The ESI for *Org. Biomol. Chem.*, 2021, DOI: 10.1039/D1OB00863C was updated on 22 June 2021. Some incorrect structures shown in the L-configuration rather than the correct D-configuration have been replaced.

Polymerase-mediated synthesis of *p*-vinylaniline–coupled fluorescent DNA for the sensing of nucleolin protein–*c-myc* G-quadruplex interactions

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1. General information

All reagents were obtained from commercial sources and used without further purification. ^{1}H and ^{13}C NMR spectra were recorded using a Bruker AV-400 spectrometer with $CDCl_3$ or DMSO-d_6 as the solvent and tetramethylsilane as the internal standard. UV–Vis spectra were recorded at room temperature using a Cary Series UV–Vis spectrophotometer (Agilent Technologies) and a quartz cuvette (path length: 1 cm); absorbance changes were measured immediately after UV irradiation of the sample solution in the cuvette. Fluorescence emission spectra were recorded at room temperature using a PF–65000 spectrofluorometer. Confocal microscopy images were recorded with all-natural oligonucleotides purchased from Bioneer (Seoul, Republic of Korea). Deoxyribonucleotide triphosphates mixture 2 mM each (dNTPs), *nPfu-special* enzyme, and buffer were purchased from Enzynomics (Bioneer, Republic of Korea). Recombinant Human Nucleolin Protein was purchased from Prospec Protein specialist, Israel.

2. Gel electrophoresis

Native polyacrylamide gel electrophoresis (nPAGE, 18%) was adopted to characterize the DNA products. The reaction mixture (10 mL) was mixed with 6x loading buffer (2.5 mL) and loaded into the well. Gel electrophoresis was performed in 1x TBE buffer (89 mM Tris, 89 mM borate, 2 mM EDTA, pH 8.3) at a constant potential of 95/80 V for 90–180 min and a current of 3 mA, followed by scanning on a gel image system. Similarly, RCA products (15 μL) were mixed with the loading buffer (6×, 2.5 μL) and run with 2% agarose gel for 40/60 min (100/50 V) in 1x TBE buffer. The gel was stained with ethidium bromide (EB) and then photographed using a ChemiDoc MP imaging system (Bio-Rad).

3. Primer extension reactions and lambda exonuclease cleavage to single-strand DNA (dU_{CN2}DNA and dU_{Py}DNA)

The template (0.3 μM) and primer (0.3 μM) were annealed at 95 °C and then left to cool to room temperature, thereby forming duplexes, and then mixed with dNTPs (2 mM each; dTTP was replaced by **dU_{CN2}TP** and **dU_{Py}TP** for the syntheses of **dU_{CN2}DNA** and **dU_{Py}DNA**, respectively) and *nPfu-special* enzyme (2U) and incubated at 37 °C for 16 h. The obtained fraction was heated at 65 °C (2 min) for deactivation of the DNA polymerase; lambda exonuclease (2U) was added and then the fraction was incubated at 37 °C for 1 h. The obtained sample was purified using a Qiagen quick oligonucleotide purification kit to obtain the single-stranded DNA (**dU_{CN2}DNA** or **dU_{Py}DNA**).

Primer 1	5´-CTC ACT ATA GGG AGC-3´
Template 1	5´[Phosphate]-CGC TAG GAC GGT ACT GGA TCA GCT CCC TAT AGT GAG-3´
Primer 2	5' – TCC AAC TAT GTA TAC – 3'
Template 2	5'[Phosphate] – TAA TAC GAC TCA CTA TAG CAA TTG CGT GCC GCT AGC CTT CCC CAC CCT CCC CAC CCT CCC CAG TAT ACA TAG TTG GA -3'
Primer 3	5' – TAA TAC GAC TCA CTA TAG CAA TTG CGT G – 3'
Templet 3	5'-TCC AAC TAT GTA TAC TGG GGA GGG TGG GGA GGG TGG GGA AGG CTA GCG GCA CGC AAT TGC TAT AGT GAG TCG TAT TA-3'
dU$_{Py}$*c-myc*DNA	5'-TCC AAC TAT GTA TAC dU$_{Py}$GG GGA GGG dU$_{Py}$GG GGA GGG dU$_{Py}$GG GGA AGG CTA GCG GCA CGC AAT TGC TAT AGT GAG TCG TAT TA-3'

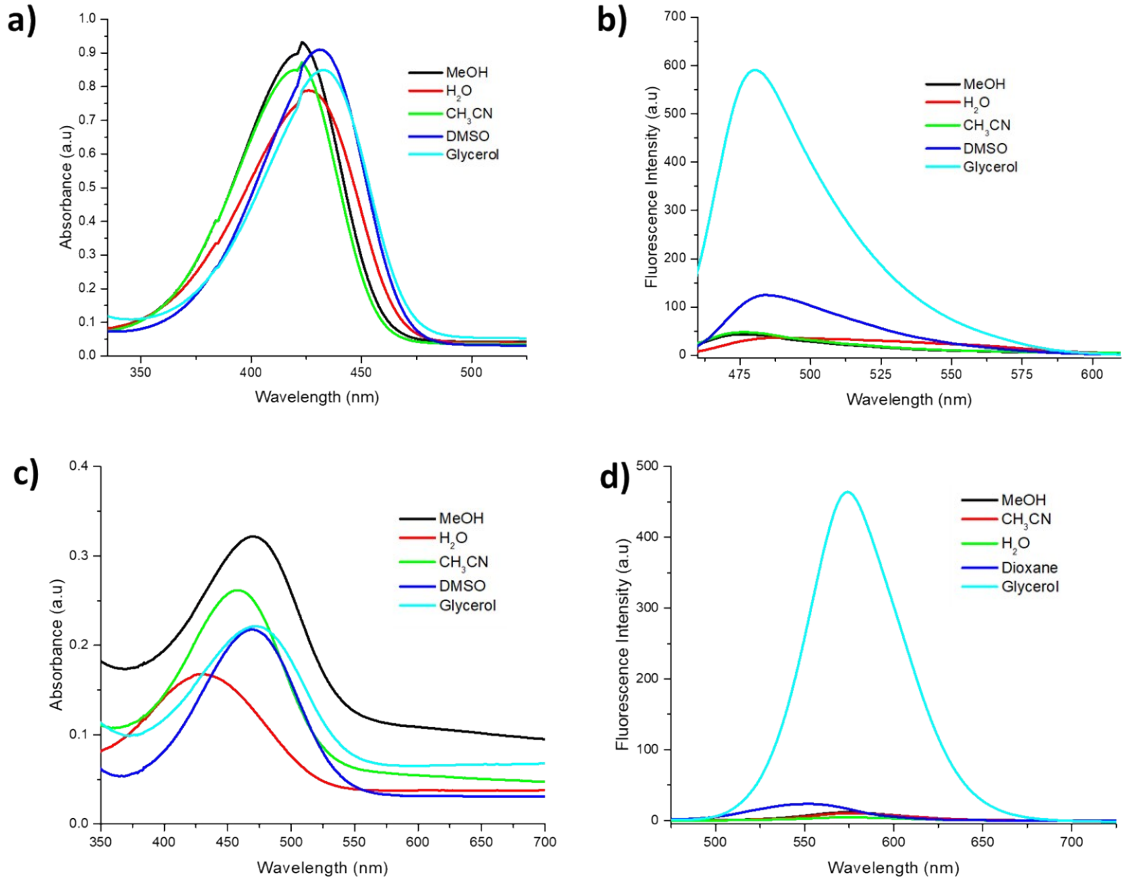


Figure S1: Photophysical properties of the nucleosides. UV–Vis and fluorescence spectra of a, b) $\mathbf{dU_{CN2}}$ and c, d) $\mathbf{dU_{Py}}$ in various solvents (16 μM).

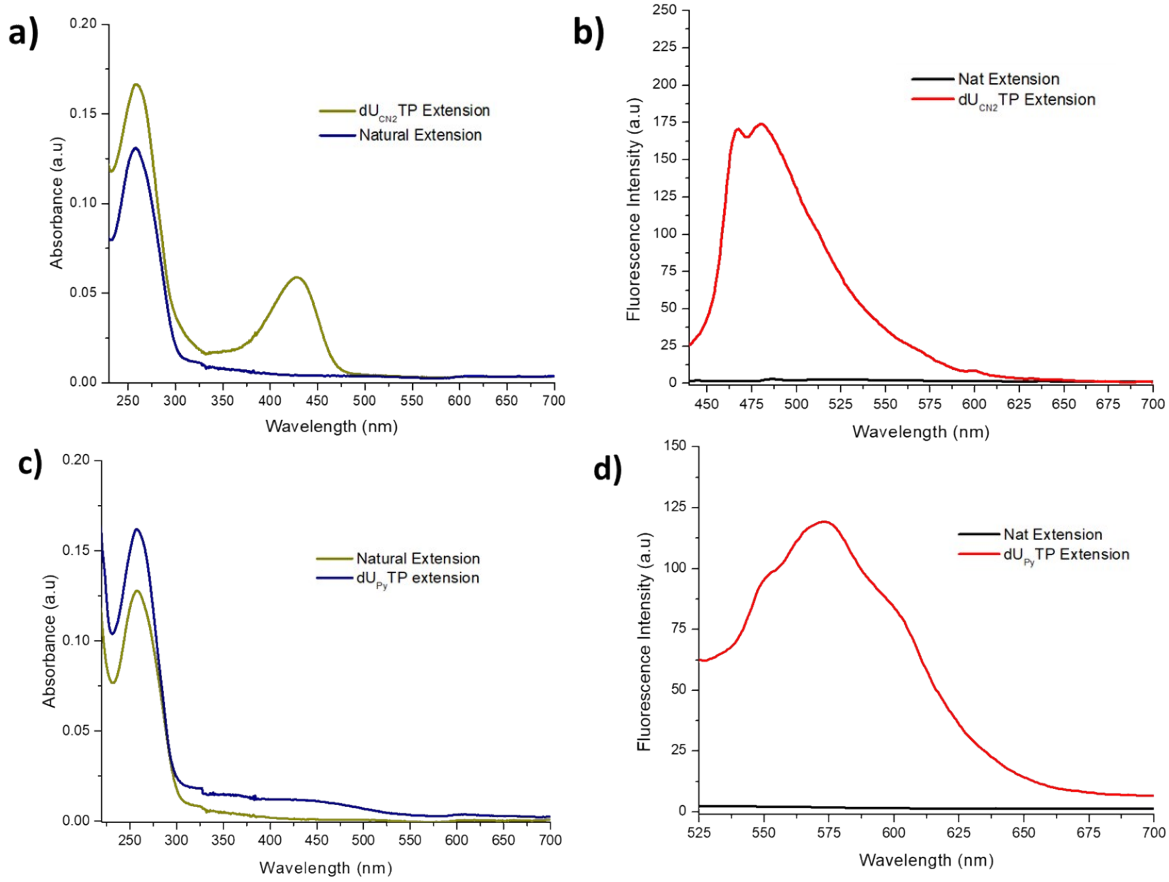


Figure S2: a, c) UV–Vis and b, d) fluorescence spectra of the primer extension products from **dU$_{CN2}$TP** and **dU$_{Py}$TP**. The obtained products were purified through an oligonucleotide purification kit (Qiagen).

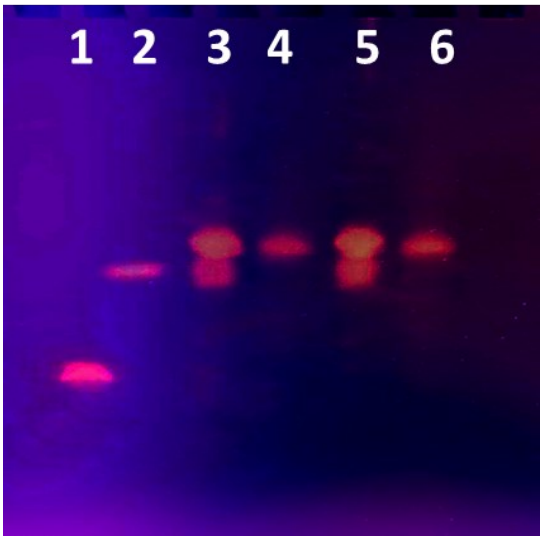
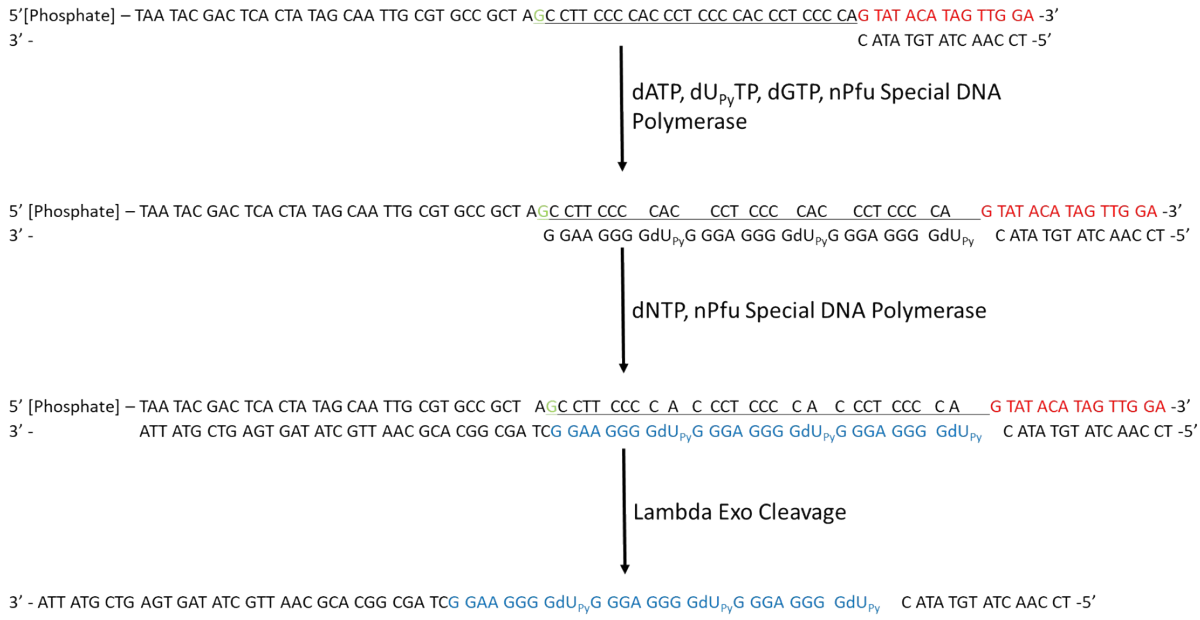


Figure S3: PAGE analysis of primer extension and lambda exonuclease cleavage of **$dU_{CN2}TP$** and **$dU_{Py}TP$**. Lane 1: **Primer 1**; lane 2: **Template 1**; lane 3: primer extension with **$dU_{CN2}TP$**; lane 4: primer extension with **$dU_{CN2}TP$** and then lambda exonuclease; lane 5: primer extension with **$dU_{Py}TP$**; lane 6: primer extension with **$dU_{Py}TP$** and then lambda exonuclease.

4. Stepwise approach to synthesize the dU_{Py}*c-myc*DNA



The template 2 (0.3 μM) and primer 2 (0.3 μM) were annealed at 95 °C and then left to cool to room temperature, thereby forming duplexes, and then mixed with dGTP, dATP and dTTP (2 mM each; dTTP

was replaced by **dU$_{Py}$TP** for the syntheses of **dU$_{Py}$*c-myc*DNA**) and *nPfu-special* enzyme (2U) and incubated at 37 °C for 16 h. The obtained sample was purified using a Qiagen quick oligonucleotide purification kit, again then mixed with dNTPs (2 mM each) and *nPfu-special* enzyme (2U) and incubated at 37 °C for 1h. The obtained fraction was heated at 65 °C (2 min) for deactivation of the DNA polymerase; lambda exonuclease (2U) was added and then the fraction was incubated at 37 °C for 1 h. The obtained sample was purified using a Qiagen quick oligonucleotide purification kit to obtain the **dU$_{Py}$*c-myc*DNA**.

5. Binding study using SSB protein

SSB protein from *E. coli* was purchased from Enzynomics (Bioneer, Republic of Korea). The single-stranded DNA (1 μM) was mixed with 0.5 equivalents of SSB protein (0.5 μM) in a 1-mL quartz cuvette at room temperature in 50 mM phosphate buffer (pH 7.4). Three parallel samples were prepared for exact comparison (ssDNA mixed with SSB protein, ssDNA diluted with phosphate buffer, and ssDNA mixed with BSA protein).

6. Protein–DNA interactions of single-stranded dU$_{CN2}$DNA and dU$_{Py}$DNA

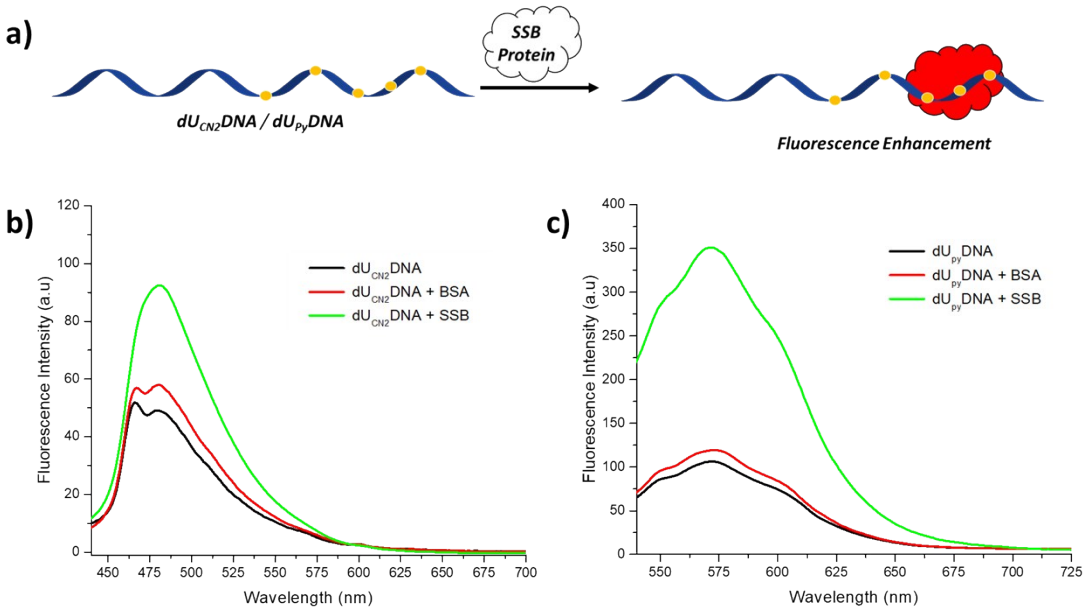


Figure S4. a) Schematic representation of FMR-containing oligonucleotides binding with SSB protein. b, c) Fluorescence spectra of b) **dU$_{CN2}$DNA** (1 μM) and c) **dU$_{Py}$DNA** (1 μM) in the absence and presence of SSB protein (0.5 μM) with excitation at 425 and 474 nm, respectively (see the Supporting Information for details).

7. ^{32}P Labeling experiment for testing primer extension efficiency

Primer 1 (0.2 μM, 2 μL), T4-PNK buffer (1x), ^{32}P-ATP (2 mM), dd H_2O, and T4-PNK enzyme (3U) were incubated at 37 °C for 1 h. The sample was purified using a QI-quick oligonucleotide purification kit and finally eluted with elution buffer (200 μL) to obtain the stock of ^{32}P-primer 1. The ^{32}P-primer 1–template (0.2 μM, 2 μL) duplex was annealed at 95 °C and then slowly cooled to room temperature.

8. ^{32}P Labeling experiment for monitoring the Nucleolin acitivity

Primer 3 (0.2 μM, 2 μL), T4-PNK buffer (1x), ^{32}P-ATP (2 mM), dd H_2O, and T4-PNK enzyme (3U) were incubated at 37 °C for 1 h. The sample was purified using a QI-quick oligonucleotide purification kit and finally eluted with elution buffer (200 μL) to obtain the stock of ^{32}P- primer 3 The ^{32}P-primer 3–template (0.2 μM, 2 μL) duplex was annealed at 95 °C and then slowly cooled to room temperature.

9. Binding study using Nucleolin protein

Nucleolin protein Human Recombinant was purchased from Prospec Protein Specialists (Isreal). The **dU_{Py}*c-myc*DNA** (0.3 μM) was annealed with the primer3 (0.3 μM) at 95^{o}C to get ***A*** to this 25mM KCl was added and mixed with 0.5μg of Nucleolin protein in a 1-mL quartz cuvette at room temperature in 50 mM phosphate buffer (pH 7.4). Four parallel samples were prepared for exact comparison (Only ***A***, ***A*** with 25 mM KCl (***C***), ***A*** with 25 mM KCl and Nucleolin (***E***), and only Nucleolin).

The annealed duplex (***A***), duplex with 25 mM KCl (***C***), duplex with 25 mM KCl and 0.5 μg of Nucleolin (***E***) were all subjected to primer extension with 2mM dNTPs and nPfU special enzyme (2U) at 37^{o}C for 60 min to obtain their corresponding stop and extended products (***B***, ***D***, ***F***). Finally to the stop product form duplex with 25 mM KCl and 0.5 μg of Nucleolin (***F***) was added with 1 μM of c-myc (pu27) in 25 mM KCl at 37^{o}C for 10 minutes to create competition for Nucleolin binding.

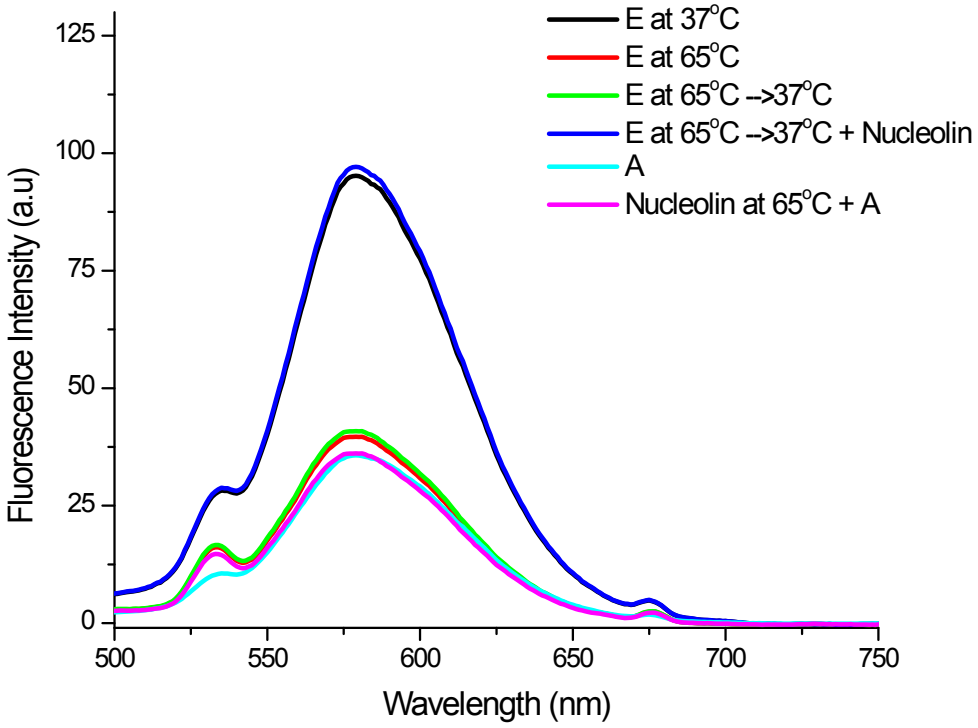


Figure S5. d) Fluorescence spectra of **dU$_{Py}$*c-myc*DNA** in the absence and presence of Nucleolin at various temperature conditions. The fluorescence of the duplex with with 25 mM KCl and Nucleolin (***E***) incubated at 37°C and at 65°C for 10 minutes were measures (black and red); the red line was further cooled to 37°C (green), to the green 0.5 μg of Nucleolin was added; The Nucleolin was incubated at 65°C for 10 minutes and then the duplex of **dU$_{Py}$*c-myc*DNA:** primer3 was added (pink); Excitation wavelength: 474 nm.

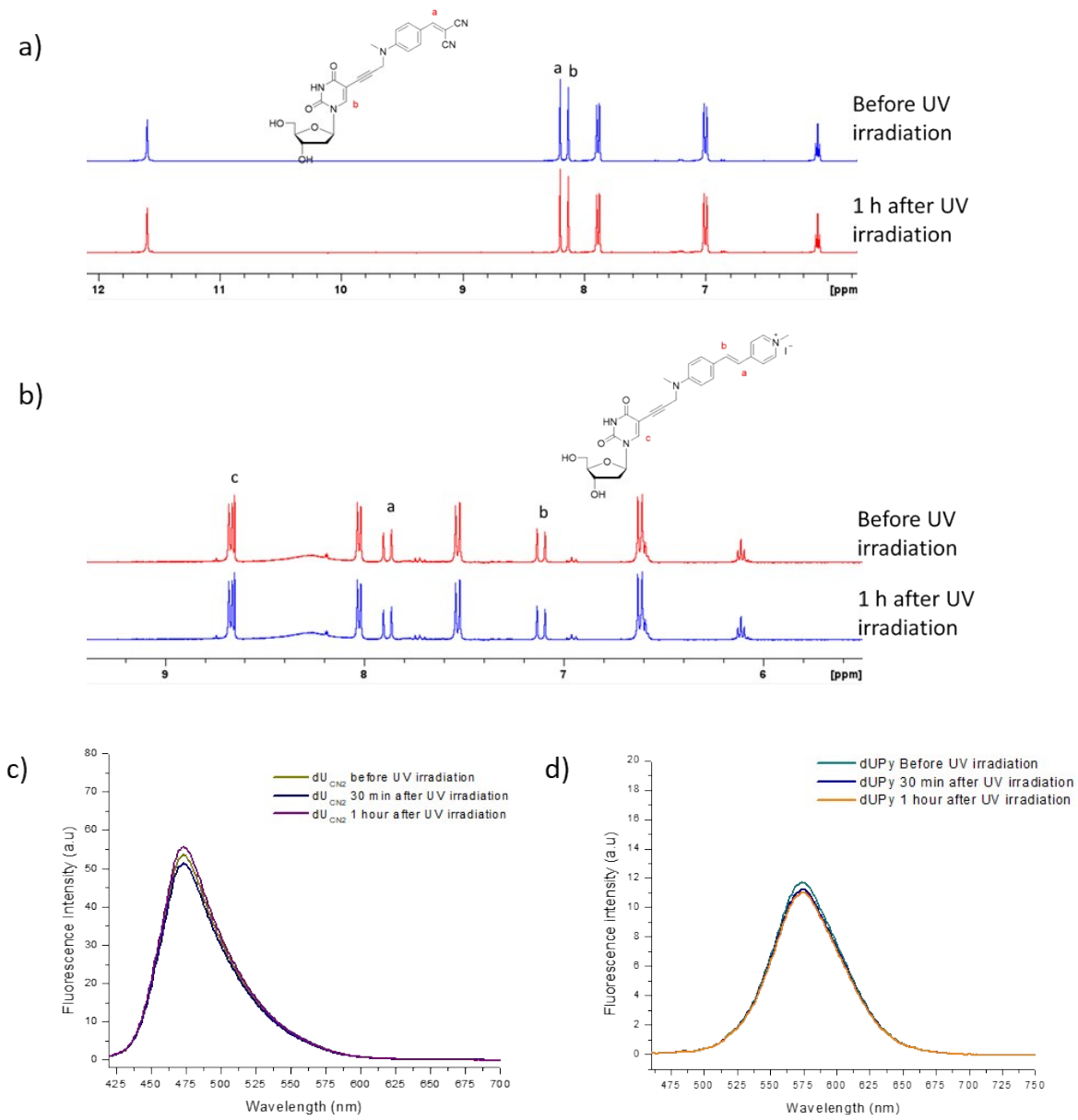


Figure S6: a) and b) 1H NMR; c) and d) Fluorescence spectrum of dUCN2 and dUPy,before and after long range UV (314 nm – 400 nm) irradiation using the Rayonet photochemical reactor to verify the photostability of the nucleosides.

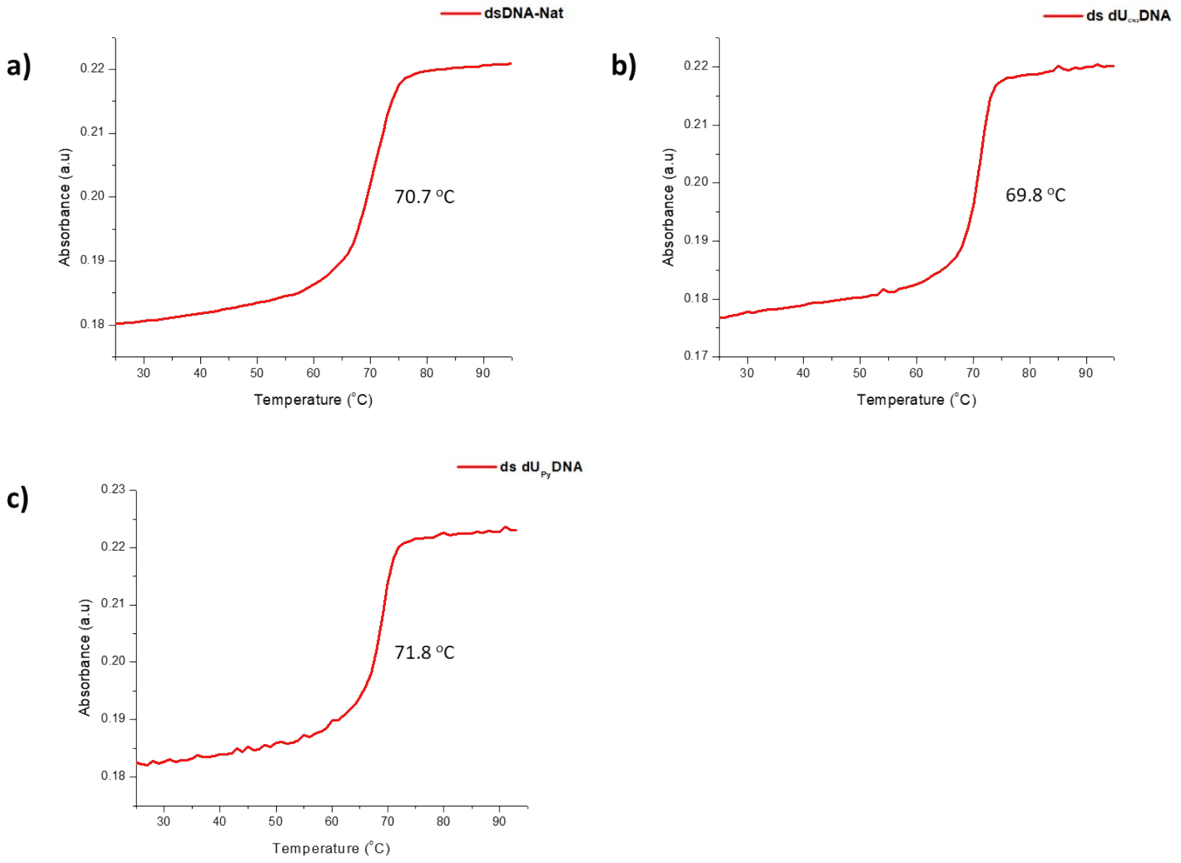


Figure S7: Melting point comparison of the Natural duplex with **dU$_{CN2}$** and **dU$_{Py}$** incorporated duplex DNAs.

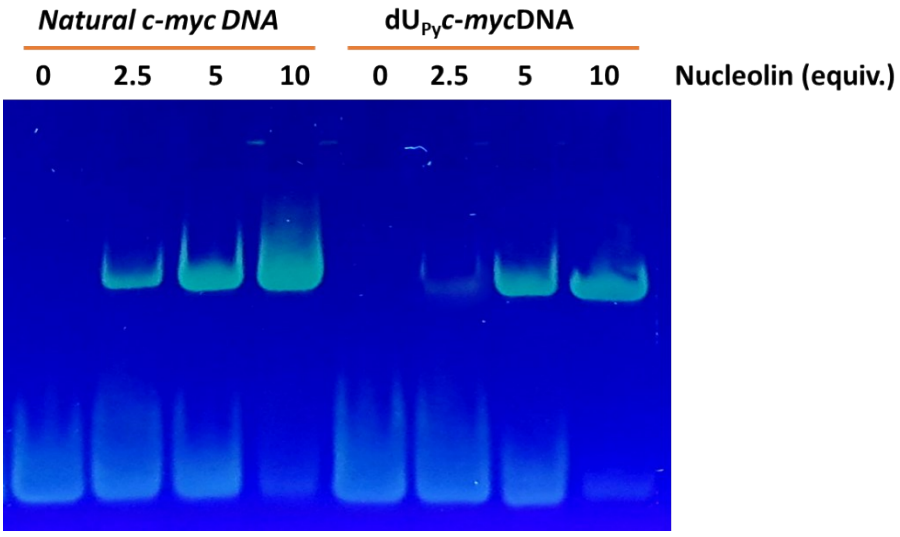
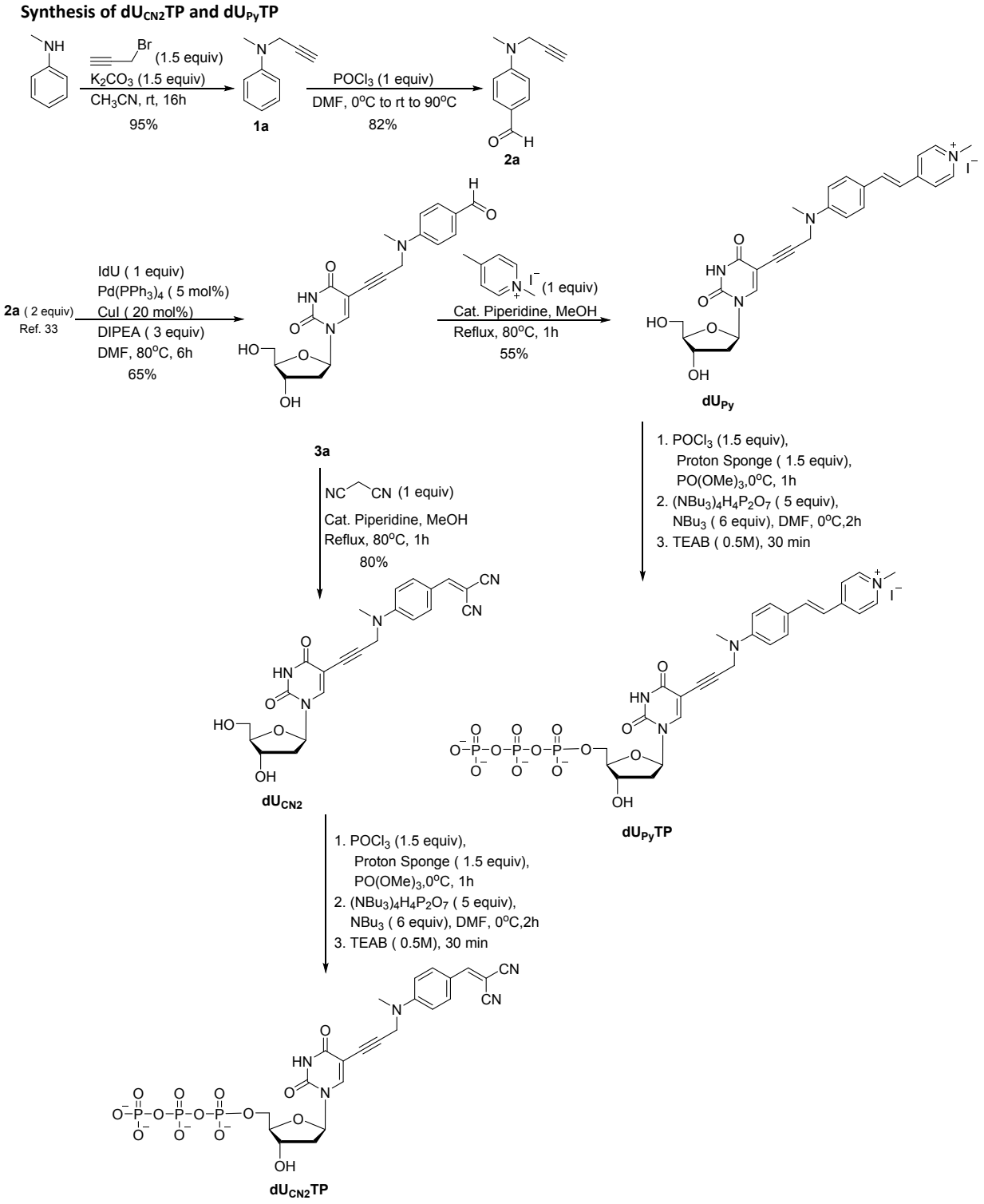


Figure S8: 5% nondenaturing EMSA PAGE after GreenStar™ (Bioneer) staining for Interaction of nucleolin with the *Natural c-myc DNA* and *dUpy c-myc DNA* (0.1 μM). Nucleolin concentration in the reactions ranged from 0 to 10 equivalent (0 to 1 μM).

10. Synthesis of $dU_{CN2}TP$ and $dU_{PY}TP$



Scheme S1. Synthesis of the FMRs $dU_{CN2}TP$ and $dU_{PY}TP$.

***N*-Methyl-*N*-(prop-2-yn-1-yl)aniline (1a).**[33] Propargyl bromide (80% in toluene, 2.54 mL, 22.8 mmol) was added dropwise at 0 °C over 10 min to a suspension of *N*-methylaniline (1.66 mL, 15.2 mmol) and K_2CO_3 (3.15 g, 22.8 mmol) in MeCN (30 mL). The mixture was stirred overnight at room temperature under a N_2 atmosphere. The solids were filtered off and the solution was evaporated to dryness under reduced pressure. The residue was purified through column chromatography (SiO_2; 5% EtOAc in hexane) to afford a pale-yellow oil (2.10 g, 95%). **^{1}H NMR (400 MHz, $CDCl_3$):** δ 7.29–7.25 (m, 2H), 6.88–6.80 (m, 3H), 4.05 (d, *J* = 2.3 Hz, 2H), 2.98 (s, 3H), 2.17 (t, *J* = 2.3 Hz, 1H); **^{13}C NMR ($CDCl_3$, 100 MHz):** δ 149.0, 129.2, 118.4, 114.3; 79.4, 72.1, 42.5, 38.6.

4-[*N*-Methyl-*N*-(prop-2-yn-1-yl) amino] benzaldehyde (2a).[33] Freshly distilled $POCl_3$ (1.25 mL, 11.0 mmol) was added dropwise to dry DMF under a N_2 atmosphere at 0 °C. The solution was stirred at same temperaturefor 30 minutes and then warmed to room temperature and stirred for an additional 20 min. The oily compound **1a** (1.60 g, 11.0 mmol) was added dropwise at 0 °C. The reaction mixture was stirred at room temperature for 1 h and then at 90 °C for 4 h. The mixture was neutralized with saturated $NaHCO_3$ while cooling in an ice bath. The resultant solid was filtered off and recrystallized from hexanes to yield **2a** (6.26 g, 82%). **^{1}H NMR (400 MHz, $CDCl_3$):** δ 9.76 (s, 1H), 7.77–7.74 (m, 2H), 6.82–6.80 (m, 2H), 4.12 (d, *J* = 2.3 Hz, 2H), 3.08 (s, 3H), 2.24 (t, *J* = 2.3 Hz, 1H); **^{13}C NMR ($CDCl_3$, 100 MHz):** δ 190.5, 153.2, 131.9, 126.5, 112.3, 78.3, 72.6, 41.8, 38.43.

4-((3-(1-(4-Hydroxy-5-(hydroxymethyl)tetrahydrofur-2-yl)-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)prop-2-yn-1-yl)(methyl)amino)benzaldehyde (3a). A solution of **2a** (0.783 g, 4.50 mmol), iododeoxyuridine (0.800 g, 2.25 mmol), $Pd(PPh_3)_4$ (0.130 g, 0.112 mmol), CuI (0.0860 g, 0.450 mmol), and DIPEA (0.736 mL, 4.50 mmol) in dry DMF (6 mL) was heated at 80 °C for 6 h. After cooling to room temperature, the mixture was evaporated to dryness and the residue purified through column chromatography (5% MeOH in CH_2Cl_2) to yield **3a** (0.584 g, 65%). **^{1}H NMR (400 MHz, $CDCl_3$):** δ 11.62 (s, 1H), 9.73 (s, 1H), 8.19 (s, 1H), 7.73 (d, *J* = 8.79 Hz, 2H), 6.95 (d, *J* = 8.79 Hz, 2H), 6.09 (t, *J* = 6.46 Hz, 1H), 5.25 (d, *J* = 4.26 Hz, 1H), 5.11 (t, *J* = 4.96 Hz, 1H), 4.49 (s, 2H), 4.24–4.20(m, 1H), 3.79 (q, *J* = 3.33 Hz, 1H), 3.61–3.56 (m, 2H), 3.09 (s, 3H), 2.13–2.10 (m, 2H). **^{13}C NMR ($CDCl_3$, 100 MHz):** δ (ppm) 190.71, 161.99, 153.65, 149.86, 144.39, 131.93, 126.15, 112.85, 98.31, 88.40, 88.06, 85.26, 76.73, 70.47, 61.33, 42.22, 38.42. HRMS (FAB): calcd for $C_{20}H_{21}N_3O_6$ [M^+] *m/z* 399.1430; found 399.1428.

2-(4-((3-(1-(4-Hydroxy-5-(hydroxymethyl)tetrahydrofur-2-yl)-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)prop-2-yn-1-yl)(methyl)amino)benzylidene)malononitrile (dU_{CN2}). Piperidine (1 drop) was added to a solution of **3a** (0.200 g, 0.500 mmol) and malononitrile (0.0330 g, 0.500 mmol) in dry MeOH and then the

mixture was heated under reflux for 1 h. After cooling to room temperature, the mixture was evaporated to dryness and the residue purified through column chromatography (3% MeOH in CH_2Cl_2) to yield **dU_{CN2}** (0.179 g, 80%). **^{1}H NMR (400 MHz, $CDCl_3$):** δ 11.62 (d, *J* = 6.99 Hz, 1H), 8.20 (d, *J* = 6.91 Hz, 1H), 8.15 (d, *J* = 6.89 Hz, 1H), 7.89 (d, *J* = 7.99 Hz, 2H), 7.01 (d, *J* = 8.23 Hz, 2H), 6.08 (q, J_1 = 6.69 Hz, J_2 = 7.0 Hz, 1H), 5.26–5.24 (m, 1H), 5.12–5.09 (m, 1H), 4.55 (d, *J* = 7.43 Hz, 2H), 4.22 (bs, 1H), 3.79–3.78 (m, 1H), 3.59–3.58 (m, 2H), 3.34 (d, *J* = 7.43 Hz, 2H), 3.15 (d, *J* = 8.11 Hz, 3H), 2.12–2.11 (m, 2H). **^{13}C NMR ($CDCl_3$, 100 MHz):** δ (ppm) 161.99, 159.79, 153.81, 149.85, 144.54, 133.92, 120.33, 116.42, 115.63, 113.30, 98.16, 88.06, 87.95, 85.28, 77.03, 71.04, 70.45, 61.32, 42.17, 38.38. HRMS (FAB): calcd for $C_{23}H_{21}N_5O_5$ [M^+] *m*/*z* 447.1543; found 447.1544.

(*E*)-4-(4-((3-(1-(4-Hydroxy-5-(hydroxymethyl)tetrahydrofur-2-yl)-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)prop-2-yn-1-yl)(methyl)amino)styryl)-1-methylpyridin-1-ium iodide (dU$_{Py}$). Piperidine (0.2 mL) was added to a solution of **3a** (0.200 g, 0.500 mmol) and N-methyl 4-methyl pyridinium iodide (0.117 g, 0.500 mmol) in dry MeOH and then the mixture was heated under reflux for 1 h. After cooling to room temperature, the mixture was evaporated to dryness and the residue purified through column chromatography (3% MeOH in CH_2Cl_2) to yield **dUPy** (0.169 g, 55%). **^{1}H NMR (400 MHz, $CDCl_3$):** δ 8.69–8.66 (m, 3H), 8.03 (d, *J* = 7.16 Hz, 2H), 7.87 (d, *J* = 16.04 Hz, 1H), 7.53 (d, *J* = 8.88 Hz, 2H), 7.10 (d, *J* = 16.04 Hz, 1H), 6.64–6.60 (m, 3H), 6.12 (t, *J* = 5.73 Hz, 1H), 5.30–5.29 (m, 1H), 5.07 (bs, 1H), 4.25–4.23 (m, 1H), 4.18 (s, 3H), 3.88–3.85 (m, 1H), 3.60–3.58 (m, 2H), 2.76 (d, *J* = 4.58 Hz, 3H), 3.24 (s, 1H), 2.23–2.13 (m, 2H). **^{13}C NMR ($CDCl_3$, 100 MHz):** δ (ppm) 193.75, 161.71, 153.92, 152.79, 150.17, 147.10, 144.73, 142.75, 130.92, 122.81, 122.42, 116.82, 112.50, 112.17, 88.37, 86.03, 70.70, 61.45, 46.77, 44.21, 30.69. HRMS (FAB): calcd for $C_{27}H_{29}N_4O_5I$ [M^+] *m*/*z* 489.2132; found 489.2135.

11. General procedure for the preparation of triphosphates

Proton sponge (1.5 equiv) and the free nucleoside (**dU_{CN2}** or **dU_{Py}**, 1 equiv) were dissolved in trimethylphosphate (0.3 M) and cooled to –20 °C. $POCl_3$ (1.5 equiv) was added dropwise and then the purple slurry was stirred at –20 °C for 2 h. Tributylamine (6.2 equiv) was added, followed by a solution of tributylammonium pyrophosphate (5.0 equiv) in DMF (0.5 M). After 5 min, the reaction was quenched through the addition of 0.5 M aqueous $Et_3NH_2CO_3$ (20 vol. equiv) and the resulting solution was lyophilized. Purification through reversed-phase (C18) HPLC (4–35% MeCN in 0.1 M $Et_3NH_2CO_3$, pH 7.5), followed by lyophilization, afforded the triphosphate as a solid.

dU_{CN2}TP:

^{31}P NMR (400 MHz, D_2O): δ –24.17 to –23.48 (m, β-P), –12.7 to –11.28 (m, α-P), –11.28 to –10.96 (m, γ-P).

MS (**LC/MS**, LRMS): calcd for $C_{23}H_{22}N_5O_{14}P_3^{2-}$ ([M]): *m/z* 685.0242; found: 685.3575.

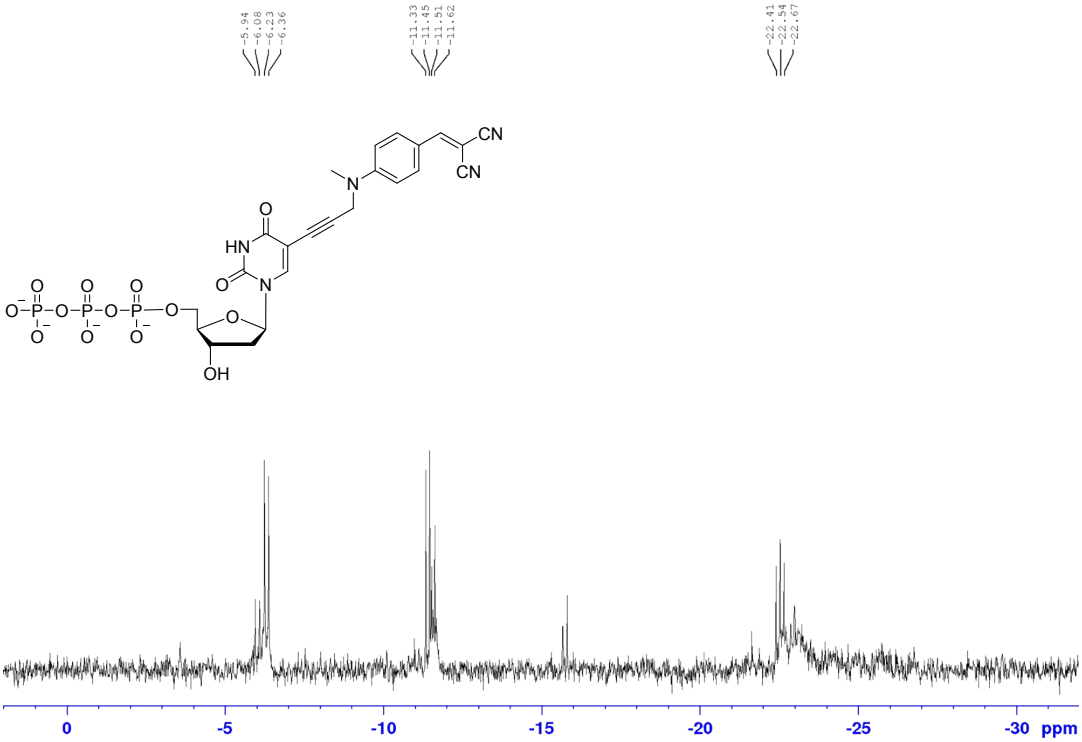


Figure S9: ^{31}P NMR spectrum of **$dU_{CN2}TP$**.

$dU_{Py}TP$:

^{31}P NMR (400 MHz, D_2O): δ –22.13 to –22.00 (m, β-P), –11.14 to –10.73 (m, α-P), –6.74 to –5.98 (m, γ-P).

MS (**LC/MS**, LRMS): calcd for $C_{27}H_{30}N_4O_{14}P_3^{2-}I^-$ ([M]): *m/z* 727.0831; found: 727.4579.

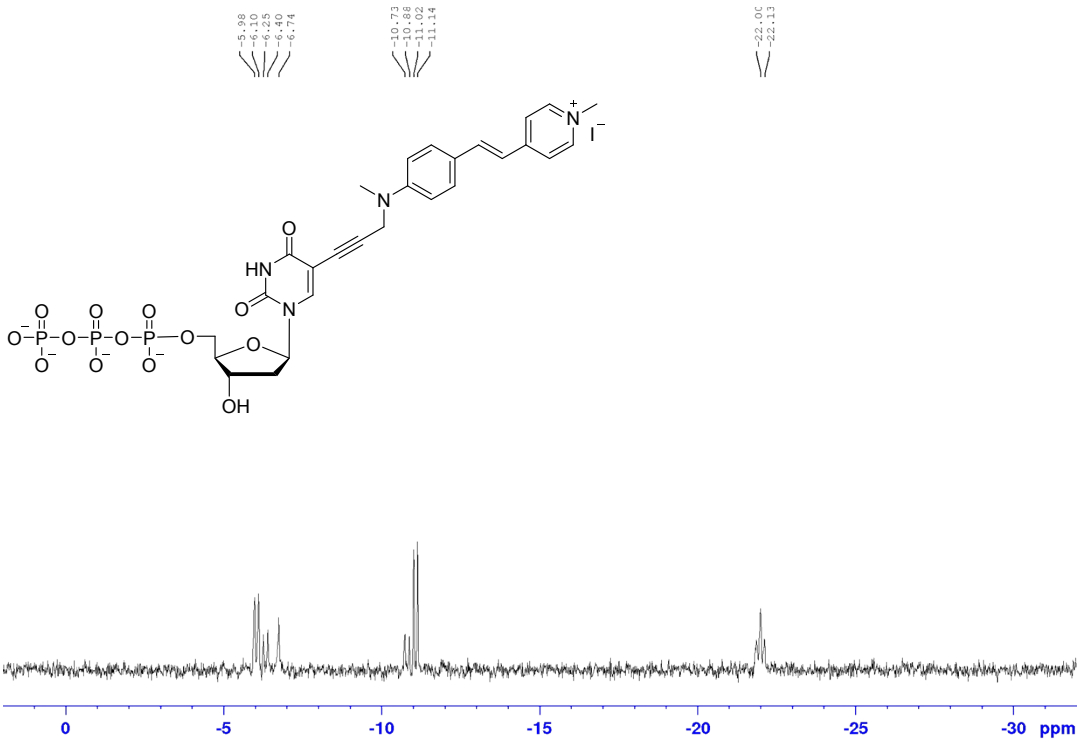


Figure S10: ^{31}P NMR Spectrum of **dU$_{Py}$TP**.

12. Photophysical properties of functional nucleotides

$$A = \varepsilon c l$$

– A is the absorbance of the compounds measured at the wavelength maximum (λ_{max}).

– ε is the extinction coefficient [M^{-1} cm^{-1}].

– c is the concentration [M].

– l is the path length of the cuvette [cm]

Compound	Absorbance at λ_{max}	Concentration (M)	Path length of cuvette (cm)	λ_{max} (nm)	Extinction coefficient (ε) (M^{-1} cm^{-1})
dU$_{CN2}$	1.0219	10×10^{-6}	1	423	102,190
dU$_{Py}$	0.3216	10×10^{-6}	1	469	32,160

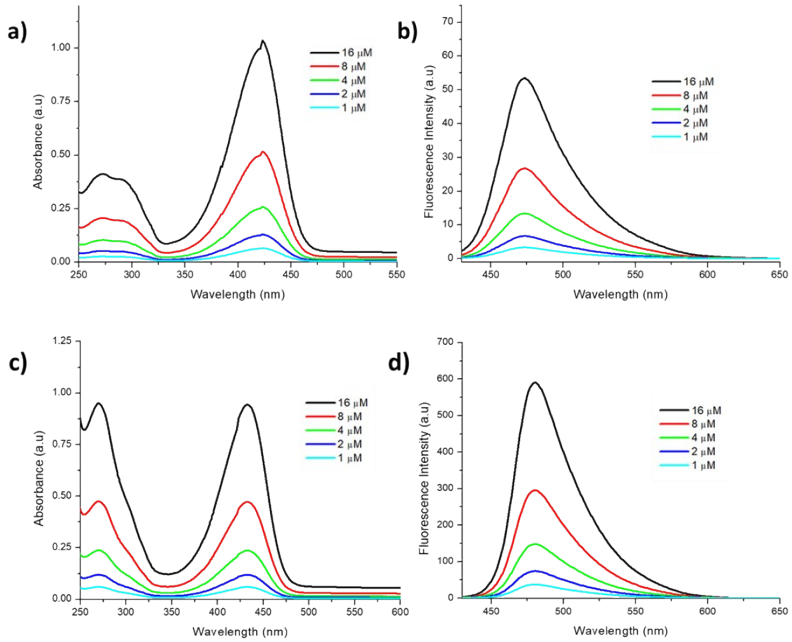


Figure S11: a, c) UV–Vis and b, d) fluorescence spectra of **dU_{CN2}** in a, b) MeOH and c, d) glycerol.

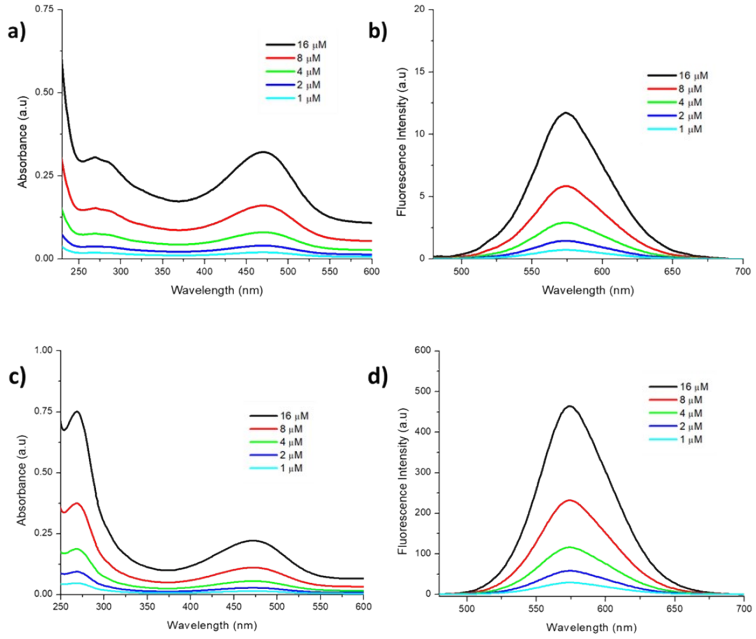


Figure S12: a, c) UV–Vis and b, d) fluorescence spectra of **dU_{Py}** in a, b) MeOH and c, d) glycerol.

Concentration	**dU_{CN2}** in MeOH		**dU_{CN2}** in glycerol		**dU_{Py}** in MeOH		**dU_{Py}** in glycerol	
	Fl. area	Abs	Fl. area	Abs	Fl. area	Abs	Fl. area	Abs
16 μM	0.03251	1.0219	0.31708	0.9437	0.00799	0.3216	0.30914	0.2213
8 μM	0.01631	0.5109	0.15764	0.4718	0.00393	0.1578	0.15457	0.1191
4 μM	0.00815	0.2547	0.07935	0.2382	0.00203	0.0813	0.07654	0.0571
2 μM	0.00407	0.1256	0.03963	0.1165	0.00097	0.0398	0.03947	0.0288
1 μM	0.00204	0.0645	0.01984	0.0587	0.00050	0.0201	0.01979	0.0138
Slope	0.0318		0.33586		0.3476		1.3944	
Quantum yield (ϕ)	0.003		0.061		0.031		0.127	

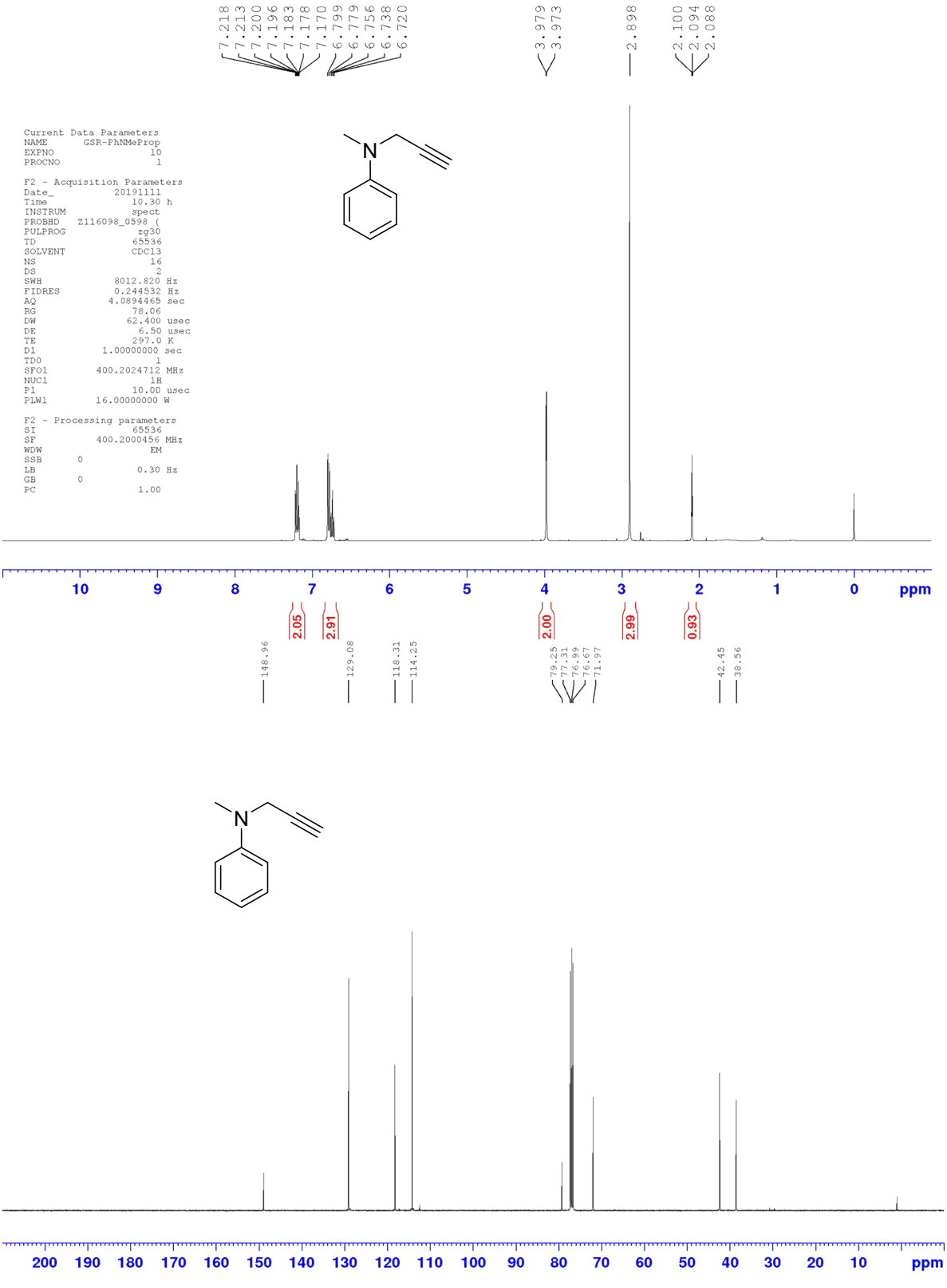


Figure S13: ^{1}H and ^{13}C NMR spectra of **1a** in $CDCl_3$.

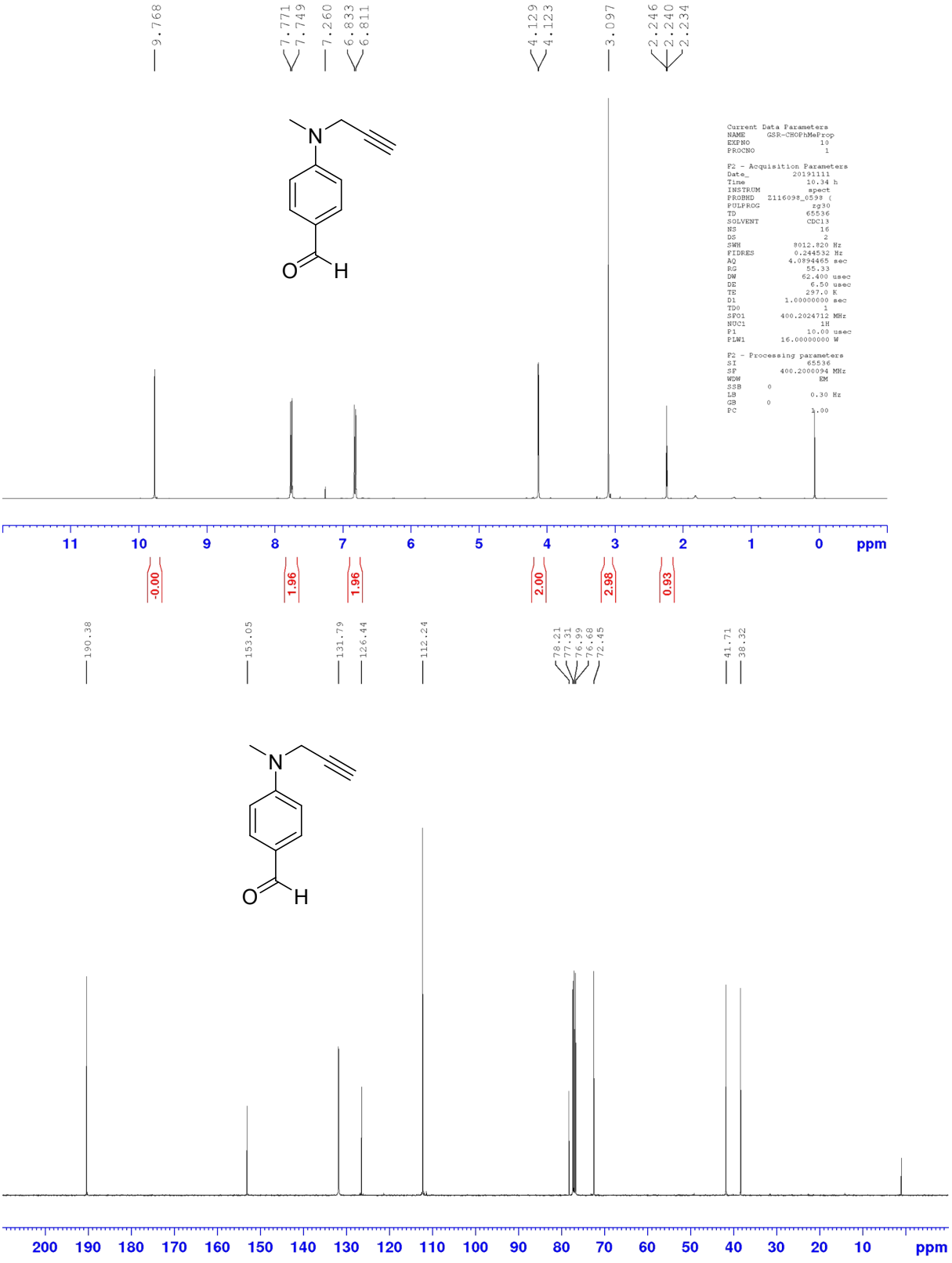


Figure S14: ^{1}H and ^{13}C NMR spectra of **2a** in $CDCl_3$.

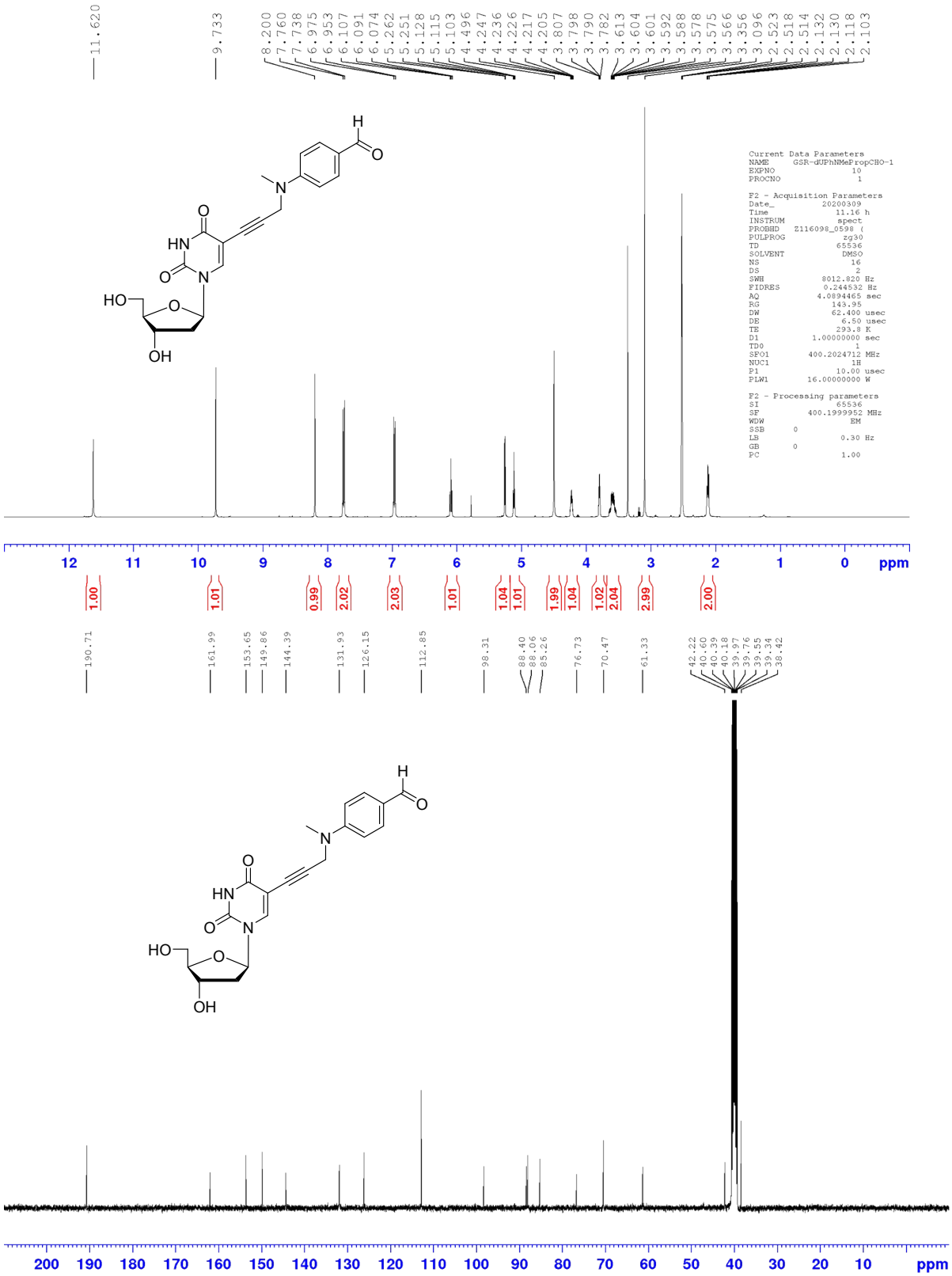


Figure S15: ^{1}H and ^{13}C NMR spectra of **3a** in DMSO-d_6.

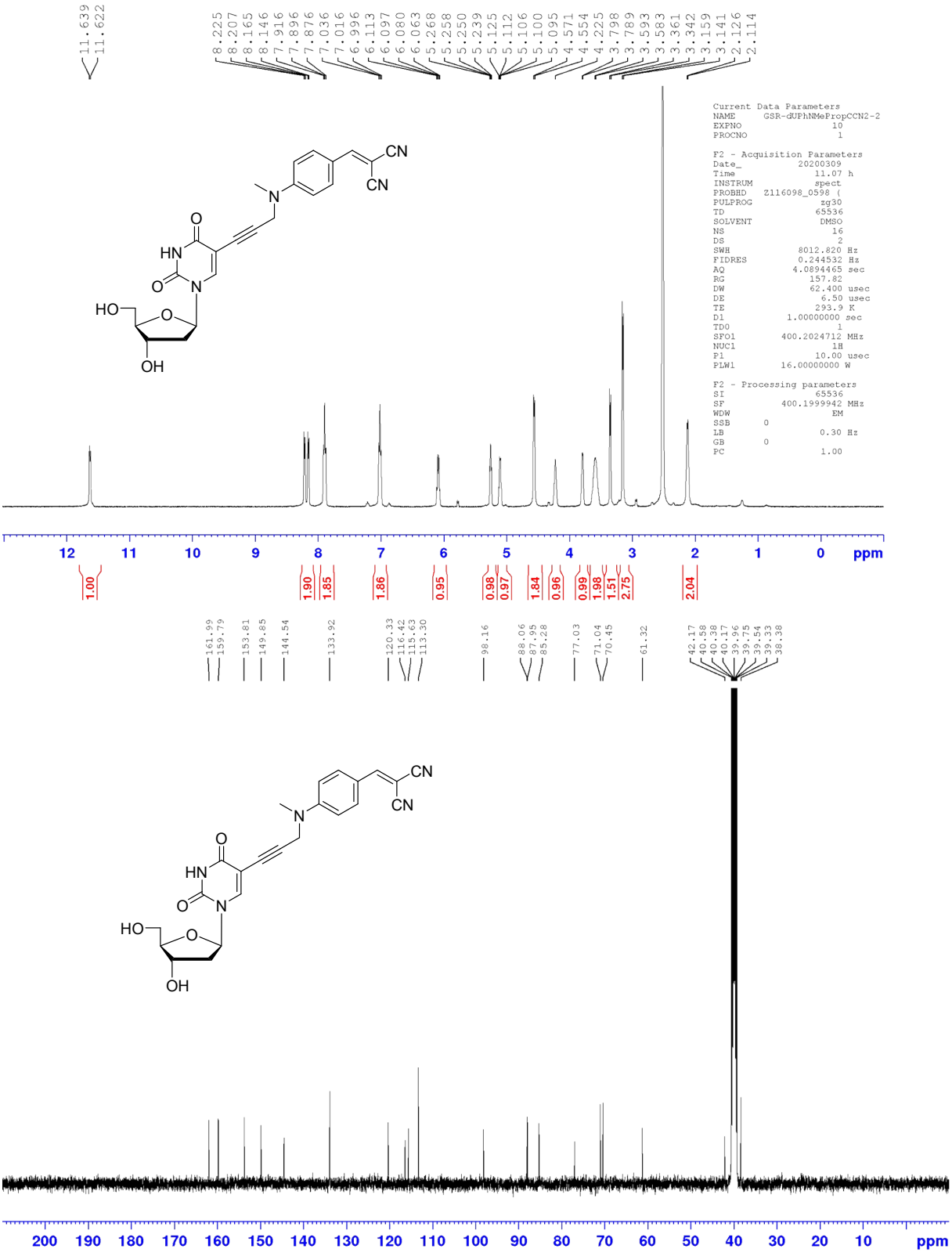


Figure S16: ^{1}H and ^{13}C NMR spectra of $\mathbf{dU_{CN2}}$ in DMSO-d_6.

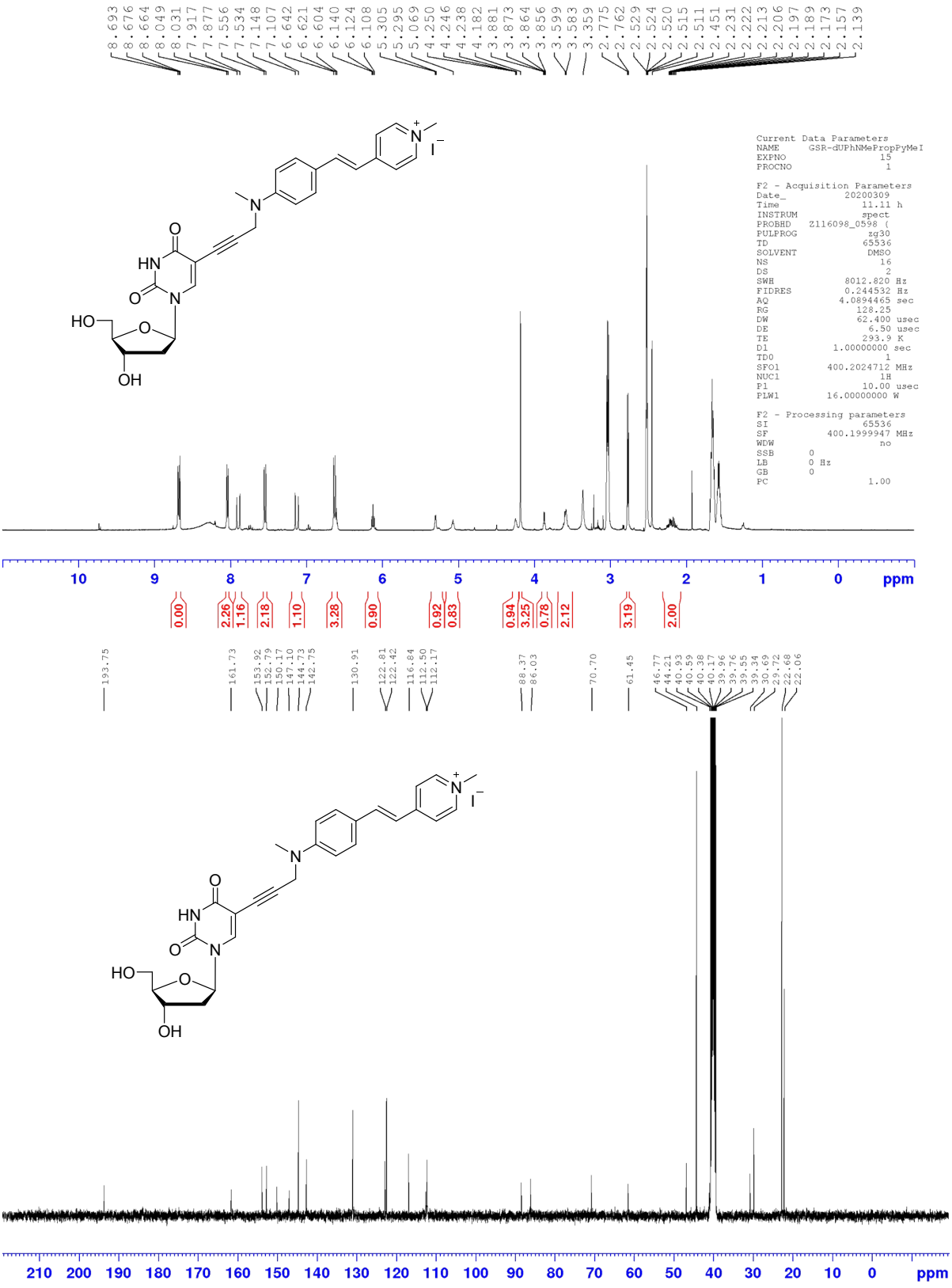


Figure S17: ^{1}H and ^{13}C NMR spectra of **dU_{Py}** in DMSO-d_6 (contains piperidine).