Supporting Information: Competitive Inhibition of Uracil DNA Glycosylase by a Modified Nucleotide Whose Triphosphate is a Substrate for DNA Polymerase

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General Methods. Unless otherwise specified, chemicals were purchased from Aldrich or Fisher Scientific and enzymes was obtained from New England Biolabs. Human UDG was isolated as previously described.[1] Oligonucleotides were synthesized via standard automated DNA synthesis techniques using an Applied Biosystems model 394 instrument. Radiolabeling was carried out according to the standard protocols.[2] [γ-^{32}P]ATP was purchased from Amersham Pharmacia Biotech. Quantification of radiolabeled oligonucleotides was carried out using a Molecular Dynamics Phosphorimager equipped with ImageQuant Version 5.1 software. ESI-MS spectra were collected using a Thermoquest LCQ-Deca Ion Trap instrument.

Kinetic study of incorporation of 2 by Klenow exo$^+$ and Klenow exo$^-$. The primer-template

duplex **3** was formed by hybridizing the radiolabeled primer (1 μM) and the cold template (1 μM) in 10 mM NaCl and 10 mM Tris•HCl (pH 7.5). The DNA was denatured at 90°C, slowly cooled to room temperature, and kept at 0 °C for overnight. Klenow exo$^-$ (0.44 pmol, 0.6 U) or Klenow exo$^+$ (0.147 pmol, 0.2 U) was treated with inorganic pyrophosphatase (0.3 U) in a solution of 1 × BSA (0.1 mg/mL), 1 × buffer 2 (NEB, 10 mM Tris•HCl, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9) in a total volume of 24 μL at room temperature for 10 min. The pre-treated enzyme solution (21.6 μL) that contains 0.4 pmol Klenow exo$^-$ or 0.133 pmol Klenow exo$^+$ was mixed with **3** (20 μL of the 1 μM solution) in 1 × BSA and 1 × buffer 2 in a total volume of 200 μL. The polymerase (2 nM Klenow exo$^-$ or 0.667 nM Klenow exo$^+$)/primer/template (100 nM) solution (5 μL) was added to 2 × dNTP (5 μL, 5-75 nM for dTTP, 2-45 μM for **2**). The reaction was incubated at room temperature for 5 min. Formamide loading buffer (5 μL, 95%, 10 mM EDTA) was added to quench the reaction. The mixture was heated to 90°C for 2 min and cooled on ice. An aliquot of the mixture was loaded on to a 20% denaturing polyacrylamide gel. The intensities of the starting material band (I_0) and the product band (I_1) were quantified. The rates of reaction were calculated from $r = 20I_0/[(I_0 + 0.5I_1)]$ %•min^{-1}. Apparent K_m and V_{max} were derived by fitting the rate-concentration plot to a hyperbola curve ($r = V_{max}[dNTP]/(K_m + [dNTP])$.

Full-length extension reactions. The experimental conditions are similar to those in the kinetic study except that 5 nM Klenow exo$^-$ and 50 μM of dNTP (mixture of dATP, dGTP, dCTP and dTTP or **2**) were used. At each desired time, an aliquot of the reaction mixture (5 μL) was

transferred to a new tube and quenched with formamide loading buffer (5 μL, 95%, 10 mM EDTA). The mixture was heated to 90°C for 2 min and chilled on ice. An aliquot of the mixture was loaded on to a 20% denaturing polyacrylamide gel. See Supporting information Figure 11 for primer/template sequences and autoradiography of the PAGE.

Kinetic study of UDG inhibition by measuring the apparent Km at different inhibitor concentrations. Duplex **4a** was formed by hybridizing the dU-containing strand (cold/hot DNA mixture, 9:1, 1 μM) and the complementary strand (1 μM) in 10 mM NaCl and 10 mM Tris•HCl (pH 7.5). The DNA was denatured at 90°C, slowly cooled to room temperature, and kept at 0 °C for 2 h. A 10 μL solution of **4a** (3-60 nM) was incubated with UDG (10 pM), DTT (1 mM), EDTA (1 mM), NaCl (1 mM), and Tris•HCl (20 mM, pH 8.0) at 37 °C for 5 min. The reaction was quenched by adding NaOH (0.3 M, 5 μL) and heated at 55°C for 30 min. The solution was neutralized with acetic acid (1 M, 1.5 μL) and concentrated in the Speedvac. Formamide loading buffer (20 μL, 95%, 10 mM EDTA) was added and the solution was heated to 90°C for 2 min and cooled on ice. An aliquot of DNA was loaded on to a 20% denaturing polyacrylamide gel. The reaction rates at different substrate concentrations were quantified. K_m and V_{max} were derived by fitting the reaction rate-concentration plot to a hyperbola curve using the equation $r = V_{max}[\mathbf{4a}]/(K_m + [\mathbf{4a}])$.

The inhibitor **4b** was hybridized under the same conditions as **4a**. Reactions in the presence of the **4b** were carried out at the same enzyme concentration and buffer conditions. The substrate concentration range and the reaction time were increased accordingly (Table 1). The apparent K_m (K_m') and V_{max} were derived by hyperbola curve-fitting. The inhibition constant K_i

was derived from the K_m' versus [**4b**] plot using the equation slope = K_m/K_i.

Table 1. Experimental conditions for the competitive inhibition assay.

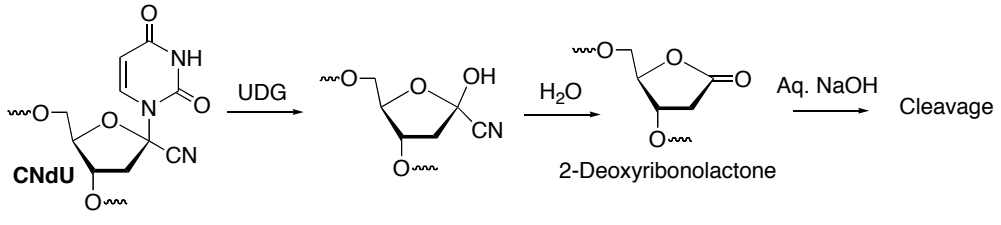
inhibitor concentration (nM)	substrate concentration range (nM)	reaction time (min)
0	3-60	5
1	5-60	5
2	5-60	7
3	5-100	7
4	10-150	10
5	10-150	10

Kinetic study of UDG inhibition by measuring the relative rate constants.[3] A 10 μL solution of radiolabeled single-stranded dU-containing DNA substrate (16-mer, sequence: 5'-d(GAA GAC CTU GGC GTC C), 10 nM) was incubated with UDG (20 pM for *E. coli* UDG, 200 pM for human UDG), DTT (1 mM), EDTA (1 mM), Tris•HCl (20 mM, pH 8.0), NaCl (1 mM) and inhibitor **4b** (0-20 nM when NaCl is 1 mM) at room temperature for 5 min. The reaction was quenched by adding NaOH (0.3 M, 5 μL) and heated at 55°C for 30 min. The solution was neutralized with acetic acid (1 M, 1.5 μL). Formamide loading buffer (3 μL, 95%, 10 mM EDTA) was mixed with an aliquot of DNA (3 μL) and loaded on to a 20% denaturing polyacrylamide gel. The reaction rates r_i at different substrate concentrations were quantified. K_i was derived by fitting the reaction rate-concentration plot to a hyperbola curve using the equation $r_i/r_0 = 1/(1 + [\mathbf{4b}]/K_i)$.

Inhibition studies by CNdU monomer were carried out at the same conditions except that the concentration range of the inhibitor was 0-500 μM for *E. coli* UDG.

Kinetic studies of human UDG inhibition by dU and CUdU nucleoside using fluorescence spectroscopy. The fluorescence of single-stranded DNA 5'-d(AUPAA) (0.5 μM, P stands for 2-aminopurine, λ_{exc} = 320 nm, λ_{emi} = 370 nm) in a solution of DTT (1 mM), EDTA (1 mM), Tris•HCl (20 mM, pH 8.0), NaCl (1 mM), and **CNdU** or **dU** (0-1 mM) was monitored after human UDG (final concentration 700 pM) was added. The fluorescence intensity data was collected every 3 seconds on a FluoroMax-3 fluorimeter. The rate of fluorescence increase r_i was derived from the slope of the time-course fluorescence spectrum between 200 s and 400 s. K_i was derived by fitting the reaction rate-concentration plot to a hyperbola curve using the equation $r_i/r_0 = 1/(1 + [\mathbf{CNdU}]/K_i)$.

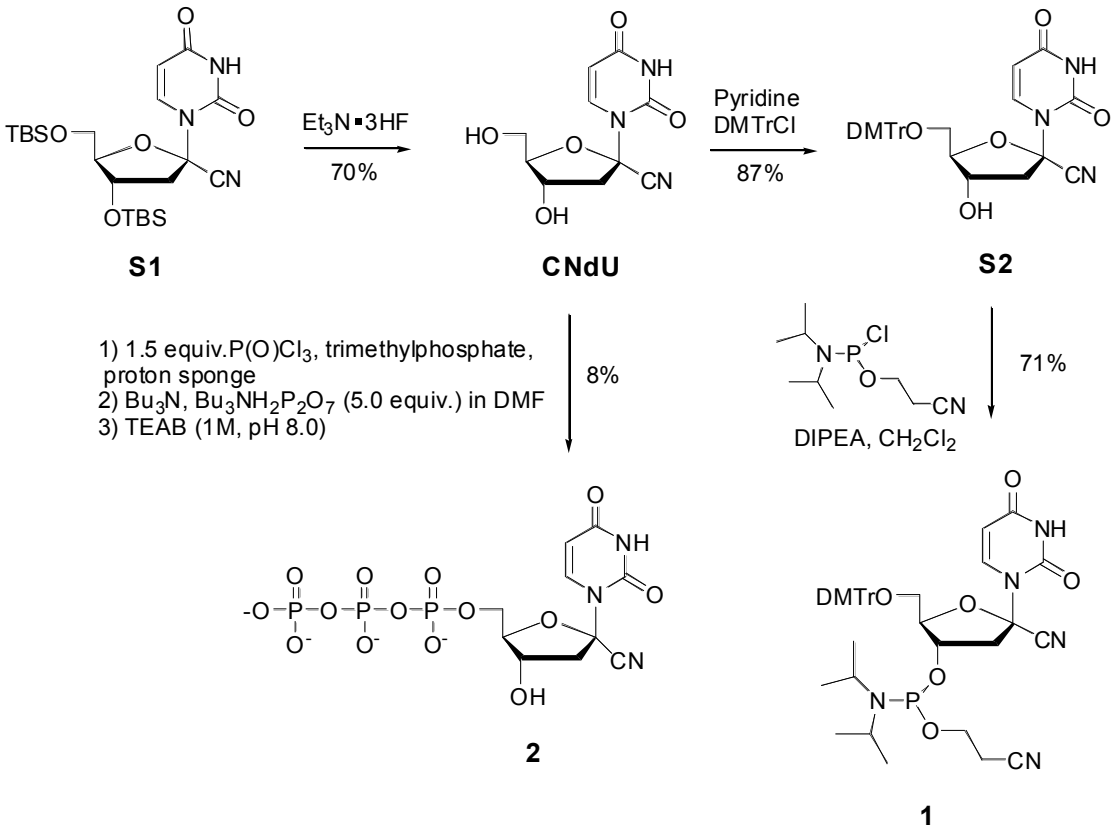
CNdU stability in the presence of UDG. A 50 μL solution of radiolabeled **4b** (10 nM), was incubated with *E. coli* UDG (5 nM), DTT (1 mM), EDTA (1 mM), and Tris•HCl (20 mM, pH 8.0) at 37 °C for 24 h. The reaction was quenched by adding NaOH (1 M, 5 μL) and heated at 55°C for 30 min. The solution was neutralized with acetic acid (1 M, 5 μL). An aliquot of the solution (5 μL) was mixed with formamide loading buffer (3 μL, 95%, 10 mM EDTA) and heated to 90°C for 2 min and cooled on ice before loaded on to a 20% denaturing polyacrylamide gel.



UV melting temperatures. DNA **5a** and **5b** (0.5-5 μM for each strand) were hybridized in 10

mM PIPES (pH 7.0), 10 mM $MgCl_2$ and 100 mM NaCl in a total volume of 200 μL by heating at 90 °C for 5 minutes, slowly cooling to room temperature, and storing at 0 °C overnight. The temperature was increased from 25 °C to 65 °C at 0.5 °C/min. Readings were taken every 0.2 °C. The melting temperatures were derived from the first derivative of the melting curves. ΔH and ΔS were derived from Van't Hoff plots using the equation $1/T_m = (R/\Delta H)\ln[C]_{total} + (\Delta S\text{-}R\ln 4)/\Delta H$. $[C]_{total}$ is the total concentration of the two DNA strands.

Syntheses of CNdU, 1 and 2



CNdU. A solution of **S1**[4] (182 mg, 0.38 mmol) and triethylamine trihydrofluoride (0.61 mL, 3.8 mmol) in THF (4 mL) was stirred at room temperature overnight. The solvent was evaporated and the crude product was purified by column chromatography (methylene chloride/methanol, 15:1) to give **CNdU** (60 mg, 0.24 mmol, 62%). ^{1}H NMR (CD_3OD) δ 8.01 (d, 1H, J = 8 Hz), 5.70

(1H, d, J = 8 Hz), 4.38 (m, 2H), 3.75 (dd, 1H, J = 12, 4 Hz), 3.65 (dd, 1H, J = 12, 4 Hz), 3.09 (dd, 1H, J = 14, 2 Hz), 2.73 (dd, 1H, J = 14, 2 Hz). ^{13}C NMR (CD_3OD) δ 166.2, 151.4, 140.1, 118.0, 102.5, 92.7, 89.1, 72.3, 62.1, 50.1. IR (HBr) 3040 (br), 2974, 2938, 2763, 2736, 2678, 2488, 1718, 1686, 1471, 1430, 1391, 1288, 1169, 1115, 735 cm^{-1}. ESI-HRMS (MH^+) calc'd for $C_{10}H_{12}N_3O_5$ 254.0771, found 254.0772.

Compound S2. A solution of **CNdU** (50 mg, 0.20 mmol), dimethoxytrityl chloride (100 mg, 0.30 mmol) in pyridine (2 mL) was stirred at 25 °C for 1 h. The reaction was quenched with 1 mL methanol and the solvent was evaporated. The crude product was purified column chromatography (hexanes/ethyl acetate 1:3) to give **S2** (95 mg, 0.17 mmol, 87%). ^{1}H NMR ($CDCl_3$) δ 7.74 (d, 1H, J = 8 Hz), 7.28 (m, 9H), 6.84 (m, 4H), 5.61 (1H, d, J = 8 Hz), 4.55 (m, 2H), 3.78 (m, 7H), 3.36 (m, 3H), 3.09 (dd, 1H, J = 14, 2 Hz), 2.72 (dd, 1H, J = 14, 2 Hz). ^{13}C NMR ($CDCl_3$) δ 163.3, 158.7, 149.7, 144.1, 138.3, 135.0(3), 134.9(9), 129.9(9), 129.9(6), 129.2, 128.0, 127.9, 127.2, 116.4, 113.4, 113.2, 102.4, 89.6, 87.4, 87.1, 71.7, 62.3, 55.3, 49.2. IR (HBr) 3106, 3058, 2936, 2907, 2837, 1724, 1683, 1609, 1509, 1450, 1378, 1293, 1250, 1178, 1117, 1301, 831 cm^{-1}. ESI-HRMS (MNa^+) calc'd for $C_{31}H_{29}N_3O_7Na$ 578.1898, found 578.1897.

Compound 1. N,N'-diisopropyl 2-cyanoethyl phosphoramidic chloride (60 μL, 0.27 mmol) was added to a solution of diisopropylethylamine (94 μL, 0.54 mmol) and **S2** (75 mg, 0.135 mmol) in methylene chloride (2 mL) at 0°C. The reaction was warmed to room temperature and continued for 1 h. The reaction was diluted with ethyl acetate (50 mL), and washed successively with saturated sodium bicarbonate solution and brine. The organic layer was dried over magnesium sulfate. The solvent was evaporated and the crude product was purified by column

chromatography (hexanes/ethyl acetate 1:1) to give **1** (72 mg, 0.095 mmol, 70%). ^{1}H NMR ($CDCl_3$) δ 7.78 (m, 1H), 7.30 (m, 9H), 6.84 (m, 4H), 5.63 (m, 1H), 4.48-4.62 (m, 2H), 3.30-3.95 (m, 13H), 2.90 (s, 1H), 2.68 (s, 1H), 1.40 (m, 12H). ^{31}P NMR ($CDCl_3$) δ 149.2, 149.0. IR (HBr) 3058, 2966, 2872, 2837, 1694, 1608, 1509, 1445, 1379, 1293, 1250, 1178, 1177, 1115, 1091, 1030, 978, 883, 829 cm^{-1}. ESI-HRMS (MNa^+) calc'd for $C_{40}H_{46}N_5O_8NaP$ 778.2976, found 778.2968.

Compound 2. Proton sponge (70 mg, 0.33 mmol) and **CNdU** (55 mg, 0.22 mmol) were azeotropically dried with benzene before dissolved in trimethyl phosphate (1.5 mL). Freshly distilled phosphorus oxychloride (30 μL, 0.33 mmol) was added at 0 °C. The reaction was kept at 0°C for 3h. A solution of tributylamine (530 μL) and tributylammonium pyrophosphate (522 mg, 1.10 mmol) in DMF (1 mL) was added. The reaction was continued for 5 min before quenching with 1 M TEAB (10 mL pH 7.5). The aqueous layer was washed with ethyl acetate (2 × 20 mL) and lyophilized to dryness. The crude product was dissolved in 2 mL water and loaded onto a DEAE-cellulose column. The column was washed with 40 mL water followed by 0.5 M TEAB (pH 7.5). The UV-active fractions were collected and lyophilized to dryness. The product was purified by FPLC (0.1 M TEAB, 1M TEAB (0-30%)) to give **2** as its triethylammonium salt. ^{1}H NMR (D_2O) δ 7.95 (d, 1H, J = 8 Hz), 5.83 (d, 1H, J = 8 Hz), 4.60 (m, 1H), 3.11 (q, 24H, J = 8 Hz), 2.96 (m, 1H), 2.83 (dd, 1H, J = 12, 5 Hz), 1.18 (m, 36H). ^{31}P NMR (D_2O) δ -9.8 (d, 1P, J = 22 Hz), -12.0 (d, 1P, J = 20 Hz), -23.3 (t, J = 20 Hz). ESI-MS ($M\text{-}H^+$) calc'd for $C_{10}H_{13}N_3O_{14}P_3$, 492.0, found 492.1.

Oligonucleotide synthesis. The 1'-CNdU-containing oligonucleotides in **4b** and **5b** were

synthesized on an Applied Biosystems Incorporated 394 DNA synthesizer using fast deprotecting phosphoramidites (Pac-dA-CE, iPac-dG-CE, and Ac-dC-CE). Pivaloyl anhydride/2,6-Lutidine/THF (1:1:8) was used as the capping reagent.[5] t-BuOOH in toluene (1 M) was used as oxidation reagent. Deprotection was carried out in a suspension of K_2CO_3 (14.5 mg) in methanol (700 μL) at room temperature for 3 h, or in concentrated NH_3/EtOH (3:1) at 16 °C for 9 h. The crude oligonucleotides were purified by 20% denaturing PAGE and desalted using a C-18 Sep-Pak cartridge. The oligonucleotides were characterized by ESI-MS after ammonium acetate precipitation.

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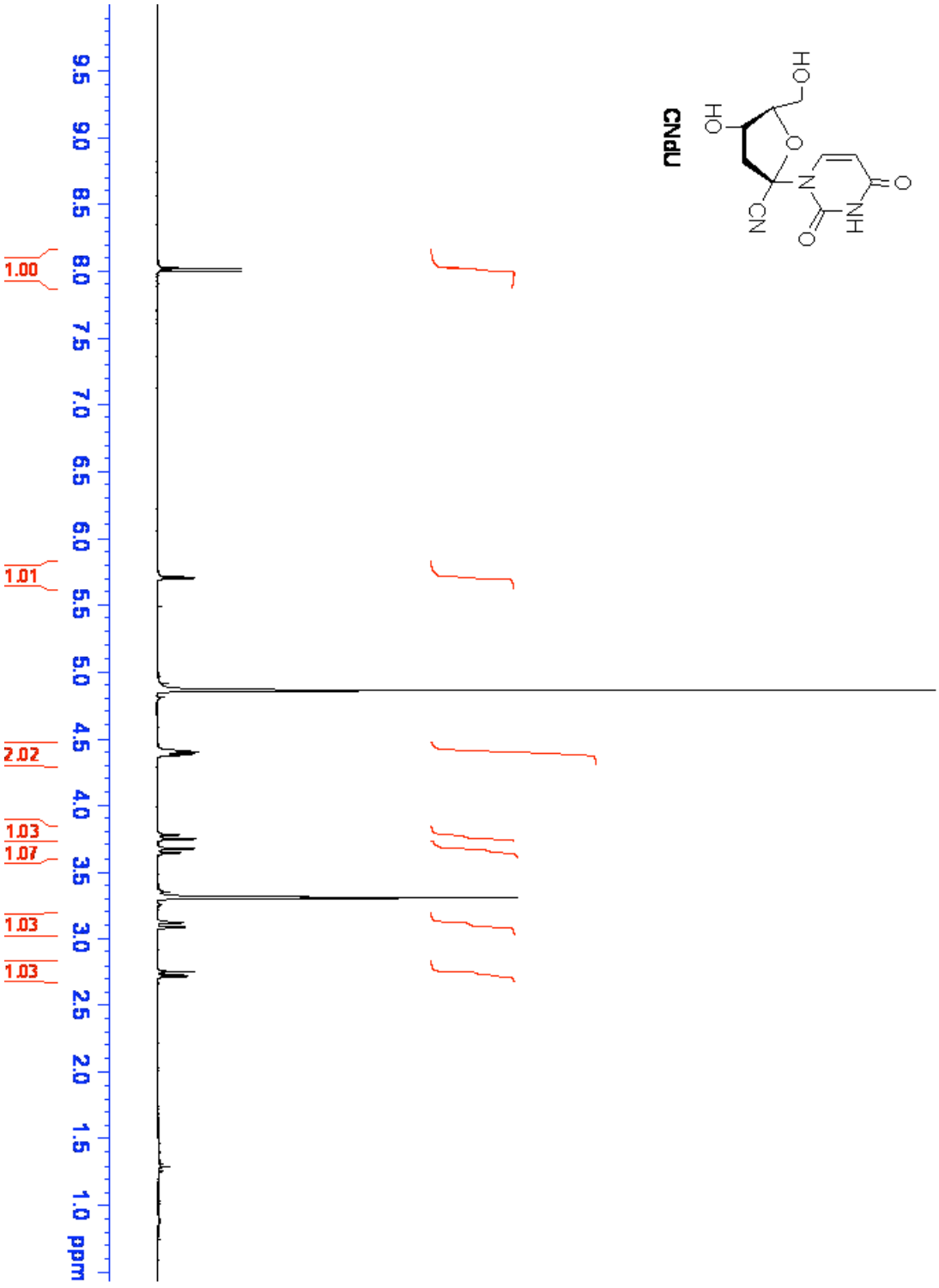
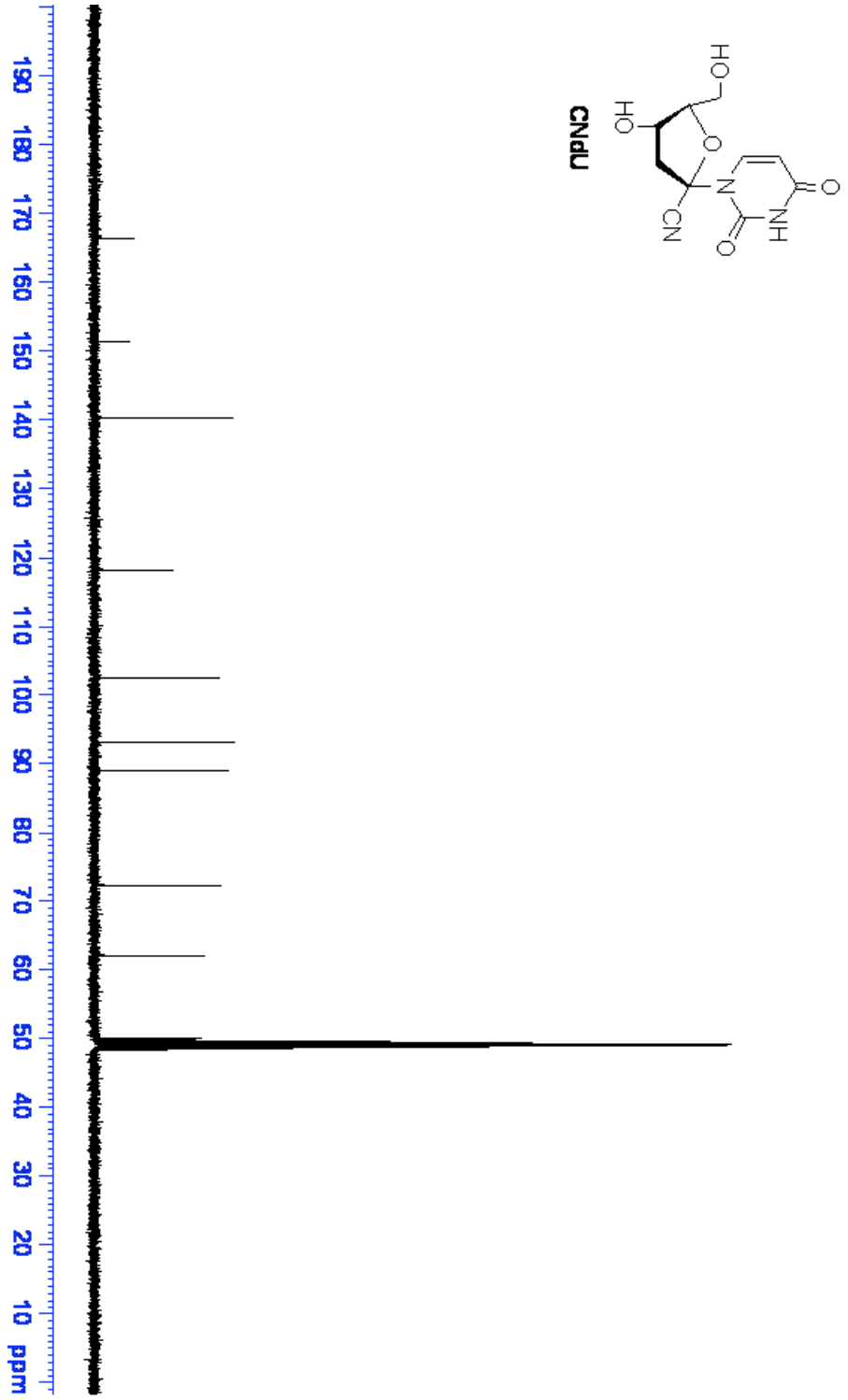
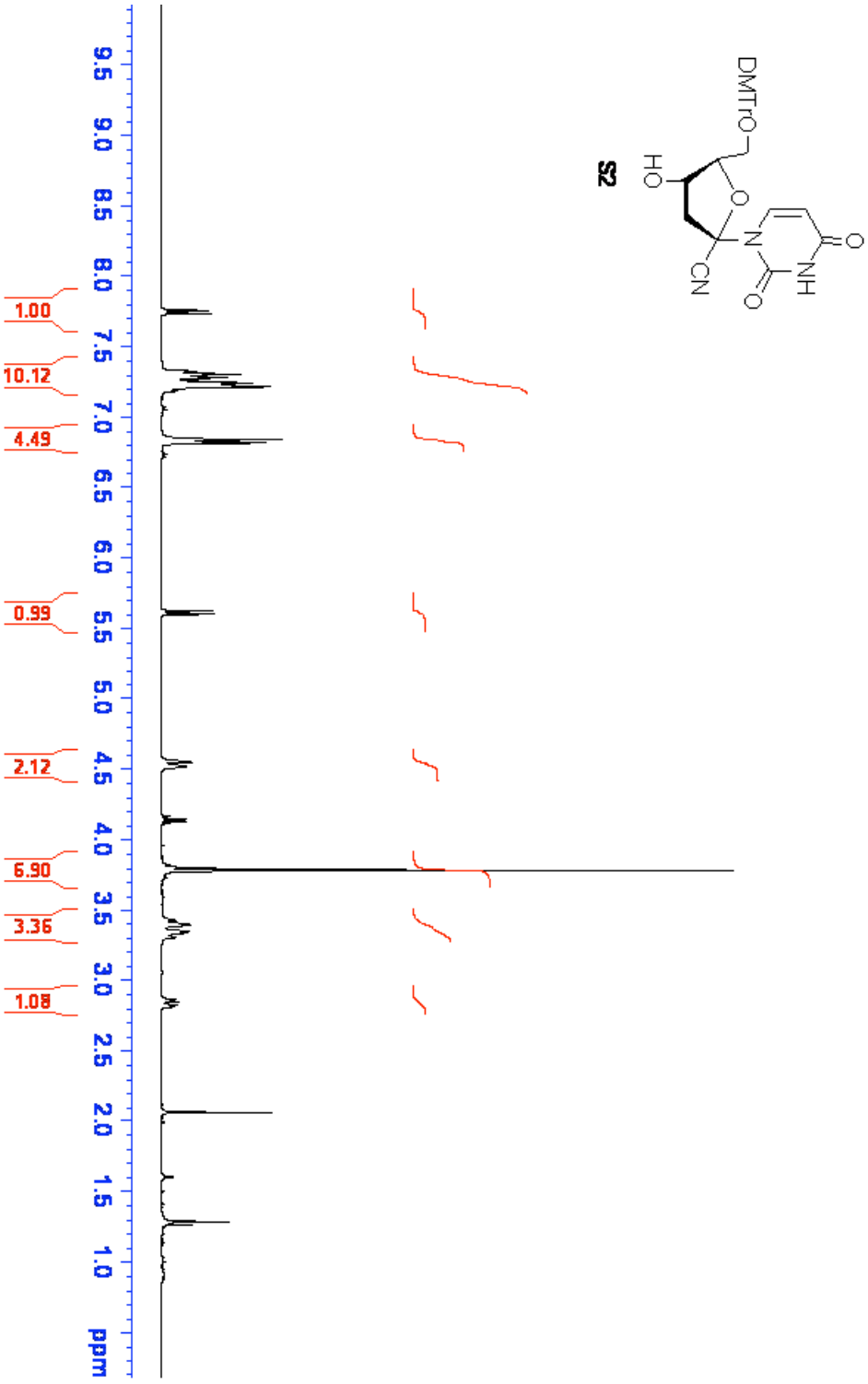


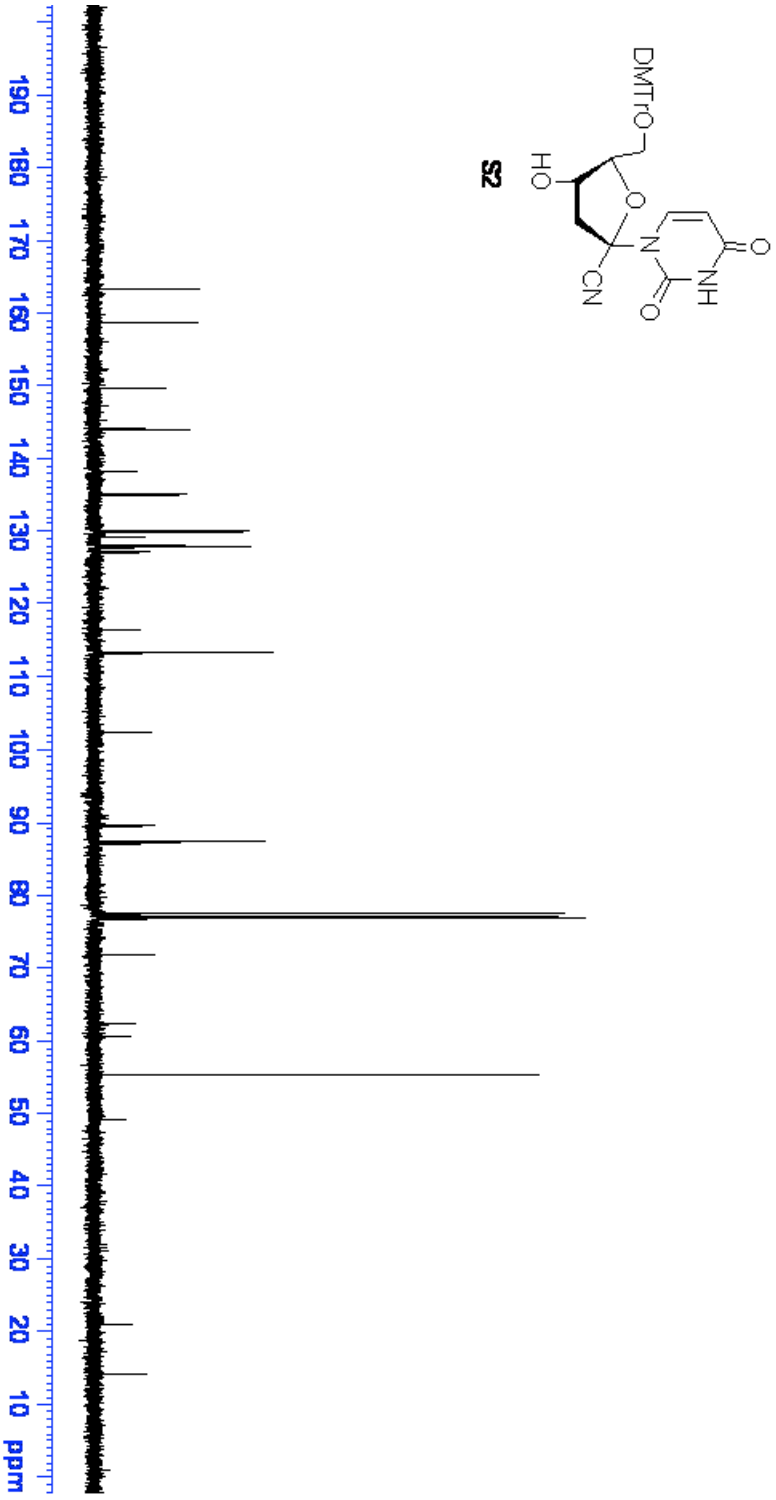
Supporting Information Figure 1. ^{1}H NMR spectrum of **CNdU** in CD_3OD.



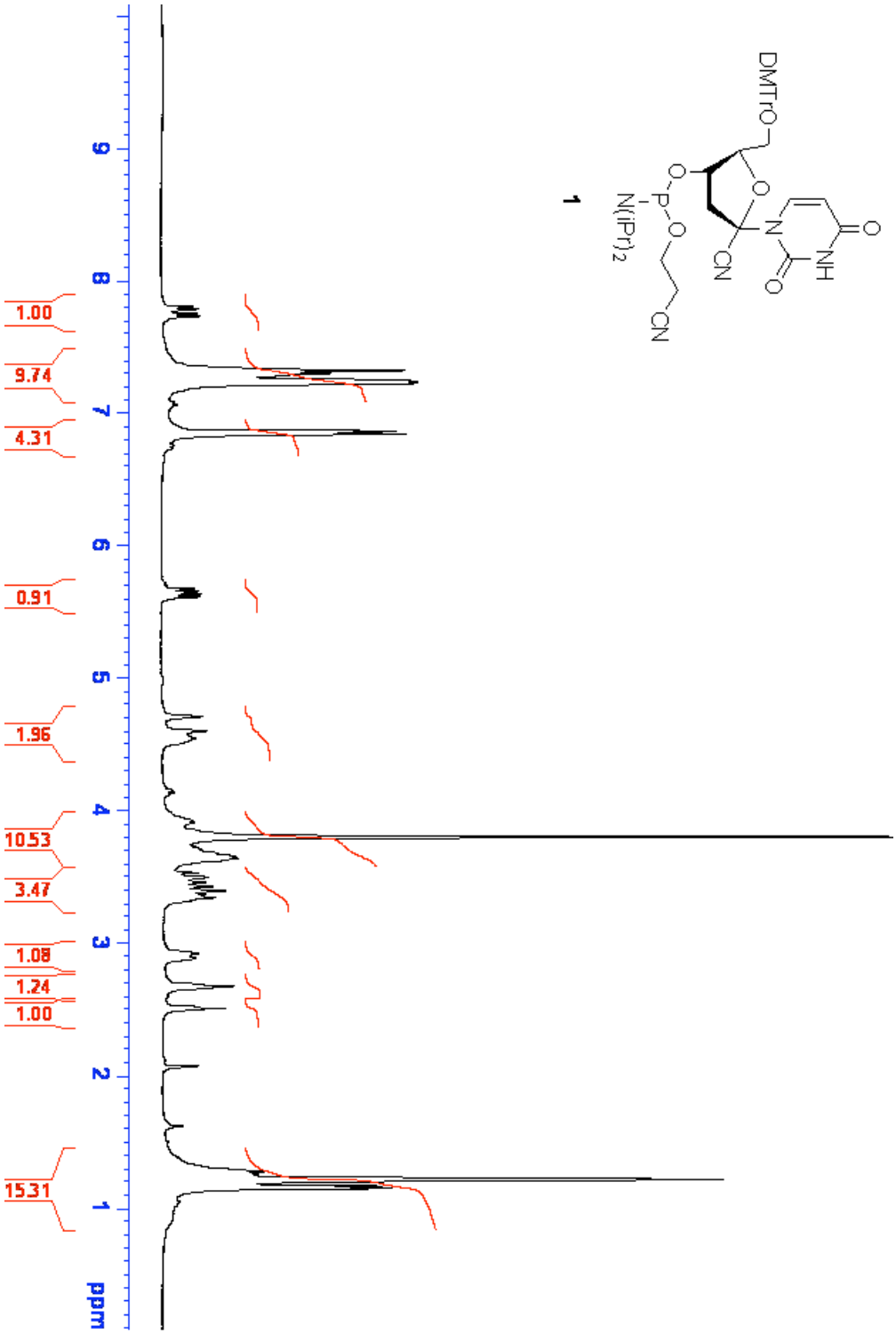
Supporting Information Figure 2. ^{13}C NMR spectrum of **CNdU** in CD_3OD.



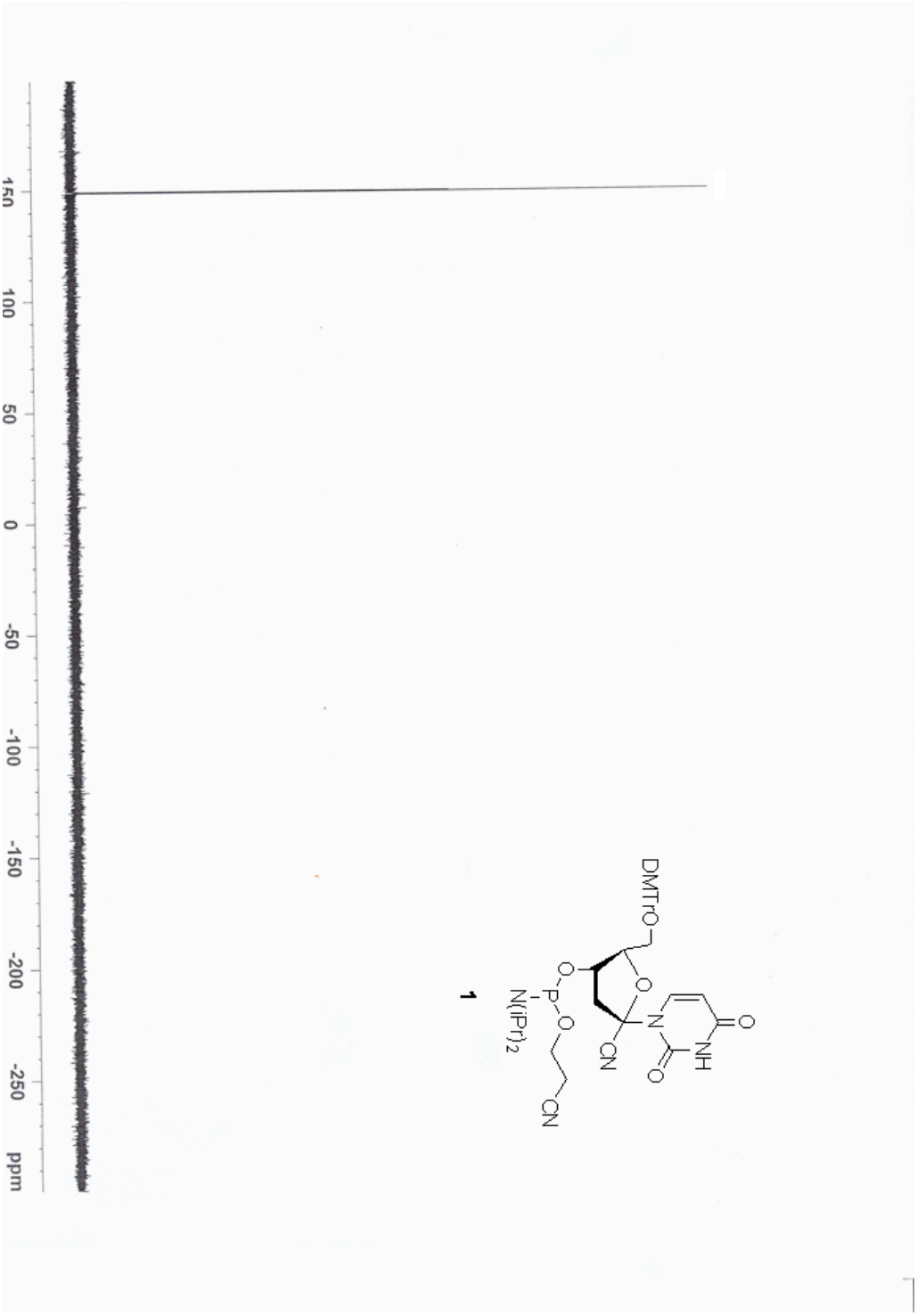
Supporting Information Figure 3. ^{1}H NMR spectrum of **S2** in $CDCl_3$.



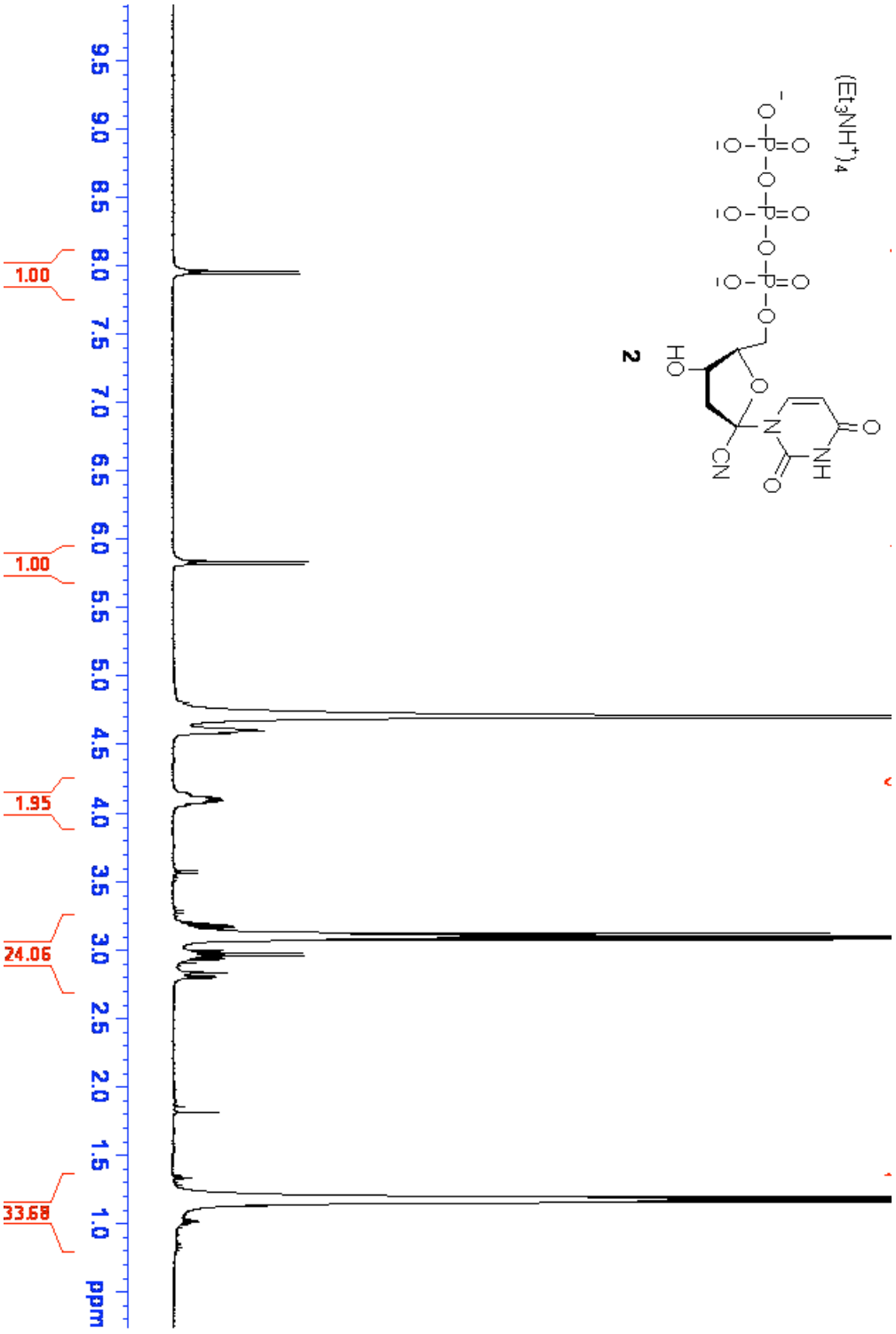
Supporting Information Figure 4. ^{13}C NMR spectrum of **S2** in $CDCl_3$.



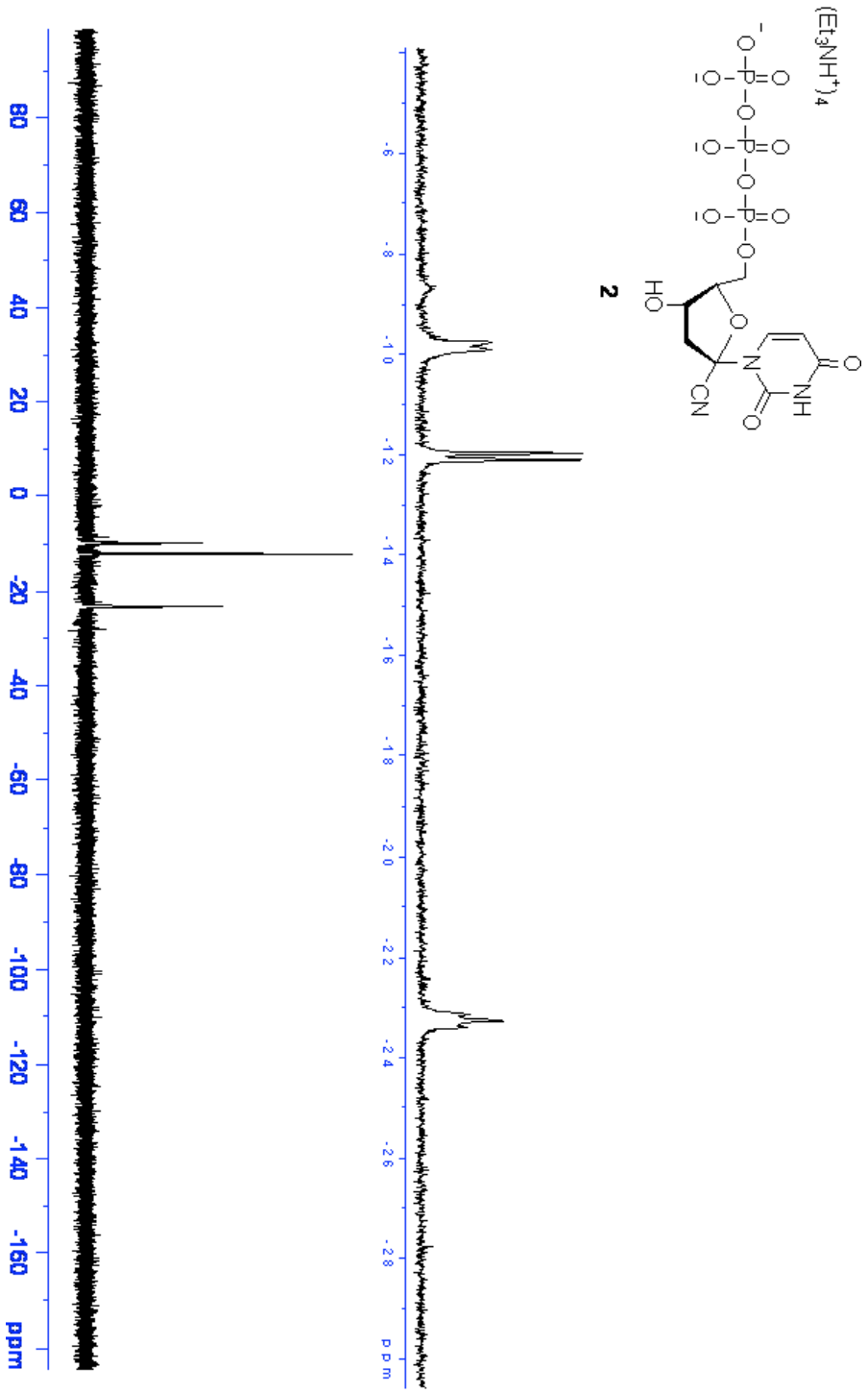
Supporting Information Figure 5. ^{1}H NMR spectrum of **1** in $CDCl_3$.



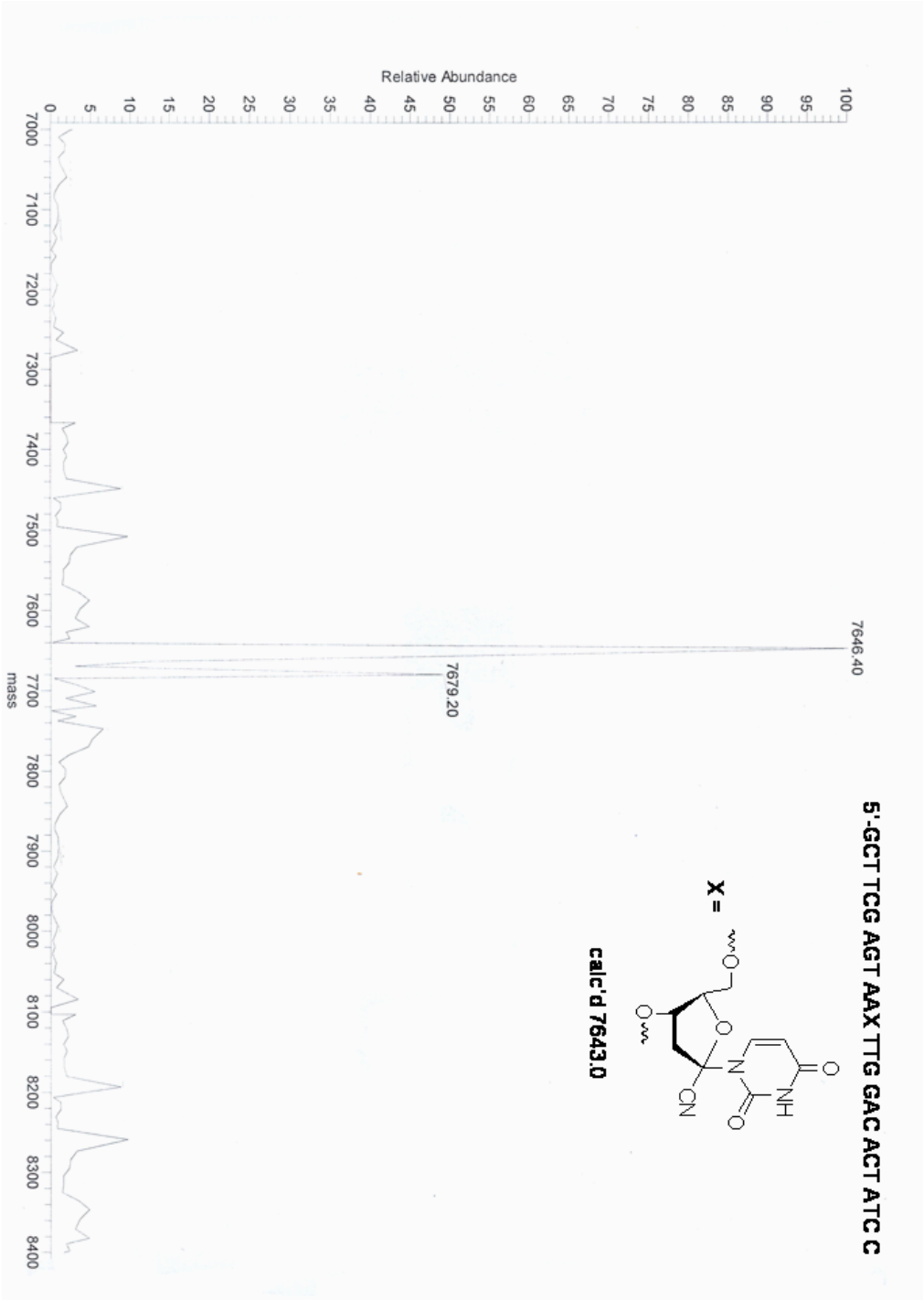
Supporting Information Figure 6. ^{31}P NMR spectrum of **1** in $CDCl_3$.



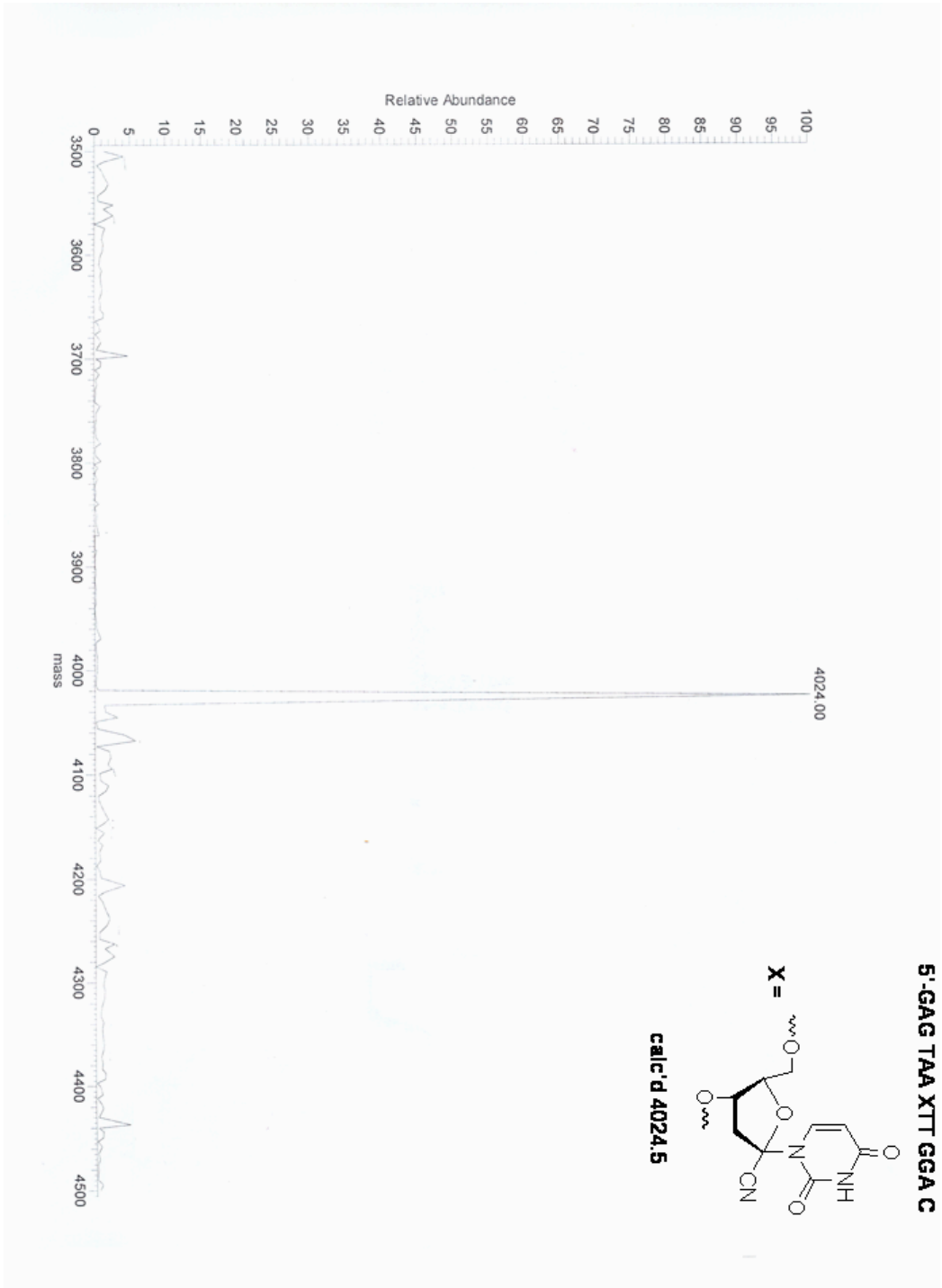
Supporting Information Figure 7. ^{1}H NMR spectrum of **2** in D_2O.



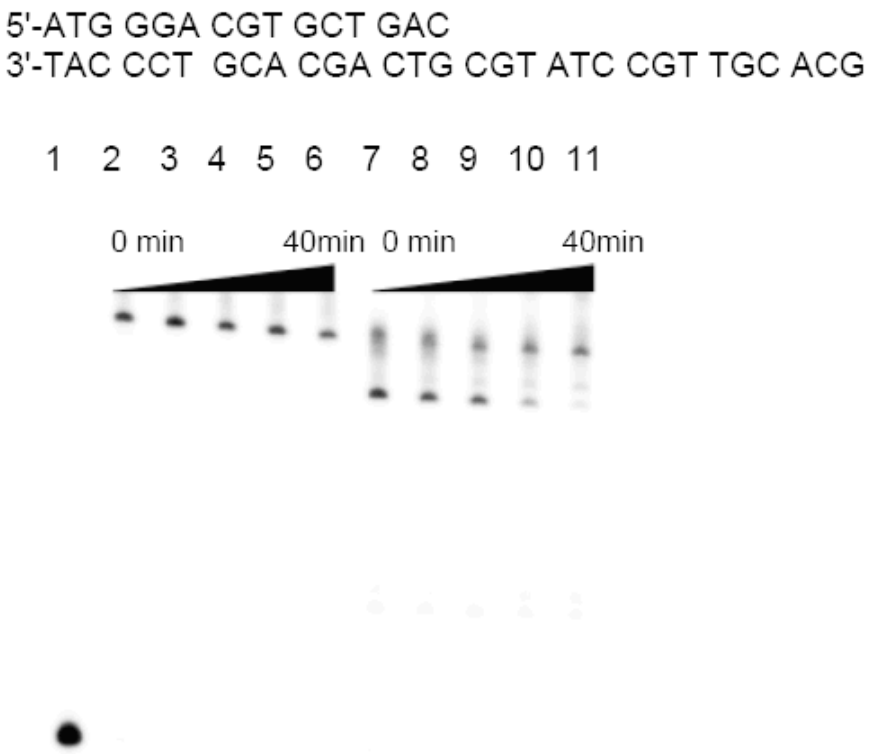
Supporting Information Figure 8. ^{31}P NMR spectrum of **2** in D_2O.



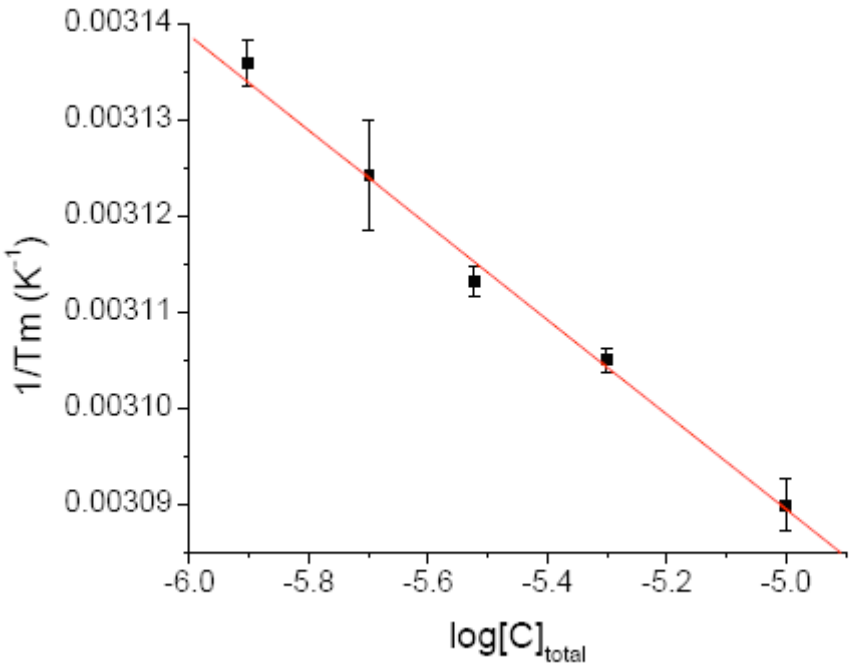
Supporting Information Figure 9. ESI-MS of the **CNdU**-containing strand of **4b**.



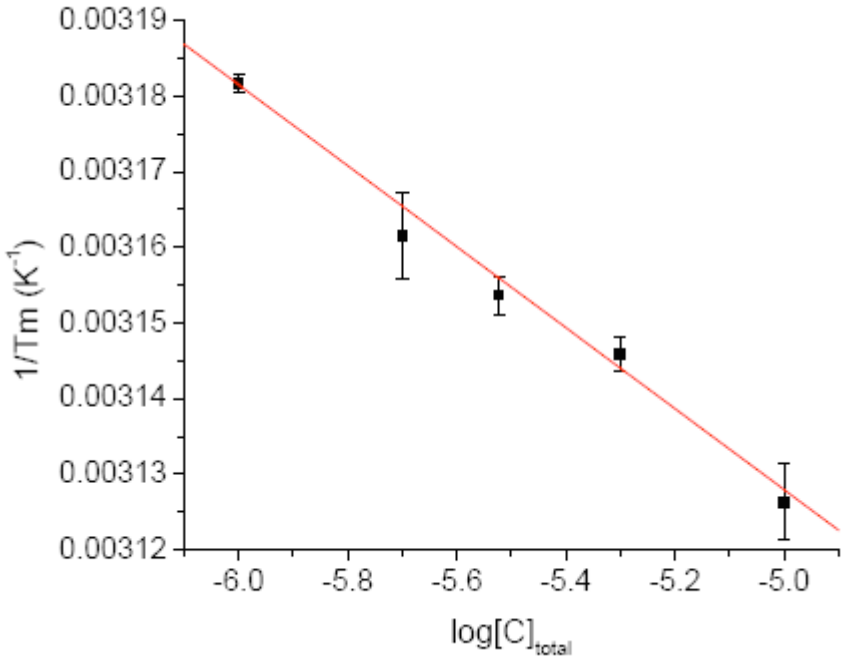
Supporting Information Figure 10. ESI-MS of the **CNdU**-containing strand of **5b**.



Supporting Information Figure 11. PAGE of full-length extension reaction. Lane 1**,** no dNTP control. Lane 2-6, dNTP (N = dA, dG, dC, T) incorporation, reaction time: 2, 5, 10, 20, 40 min. Lane 7-11, dNTP (N = dA, dG, dC, CNdU) incorporation, reaction time: 2, 5, 10, 20, 40 min.



Supporting Information Figure 12. Van't Hoff plot of DNA duplex **5a**.



Supporting Information Figure 13. Van't Hoff plot of DNA duplex **5b**.