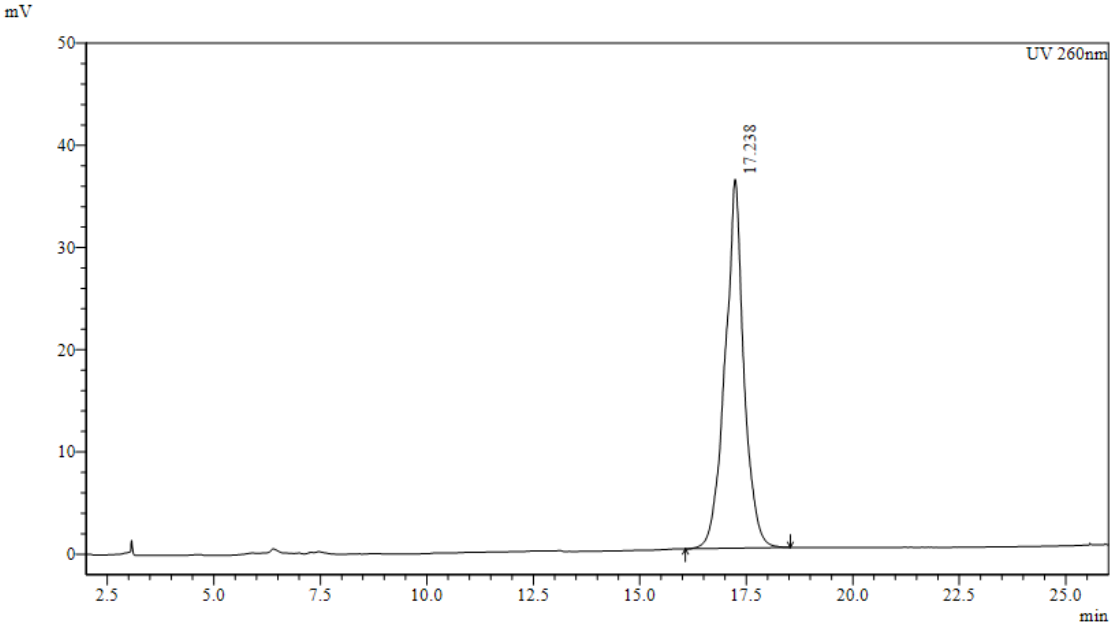


Fig S21: MALDI-TOF mass of PMO10. Calculated mass of molecular formula $[C_{119}H_{171}N_{51}O_{40}P_8K]^+$ = 3242.88, found = 3242.33



Peak Table

UV 260nm

Peak#	Ret. Time	Area	Height	Area%
1	17.238	1106739	36077	100.000
Total		1106739	36077	100.000

Fig S22: Pure HPLC Chromatogram of 9 mer **PMO 11** (5'- GTG**ZZZ**TGC-3' was obtained on Shimadzu SP-20AD system with C18 (CAPCELL PAK) column using 50mM Ammonium acetate buffer (in H_2O)-CH_3CN gradient system (10-70%) flow rate = 1 ml/min.

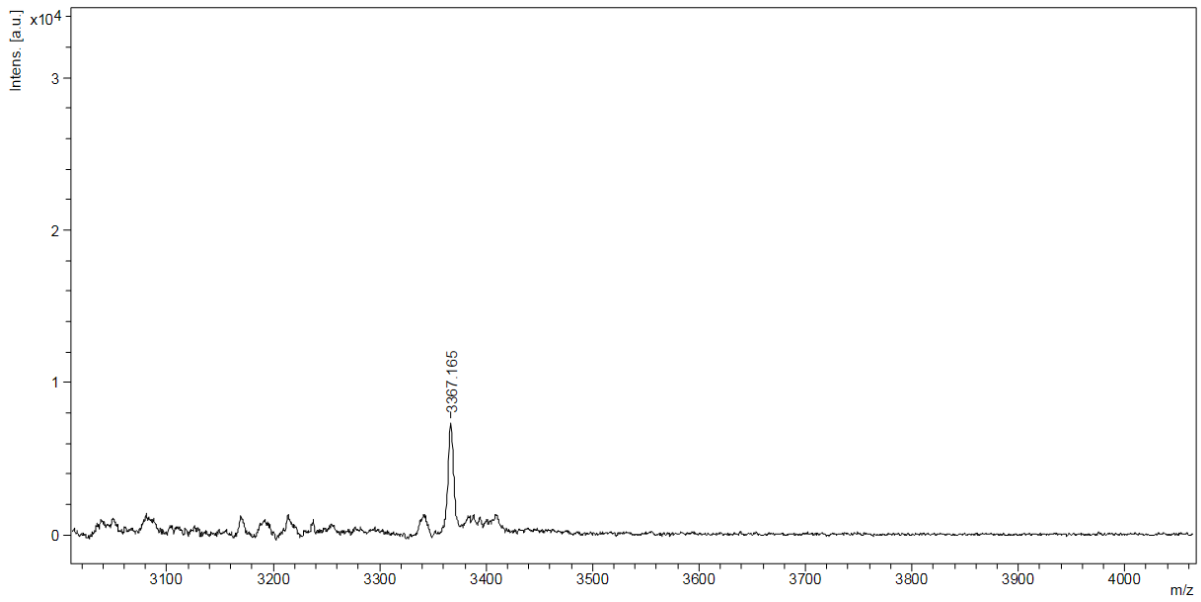
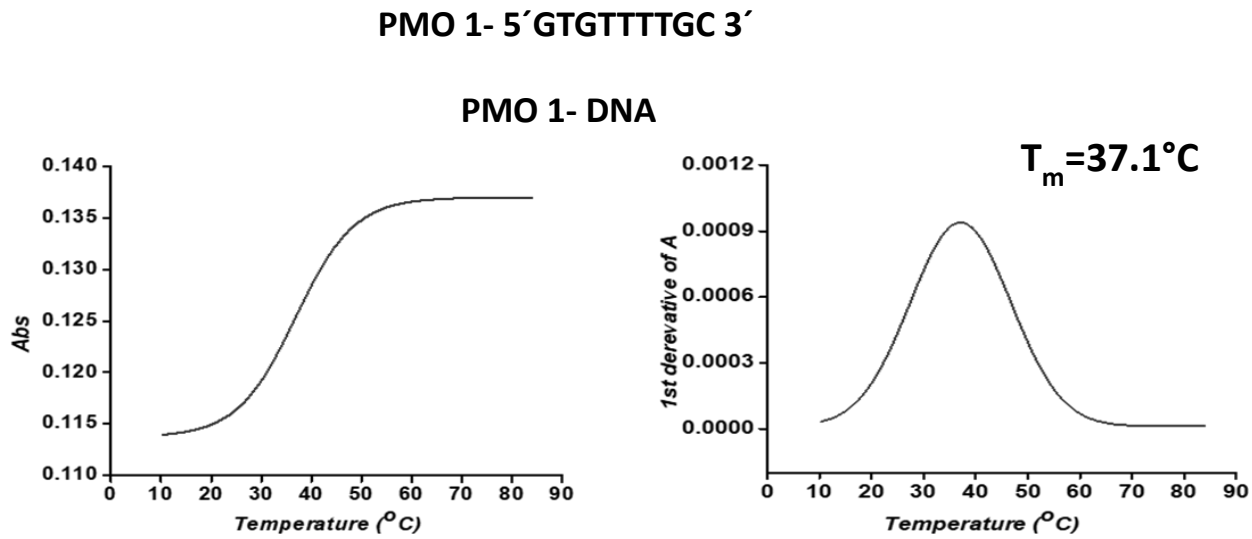


Fig S23: MALDI-TOF mass of PMO11. Calculated mass of molecular formula [$C_{126}H_{172}N_{54}O_{40}P_8K]^+$ = 3368.062, found = 3367.165.

Melting Temperature Curves:

The duplex samples were obtained by mixing both strands of interest in a 1:1 ratio at 2 µm concentration in 40 mM phosphate (NaH_2PO_4/Na_2HPO_4) / 100 mM Sodium Chloride (NaCl) buffer (pH 7) followed by heating to 85 °C for 5 min then with slow cooling to 10 °C. Absorbance at 260 nm was recorded as a function of decreasing temperature on a Cary-3500 UV-Vis spectrophotometer equipped with a Peltier cell holder using quartz cuvettes with a 1-cm path length. Temperature was ramped at 1°C/min. The T_m value was calculated from the sigmoid and first derivative plot, with Gaussian curve fitting using Origin 21.0 software

A.



B.

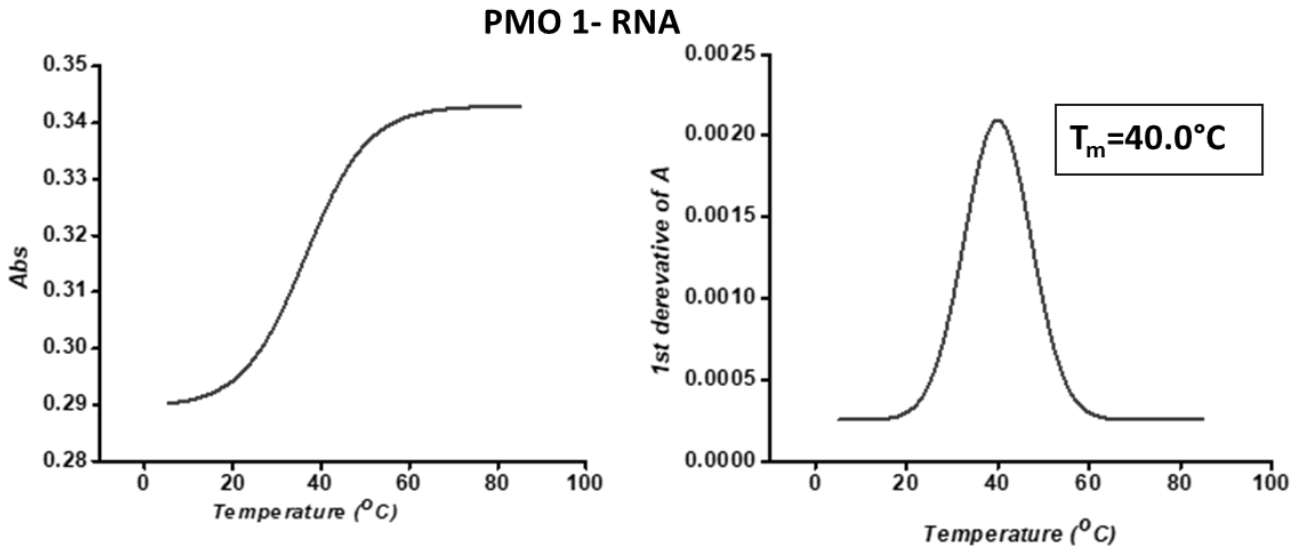
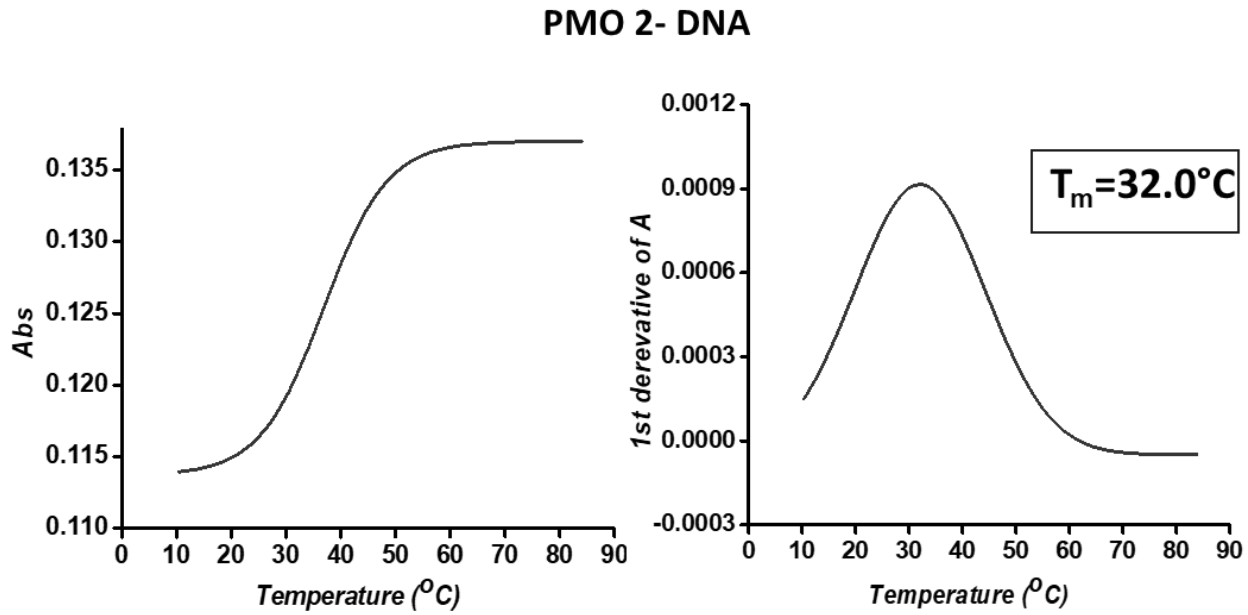


Fig S24: Thermal melting curves and first derivative plot of duplexes (A) PMO1-DNA (B) PMO1-RNA

PMO 2: 5´-GTGTWTTGC-3´

A.



B.

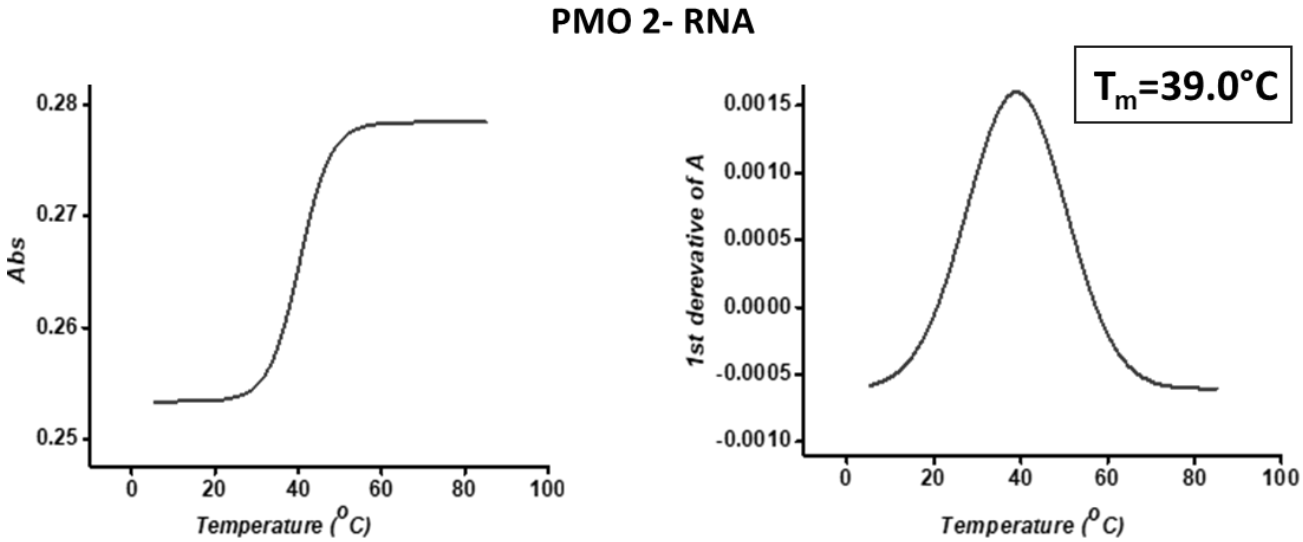
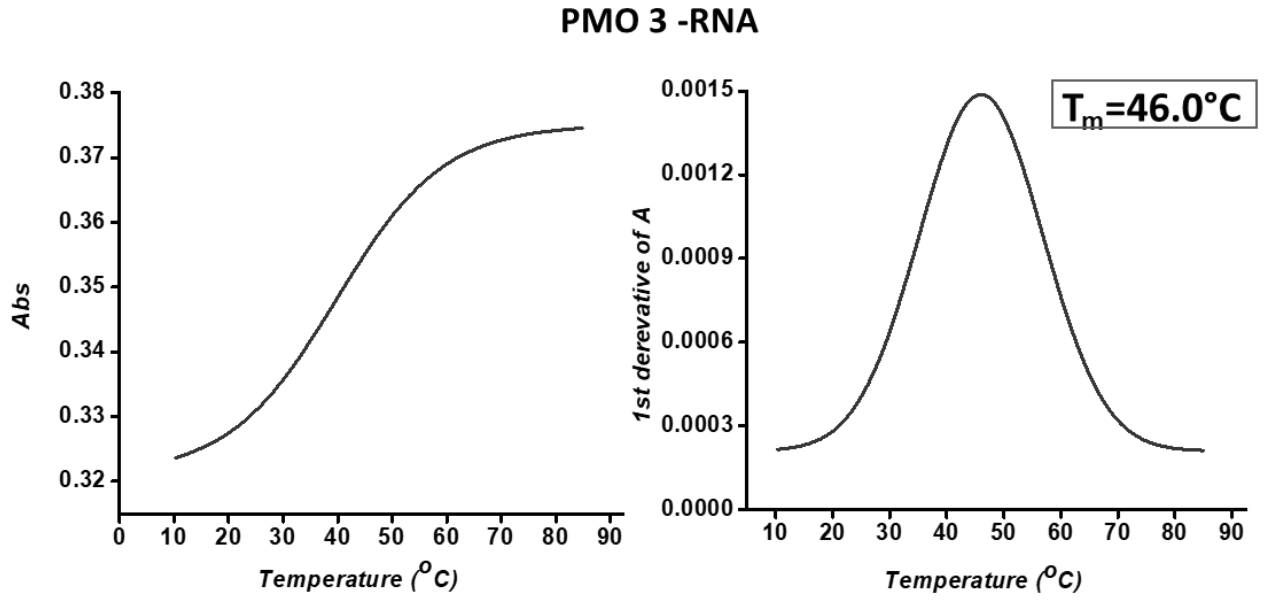


Fig S25: Thermal melting curves and first derivative plot of duplexes (A) PMO2-DNA (B) PMO2-RNA

PMO 3: 5´-GTGTWWTGC-3´

A.



B.

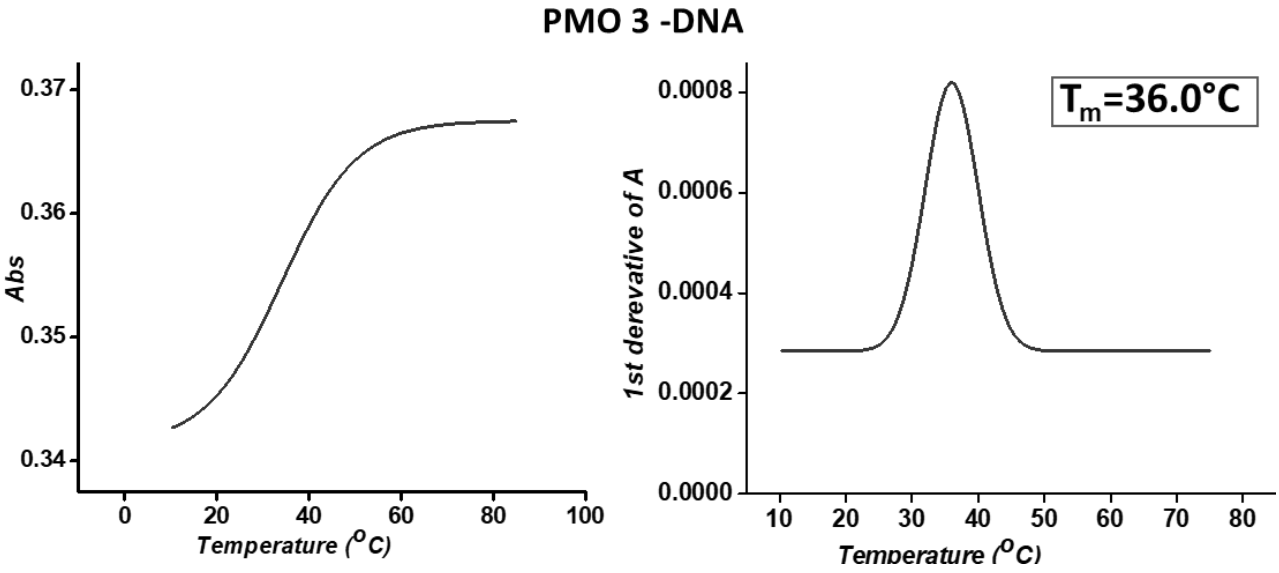


Fig S26: Thermal melting curves and first derivative plot of duplexes (A) PMO3-DNA (B) PMO3-RNA

A.

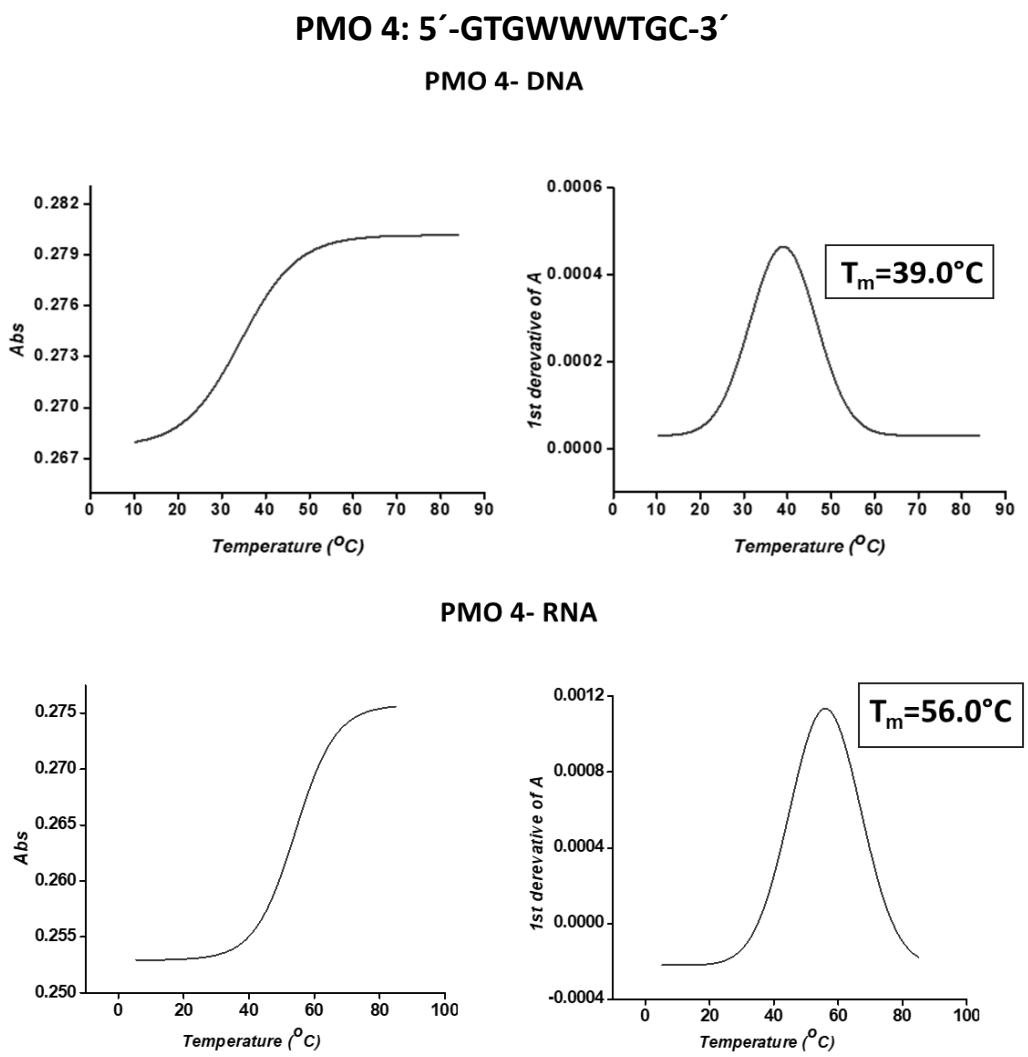


Fig S27: Thermal melting curves and first derivative plot of duplexes (A) PMO4-DNA (B) PMO4-RNA

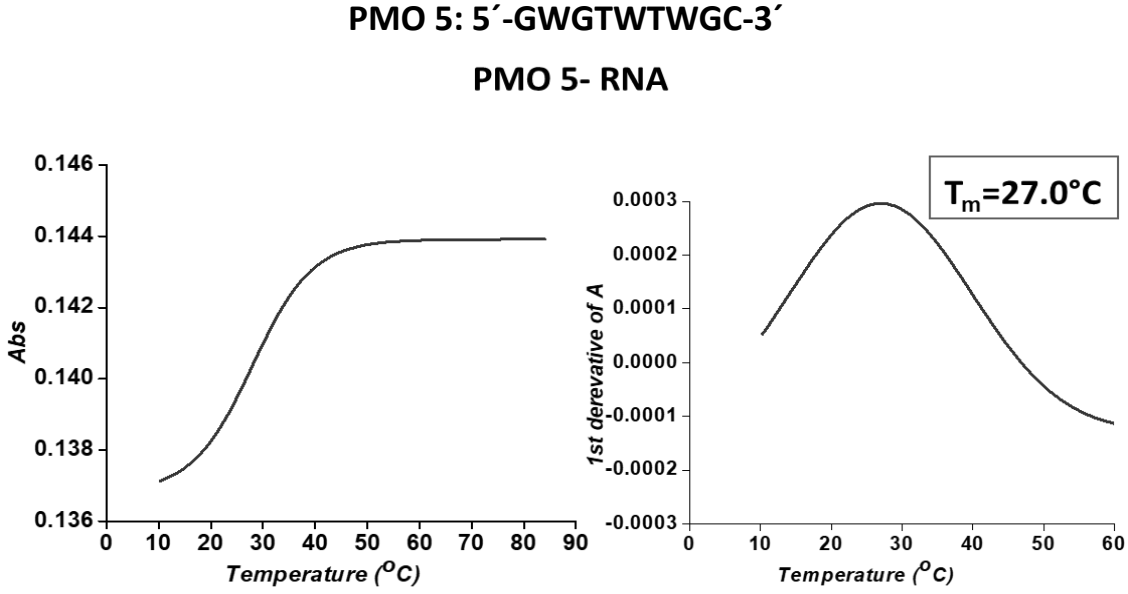
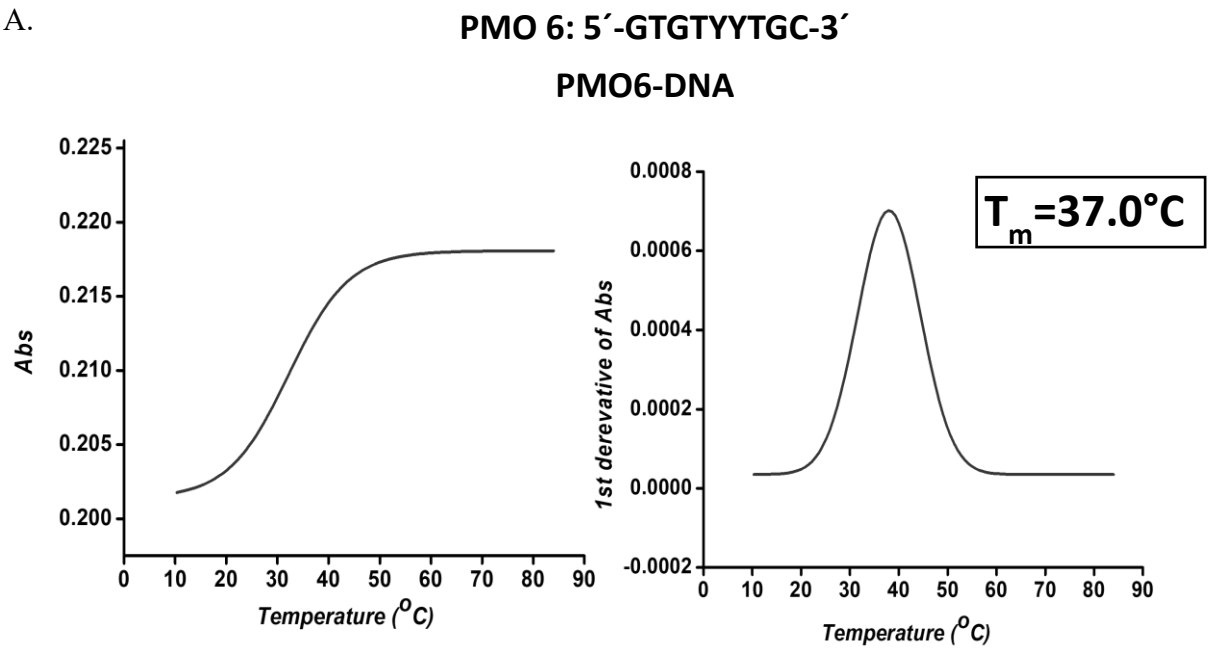


Fig S28: Thermal melting curves and first derivative plot of duplexes PMO5-RNA

A.



B

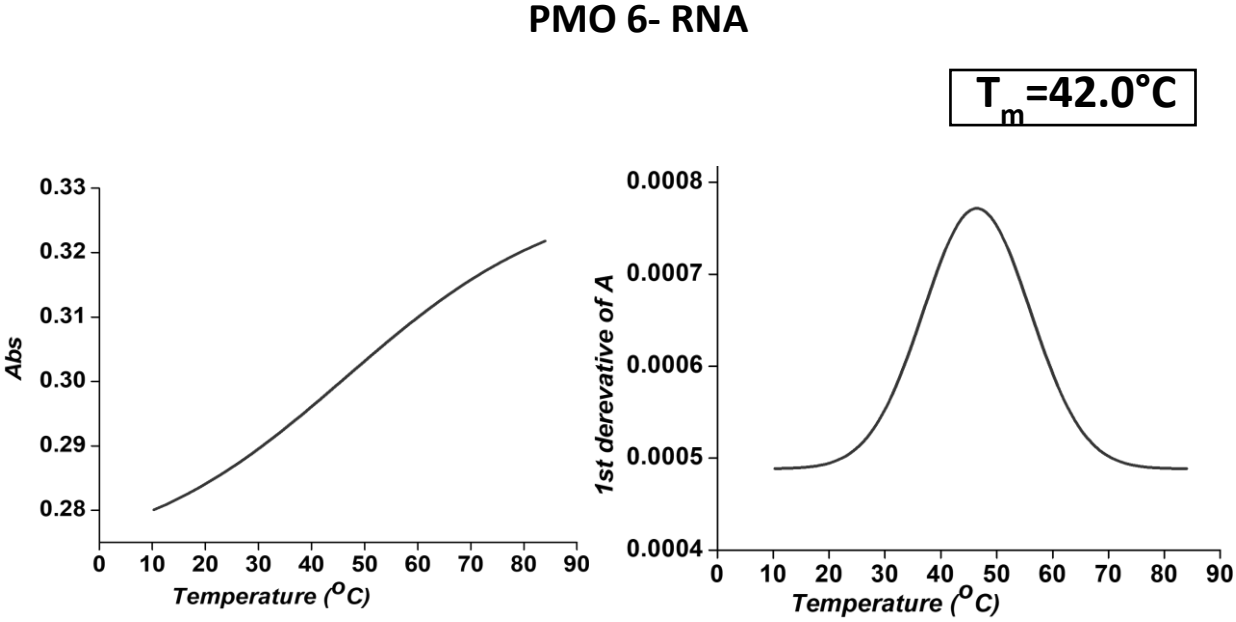
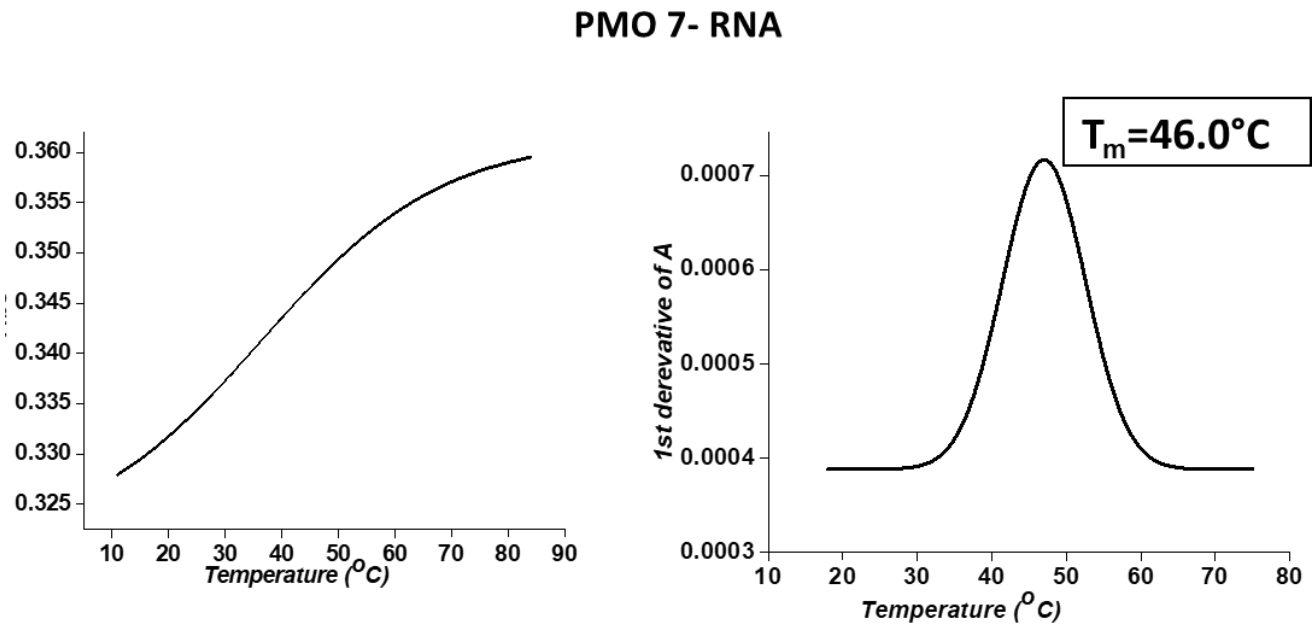


Fig S29: Thermal melting curves and first derivative plot of duplexes (A) PMO6-DNA (B) PMO6-RNA

PMO 7: 5´-GTGYYYTGC-3´

A.



B.

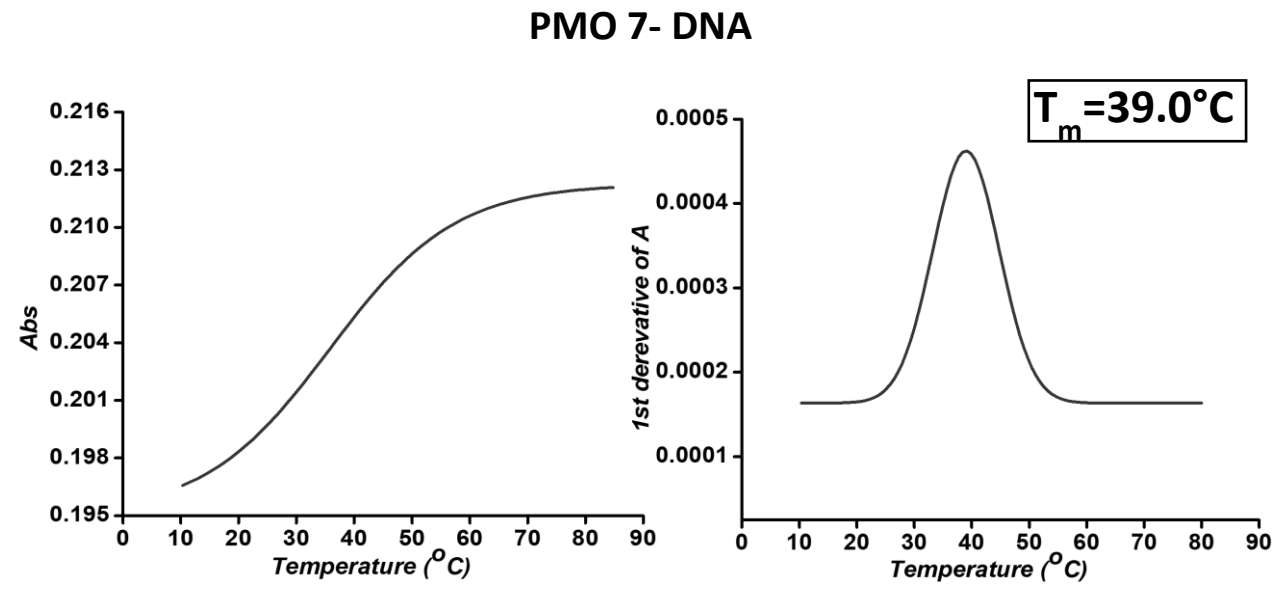
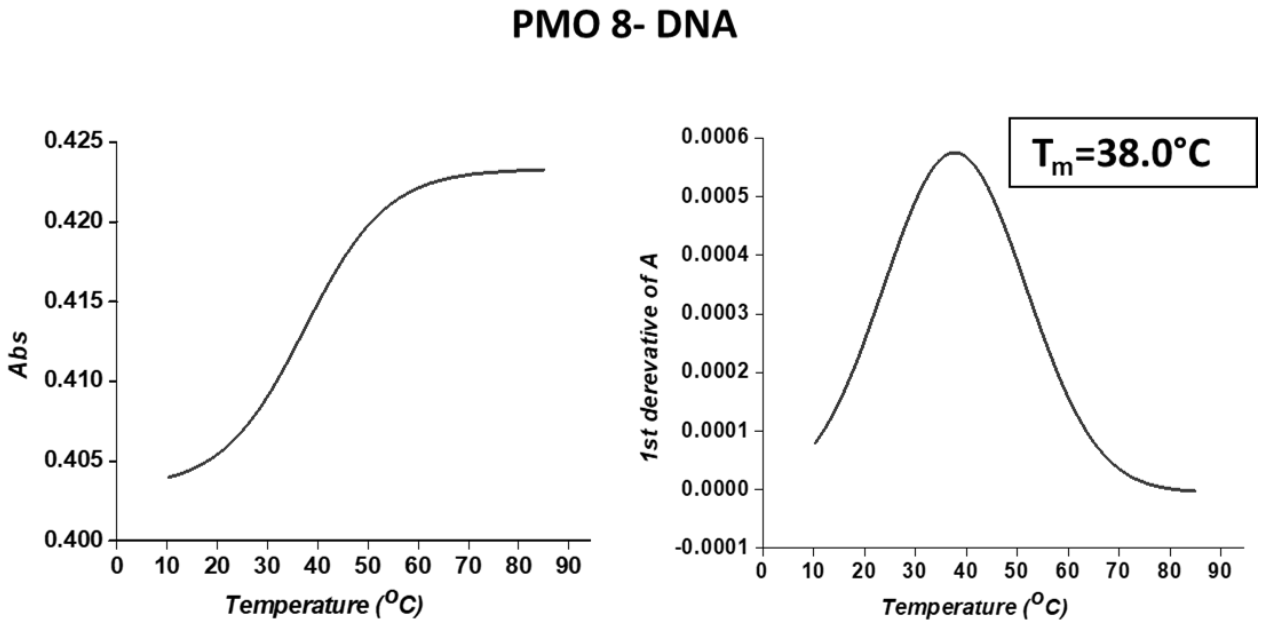


Fig S30: Thermal melting curves and first derivative plot of duplexes (A) PMO7-DNA (B) PMO7-RNA

PMO 8- 5´GTGTXXTGC 3´

A.



B.

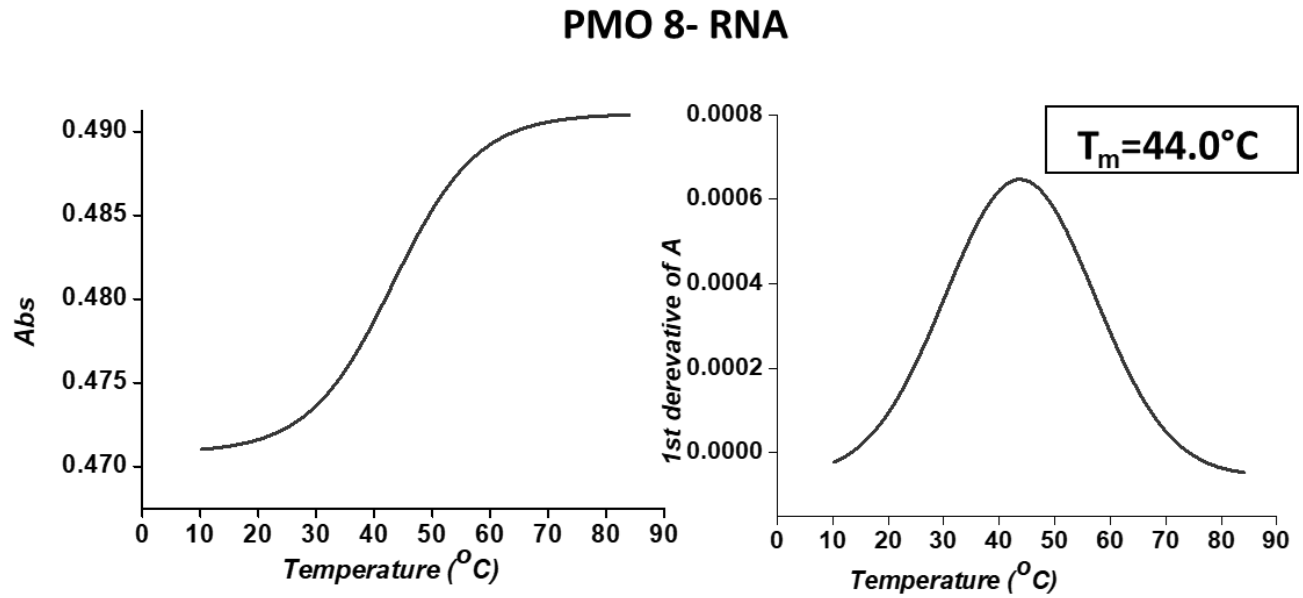
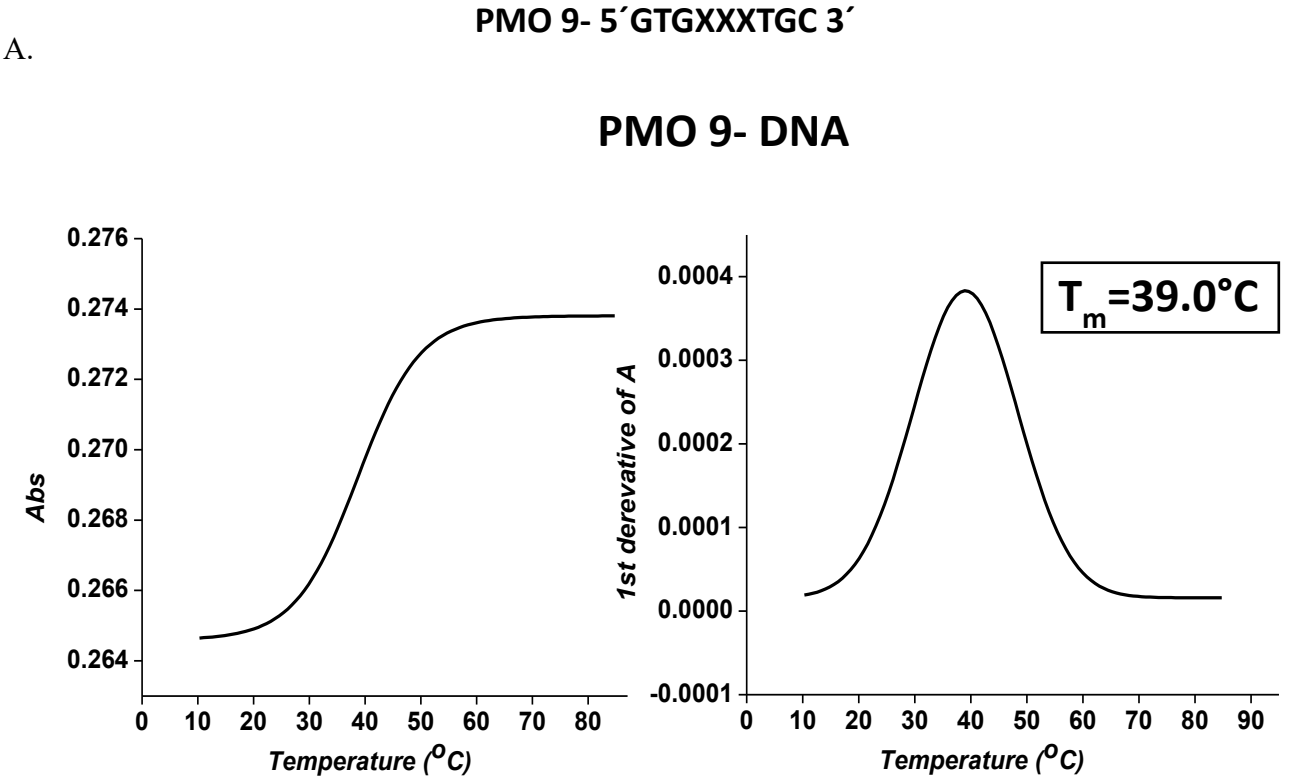


Fig S31: Thermal melting curve and first derivative plot of duplexes (A) PMO8-DNA (B) PMO8-RNA

A.



B.

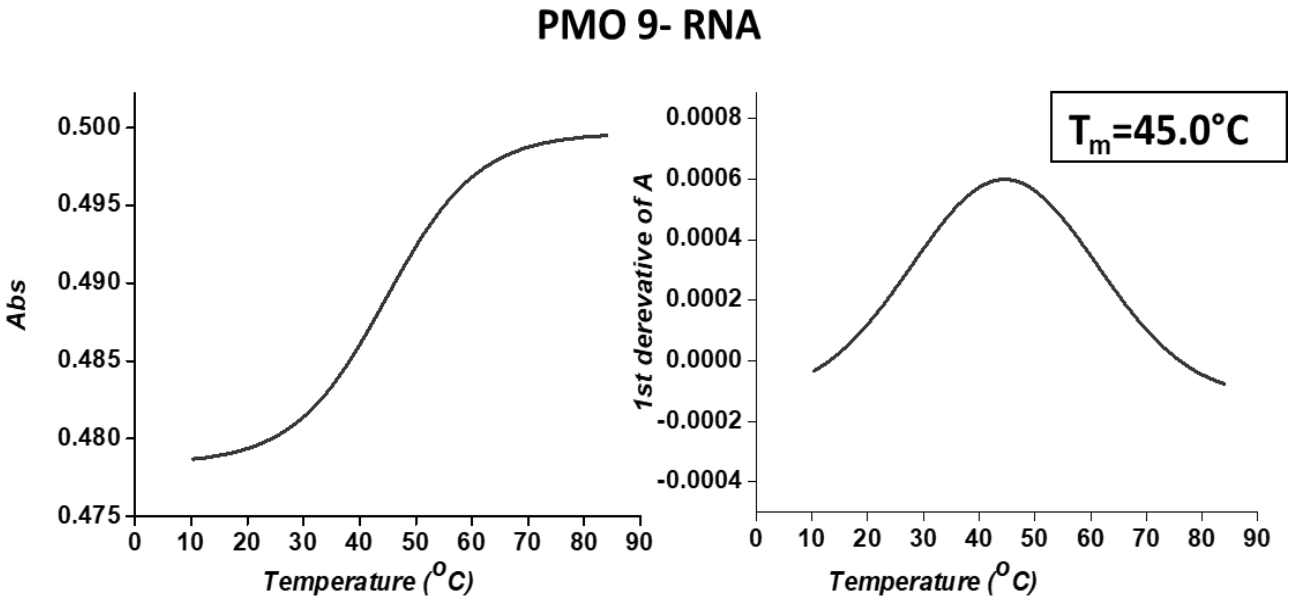
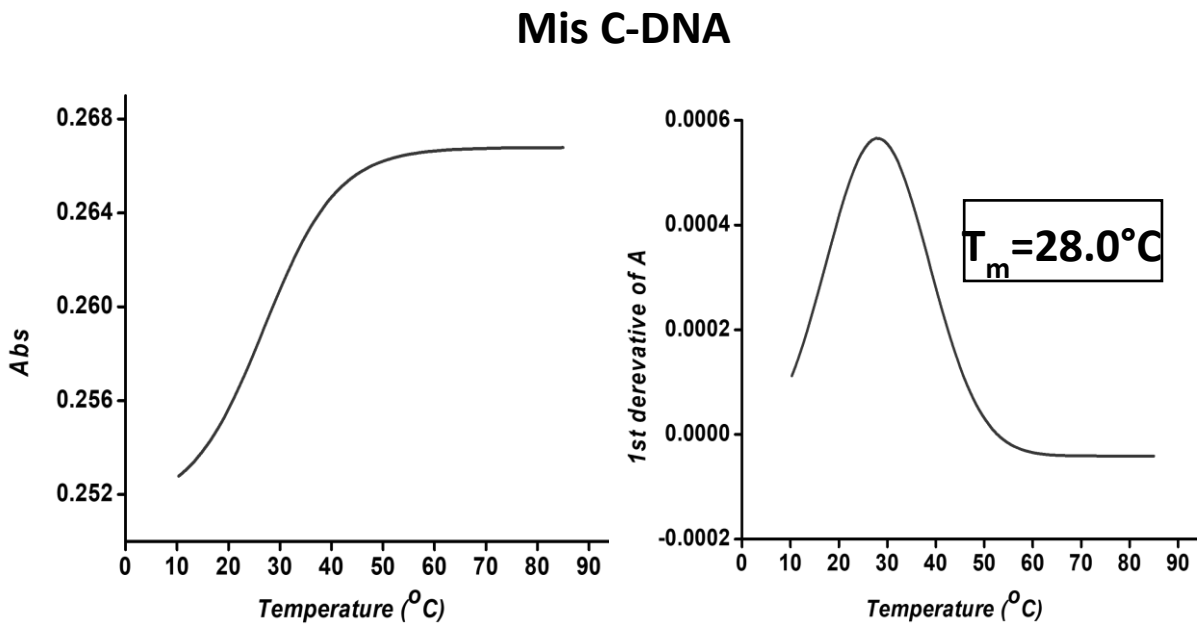


Fig S32: Thermal melting curve and first derivative plot of duplexes (A) PMO9-DNA (B) PMO9-RNA

Thermal melting studies of PMO 4 with single base mismatch sequence

A.

5´-GTGXXXTGC-3´
3´-CACAC*AACG-5´



B.

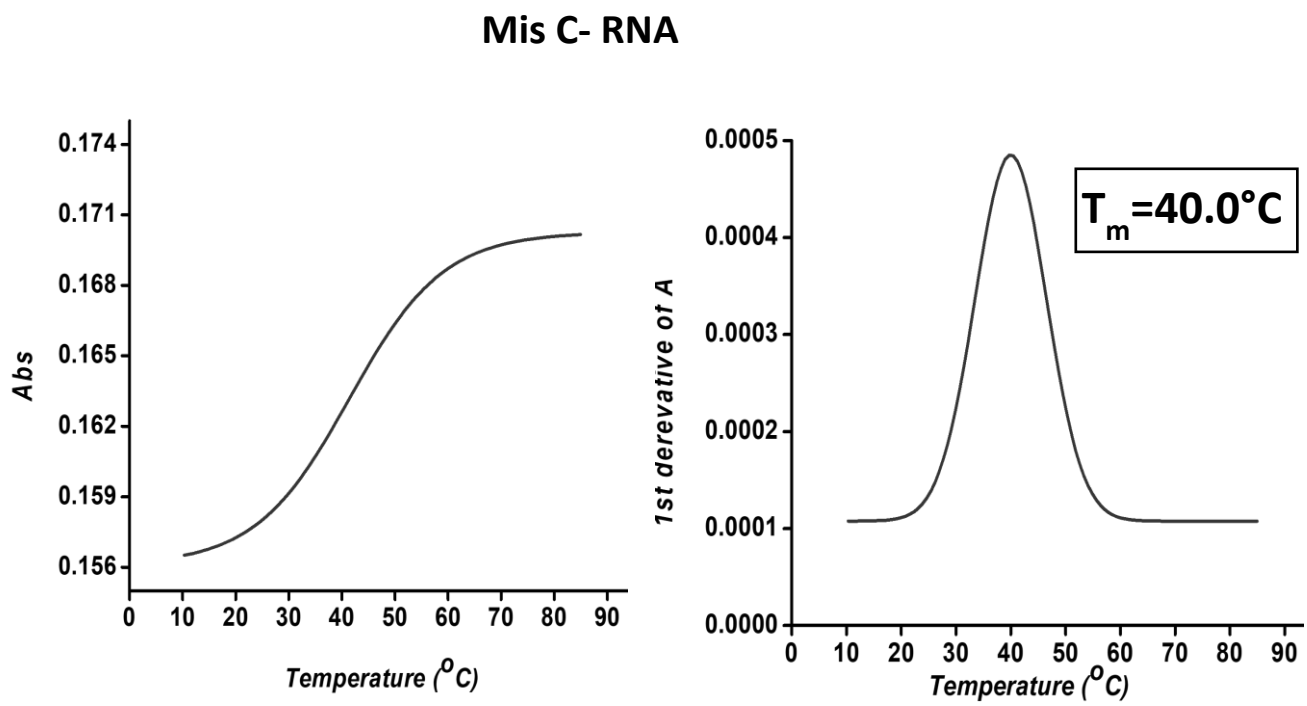
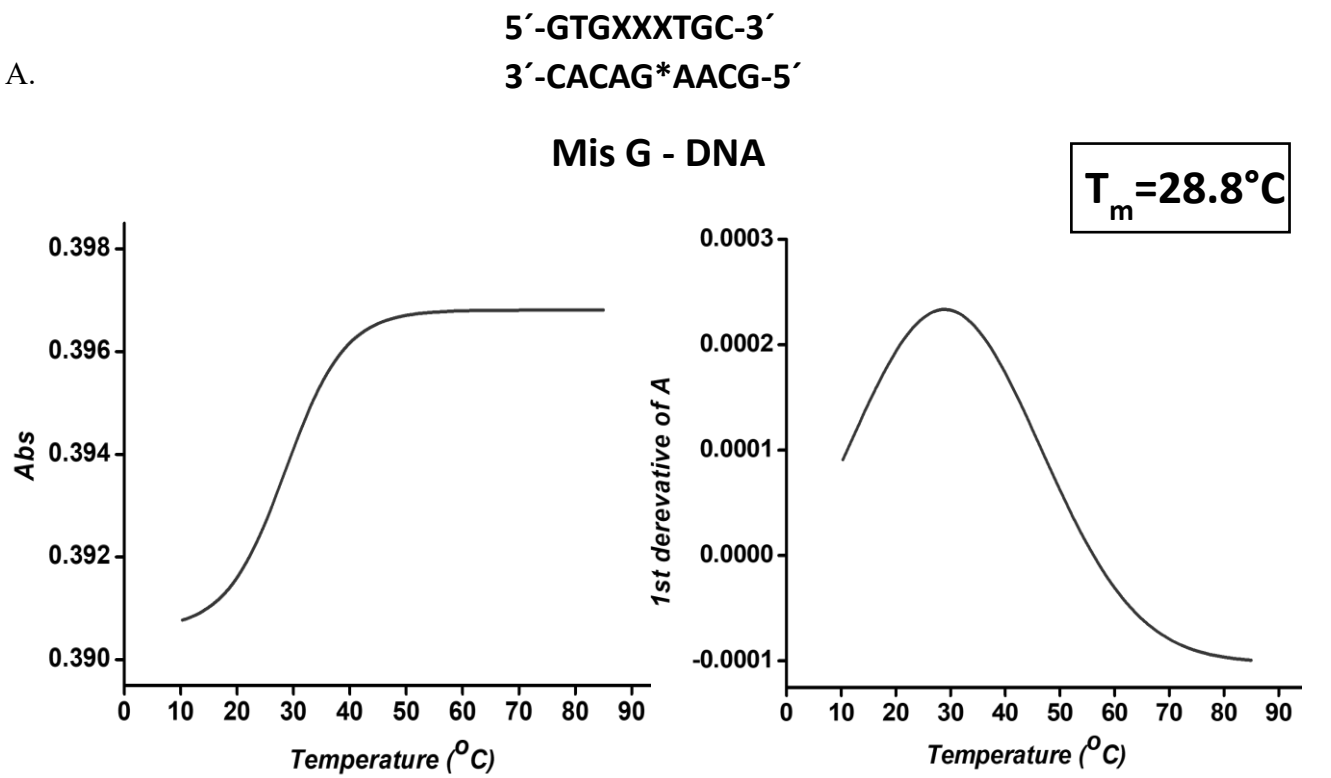


Fig S33: Thermal melting curve and first derivative plot of duplexes (A) PMO4- Mismatch C-DNA (B) PMO4- Mismatch C-RNA.

A.



B.

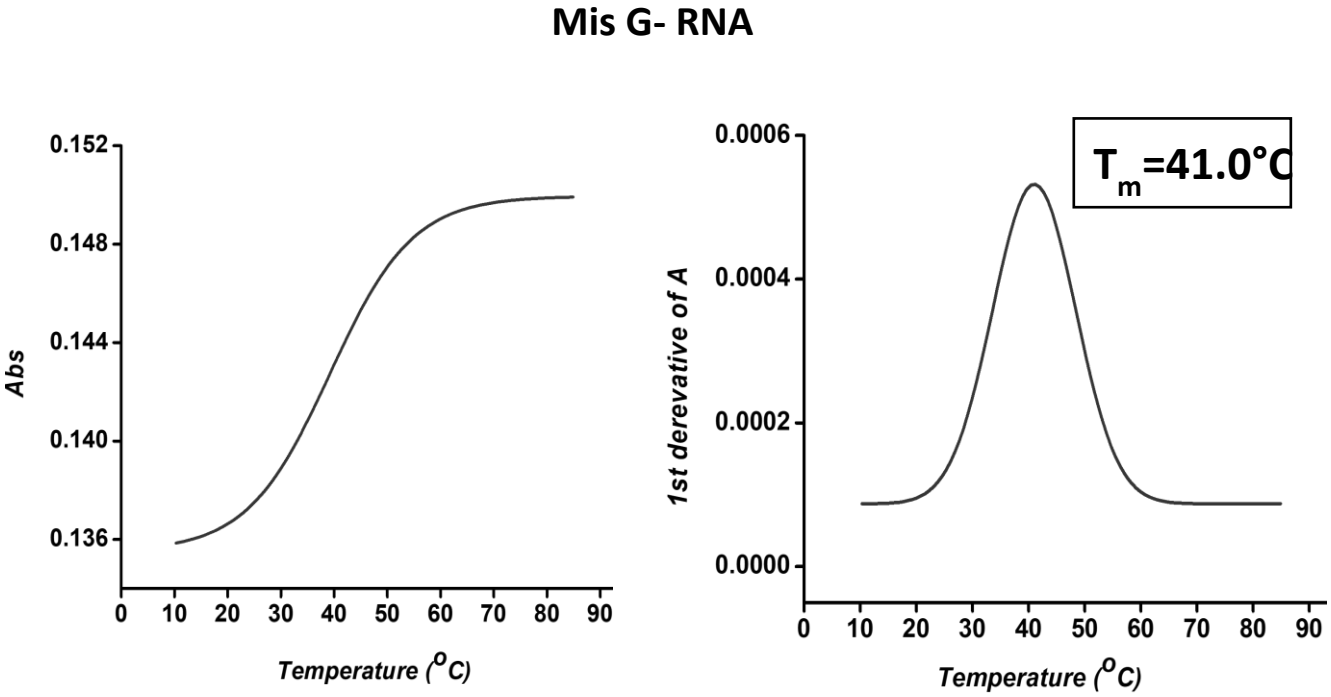
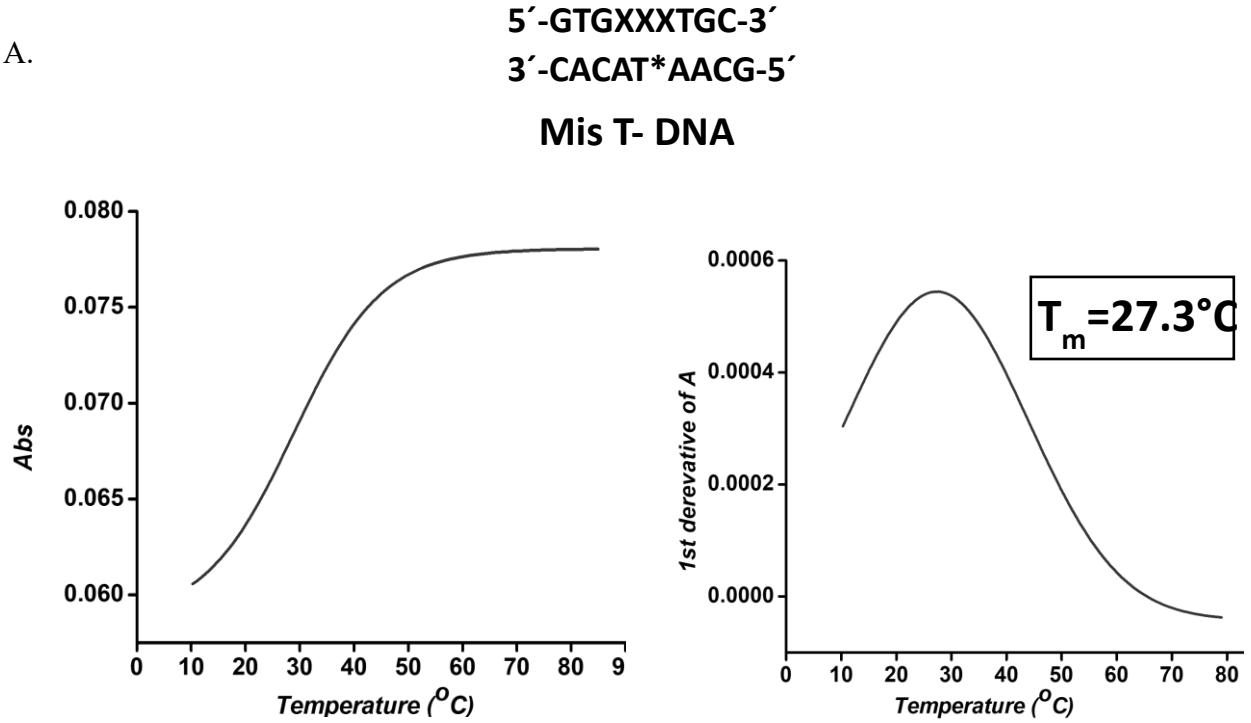


Fig S34: Thermal melting curve and first derivative plot of duplexes (A) PMO4- Mismatch G-DNA (B) PMO4- Mismatch G-RNA.

A.



B.

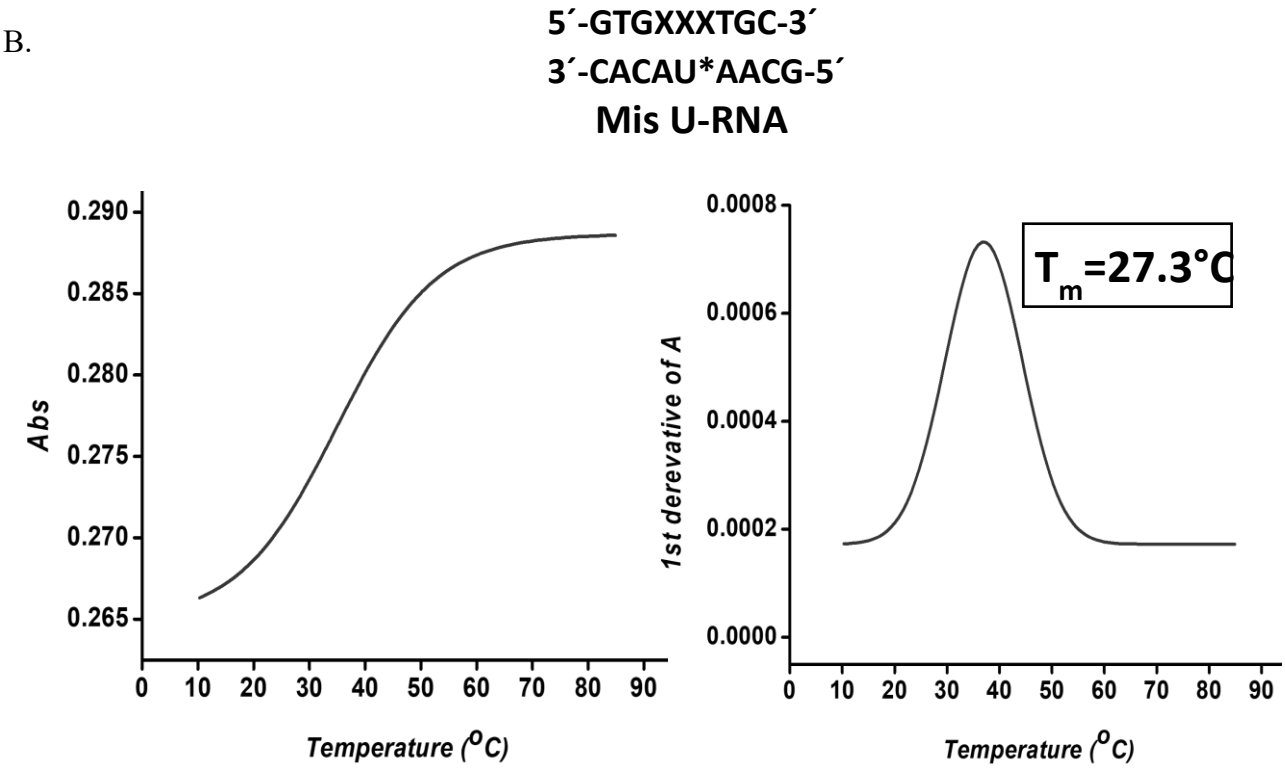


Fig S35: Thermal melting curve and first derivative plot of duplexes (A) PMO4- Mismatch T-DNA (B) PMO4- Mismatch U-RNA.

Thermal melting studies of PMO-1 with single base mismatch RNA sequence

5′-GTGTTTTGC-3′

3′-CACAG*AACG-5′

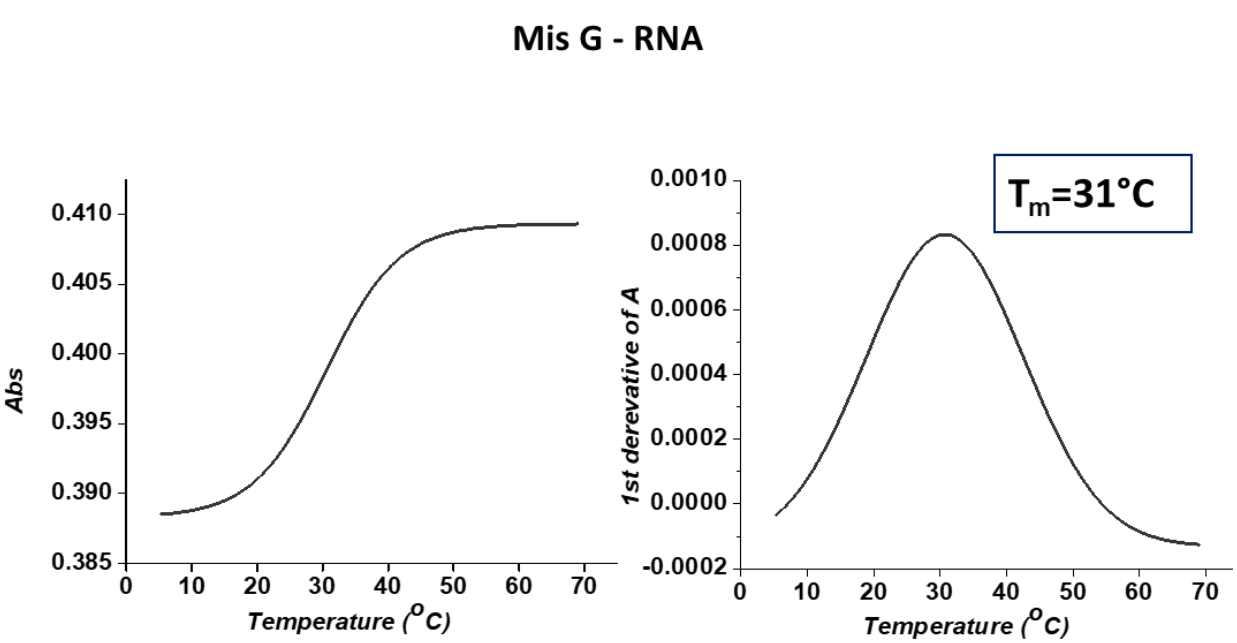


Fig S36: Thermal melting curve and first derivative plot of duplexes PMO 1- Mismatch G-RNA

5′-GTGTTTTGC-3′

3′-CACAC*AACG-5′

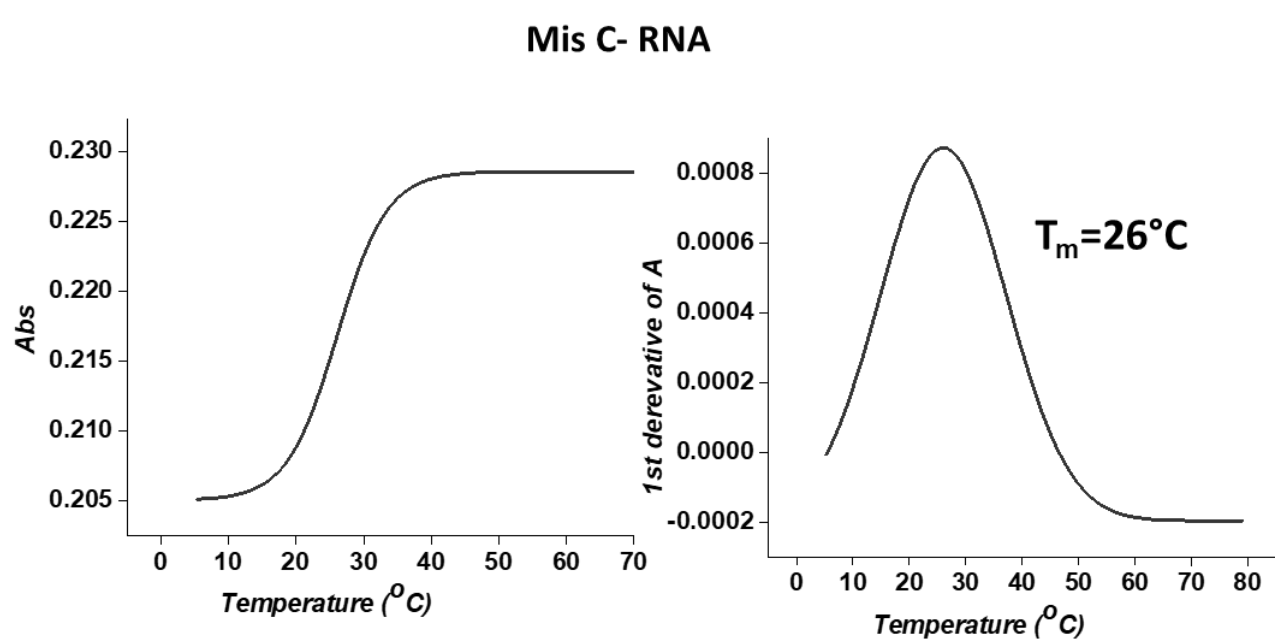


Fig S37: Thermal melting curve and first derivative plot of duplexes PMO 1- Mismatch C-RNA

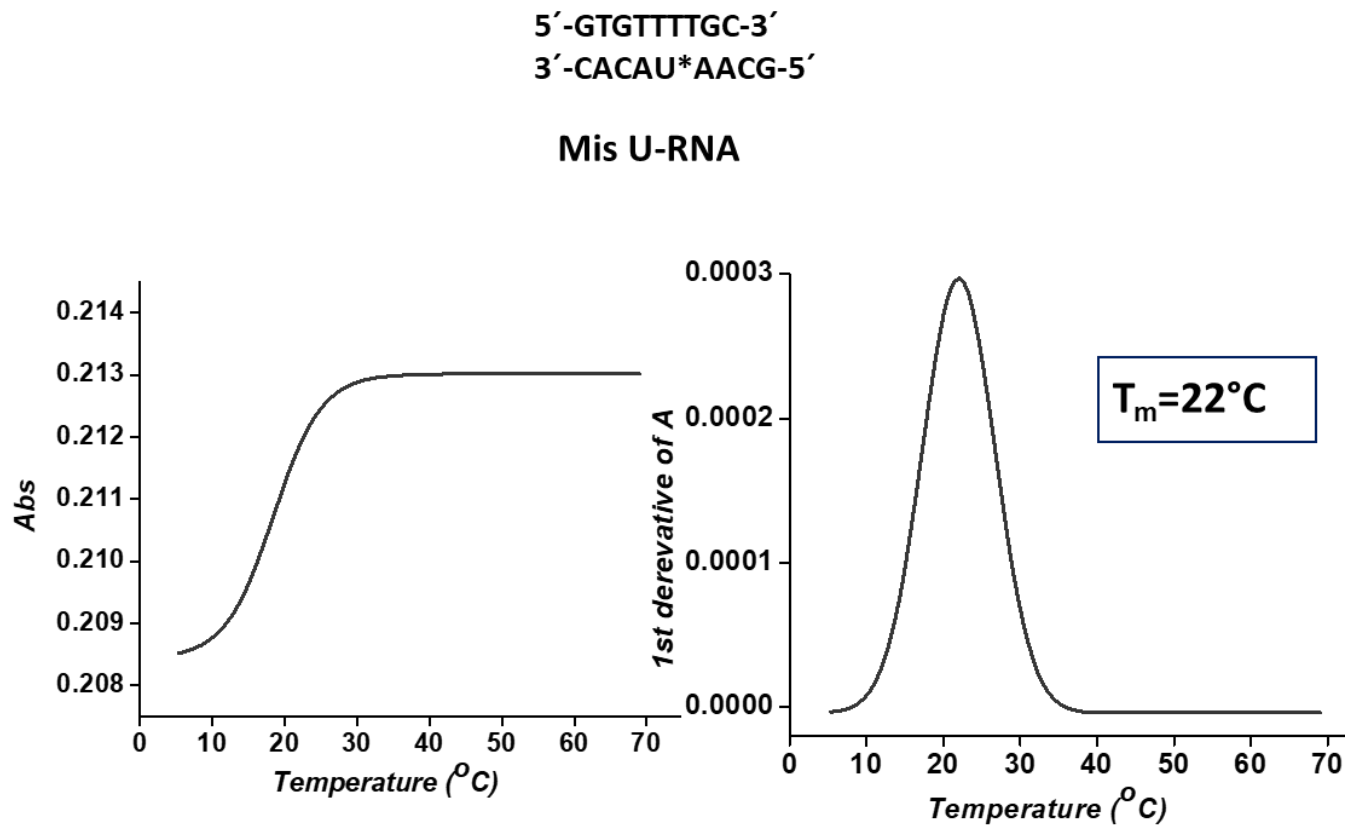


Fig S38: Thermal melting curve and first derivative plot of duplexes PMO 1- Mismatch U-RNA

References:

1. (a) J. Kundu, A. Ghosh, U. Ghosh, A. Das, D. Nagar, S. Pattanayak, A. Ghose and S. Sinha, *J. Org. Chem.*, 2022, **87**, 9466–9478. (b) A. Das, A. Ghosh and S. Sinha, *Org. Biomol. Chem.*, 2023, **21**, 1242–1253.