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Electronic Supporting Information

Synthesis and enzymatic incorporation of norbornene-modified nucleoside triphosphates for Diels-Alder bioconjugation

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List of abbreviations:

DARinv = Diels-Alder reaction with inverse-electron-demand, Norb = norbornene, DMF = *N,N*-dimethylformamide, CyHex = cyclohexane, DCM = dichloromethane, EE = ethyl acetate, MeOH = methanol, RT = room temperature, PEX = Primer extension

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General materials and methods

Reagents were purchased from Sigma-Aldrich and used without further purification. Reversed-phase HPLC purification was performed on an Agilent 1100 Series HPLC system equipped with a diode array detector using a semi-preparative Phenomenex Luna C18 5 µm column (10.0×250 mm) at a flow rate of 3 ml/min and eluting with a gradient of 100 mM triethylammonium acetate pH 7.0 (buffer A) and 100 mM triethylammonium acetate in 80% acetonitrile (buffer B). Flash purification was done on a Varian IntelliFlash 310 Discovery Scale Flash Purification System. MS experiments were performed on a Bruker microTOFQII ESI/APCI mass spectrometer. Analysis of the MS measurements was carried out using DataAnalysis (Version 4.0, SP 4) software (Bruker Daltonics). For high-resolution mass spectra, internal calibration was performed using ESI Tune-mix (Fluka, enhanced quadratic mode) or sodium formiate (HPC mode) as calibrant. Calculated molecular weights refer to the m/z values given by the Data Analysis software. NMR spectra were recorded on a Varian Mercury Plus spectrometer (300 MHz) or a Varian NMR system (500 MHz). The assignment of proton and carbon resonances is based on two-dimensional correlation experiments (COSY, GHSQC, GHMBC). Agarose gels were stained with ethidium bromide and visualised by UV illumination using an AlphaImager^{TM} 2200. Denaturing polyacrylamide gels were visualised by a Typhoon 9400 imaging system.

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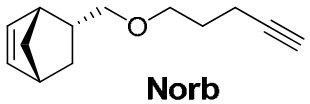
Synthesis of modified nucleosides

endo-5-((Pent-4-ynyloxy)methyl)-bicyclo[2.2.1]hept-2-ene (Norb)

Synthesis was performed according to a literature-known procedure using *endo*- instead of *exo*-bicyclo[2.2.1]hept-5-en-2-ylmethanol.[1]



Norb

Scale: 6.56 mmol, V(THF) = 28.4 mL.

Yield: 907 mg, 4.77 mmol, 73%.

Chromatography: SiO_2, CyHex/EE = 19:1, R_f = 0.38.

Habitus: Colourless oil.

^{1}H-NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ = 0.49 (ddd, *J* = 11.60, 4.40, 2.64 Hz, 1H), 1.24 (d, *J* = 8.22 Hz, 1H), 1.39-1.45 (m, 1H), 1.75-1.83 (m, 3H), 1.95 (t, *J* = 2.62 Hz, 1H), 2.30 (dt, *J* = 7.00, 2.62 Hz, 2H), 2.34-2.40 (m, 1H), 2.79 (s, 1H), 2.92 (s, 1H), 3.02 (t, *J* = 9.25 Hz, 1H), 3.16 (dd, *J* = 9.25, 6.55 Hz, 1H), 3.47 (tq, *J* = 9.63, 6.18 Hz, 2H), 5.93 (dd, *J* = 5.69, 2.91 Hz, 1H), 6.12 (dd, *J* = 5.69, 3.03 Hz, 1H).

^{13}C{^{1}H}-NMR (75 MHz, $CDCl_3$, 25 °C, TMS): δ = 15.2, 28.7, 29.08, 38.7, 42.2, 49.4, 68.3, 69.1, 74.6, 84.1, 132.5, 137.1.

MS (APCI$^+$): m/z 191.12 (calculated for $[C_{13}H_{18}O_1+H]^+$ 191.14).

5-(5-(*endo*-Bicyclo[2.2.1]hept-5-ene-2-ylmethoxy)pent-1-ynyl)-2'-deoxyuridine (dU^{Norb})

5-Iodo-2'-deoxyuridine (457 mg, 1.28 mmol 1 eq) was dissolved in 6.1 mL dry DMF, and $Pd(PPh_3)_4$ (152 mg, 122 µmol 9.5 mol%) as well as CuI (60.9 mg, 274 µmol, 21.4 mol%) were added. After degassing, Hünig's Base (degassed, 565 µL, 2.59 mmol, 2 eq) was added and the mixture stirred for 10 min at RT. Then a degassed solution of *endo*-5-((pent-4-ynyloxy) methyl) bicyclo[2.2.1]hept-2-ene (700 mg, 3.63 mmol, 2.8 eq) in 3 mL DMF was added. After stirring for 15 h at RT, solvents were evaporated and the crude product was purified by flash chromatography (SiO_2, DCM/MeOH = 15:1, R_f (**dU^{Norb}**) = 0.18).

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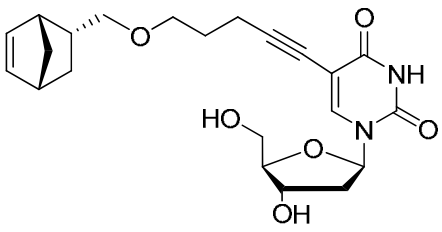

dU^{Norb}

Yield: 224 mg, 0.54 mmol, 42%.

Habitus: Yellow solid.

^{1}H-NMR (500 MHz, $CDCl_3$, 25 °C, TMS): δ = 0.47 (ddd, *J* = 11.58, 4.28, 2.58 Hz, 1H, $H_{Norb\text{-}linker}$), 1.23 (d, *J* = 8.06 Hz, 1H, $H_{Norb\text{-}linker}$), 1.41 (ddd, *J* = 8.06, 3.91, 1.98 Hz, 1H, $H_{Norb\text{-}linker}$), 1.77-1.83 (m, 3H, $H_{Norb\text{-}linker}$), 2.27-2.41 (m, 3H, $H_{Norb\text{-}linker}$, $H_{2'}$), 2.45 (t, *J* = 7.14 Hz, 2H, $H_{Norb\text{-}linker}$), 2.77 (bs, 1H, $H_{Norb\text{-}linker}$), 2.88 (bs, 1H, $H_{Norb\text{-}linker}$), 3.02 (t, *J* = 9.25 Hz, 1H, $H_{Norb\text{-}linker}$), 3.15 (dd, *J* = 9.25, 6.59 Hz, 1H, $H_{Norb\text{-}linker}$), 3.43-3.53 (m, 2H, $H_{Norb\text{-}linker}$), 3.55 (bs, 1H, OH), 3.82 (d, *J* = 10.05 Hz, 1H, $H_{5'}$), 3.88 (d, *J* = 10.05 Hz, 1H, $H_{5'}$), 3.94-3.55 (bs, 1H, OH), 4.03 (d, *J* = 3.10 Hz, 1H, $H_{4'}$), 4.49-4.55 (m, 1H, $H_{3'}$), 5.91 (dd, *J* = 5.65, 2.86 Hz, 1H, $H_{Norb\text{-}linker}$), 6.11 (dd, *J* = 5.65, 2.99 Hz, 1H, $H_{Norb\text{-}linker}$), 6.17 (t, *J* = 6.51 Hz, 1H, $H_{1'}$), 7.93 (s, 1H, H_6), 9.77 (bs, 1H, NH).

$^{13}C\{^{1}H\}$-NMR (125 MHz, $CDCl_3$, 25 °C, TMS): δ = 16.4, 28.5, 29.1, 29.7, 38.7 ($C_{Norb\text{-}linker}$), 40.5 ($C_{2'}$), 42.2, 44.0, 49.4 ($C_{Norb\text{-}linker}$), 62.0 ($C_{5'}$), 69.3 ($C_{Norb\text{-}linker}$), 71.2 ($C_{3'}$), 71.5, 74.6 ($C_{Norb\text{-}linker}$), 86.4 ($C_{1'}$), 87.3 ($C_{4'}$), 94.5 ($C_{Norb\text{-}linker}$), 100.6 (C_5), 132.4, 137.2 ($C_{Norb\text{-}linker}$), 142.9 (C_6), 149.7 (C_2), 162.3 (C_4).

MS (HR-ESI^{+}): m/z 439.1836 (calculated for $[C_{22}H_{28}N_2O_6+Na]^{+}$ 439.1840).

7-Deaza-7-(5-(*endo*-bicyclo[2.2.1]hept-5-ene-2-ylmethoxy)pent-1-ynyl)-2'-deoxyadenosine (dA^{Norb})

7-Deaza-7-iodo-2'-deoxyadenosine [2] (360 mg, 970 µmol 1 eq) was dissolved in 4.6 mL dry DMF, and $Pd(PPh_3)_4$ (114 mg, 91.5 µmol, 9.5 mol%) as well as CuI (46 mg, 206 µmol, 21.4 mol%) were added. After degassing, Hünig's Base (degassed, 423 µL, 1.94 mmol, 2 eq) was added and the mixture stirred for 10 min at RT. Then a degassed solution of *endo*-5-((pent-4-ynyloxy) methyl) bicyclo[2.2.1]hept-2-ene (509 mg, 2.68 mmol, 2.8 eq) in 2.3 mL dry DMF was added. After stirring for 15 h at RT, solvents were evaporated and the crude product was purified by flash chromatography (SiO_2, DCM/MeOH = 20:1 → 10:1 R_f (**dA^{Norb}**) = 0.24 (DCM/MeOH = 10:1)).

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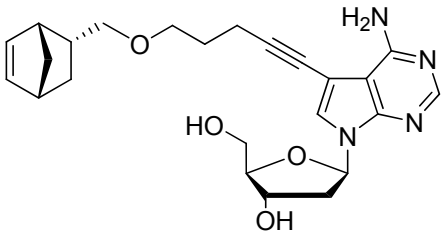

dA^{Norb}

Yield: 261 mg, 596 µmol, 62%.

Habitus: Yellow Solid.

^{1}H-NMR (500 MHz, $CDCl_3$, 25 °C, TMS): δ = 0.47 (ddd, *J* = 11.61, 4.41, 2.57 Hz, 1H, $H_{Norb-linker}$), 1.22 (d, *J* = 8.15 Hz, 1H, $H_{Norb-linker}$), 1.40 (ddd, *J* = 8.15, 4.10, 1.88 Hz, 1H, $H_{Norb-linker}$), 1.79 (ddd, *J* = 11.61, 9.15, 3.77 Hz, 1H, $H_{Norb-linker}$), 1.84 (qu, *J* = 6.70 Hz, 2H, $H_{Norb-linker}$), 2.23 (dd, *J* = 13.52, 5.83 Hz, 1H, $H_{2'}$), 2.33 (dtd, *J* = 13.21, 9.30, 4.10 Hz, 1H, $H_{Norb-linker}$), 2.53 (t, *J* = 6.70 Hz, 2H, $H_{Norb-linker}$), 2.77 (bs, 1H, $H_{Norb-linker}$), 2.87 (d, *J* = 0.57 Hz, 1H, OH), 2.89 (bs, 1H, $H_{Norb-linker}$), 2.94 (s, 1H, OH), 2.98-3.04 (m, 2H, $H_{Norb-linker}$, $H_{2'}$), 3.15 (dd, *J* = 9.30, 6.57 Hz, 1H, $H_{Norb-linker}$), 3.43-3.55 (m, 2H, $H_{Norb-linker}$), 3.75 (d, *J* = 12.00 Hz, 1H, $H_{5'}$), 3.93 (dd, *J* = 12.00, 1.79 Hz, 1H, $H_{5'}$), 4.15-4.17 (m 1H, $H_{4'}$), 4.72 (d, *J* = 5.04 Hz, 1H, $H_{3'}$), 5.86 (bs, 2H, NH_2), 5.91 (dd, *J* = 5.70, 2.89 Hz, 1H, $H_{Norb-linker}$), 6.10 (dd, *J* = 5.70, 3.03 Hz, 1H, $H_{Norb-linker}$), 6.17 (dd, *J* = 9.38, 5.83 Hz, 1H, $H_{1'}$), 7.09 (s, 1H, H_8), 8,18 (s, 1H, H_2).

$^{13}C\{^{1}H\}$-NMR (125 MHz, $CDCl_3$, 25 °C, TMS): δ = 16.4, 28.7, 29.1, 38.7, ($C_{Norb-linker}$), 40.5 ($C_{2'}$), 42.1, 43.9, 49.4 ($C_{Norb-linker}$), 63.4 ($C_{5'}$), 69.2 ($C_{Norb-linker}$), 73.1, 73.2 ($C_{3'}$, $C_{Norb-linker}$), 74.7 ($C_{Norb-linker}$), 89.1, 89.4 ($C_{1'}$, $C_{4'}$), 92.4 (C_7), 95.9 ($C_{Norb-linker}$), 105.1 (C_5), 127.5 (C_8), 132.3, 137.2 ($C_{Norb-linker}$), 147.8 (C_4), 152.0 (C_2), 157.9 (C_6).

MS (HR-ESI$^+$): m/z 461.2153 (calculated for $[C_{24}H_{30}N_4O_4+Na]^+$ 461.2159).

5-(5-(*endo*-Bicyclo[2.2.1]hept-5-ene-2-ylmethoxy)pent-1-ynyl)-2'-deoxycytosine (dC^{Norb})

5-Iodo-2'-deoxycytosine (337 mg, 970 µmol, 1 eq, Berry & Associates) was dissolved in 4.6 mL dry DMF, and $Pd(PPh_3)_4$ (114 mg, 91.5 µmol 9.5 mol%) as well as CuI (46.0 mg, 206 µmol, 21.4 mol%) were added. After degassing, Hünig's Base (degassed, 423 µL, 1.94 mmol, 2 eq) was added and the mixture stirred for 10 min at RT. Then a degassed solution of *endo*-5-((pent-4-ynyloxy) methyl) bicyclo[2.2.1]hept-2-ene (509 mg, 2.68 mmol, 2.8 eq) in 2.3 mL dry DMF was added. After stirring for 15 h at RT, solvents were evaporated and the crude product was

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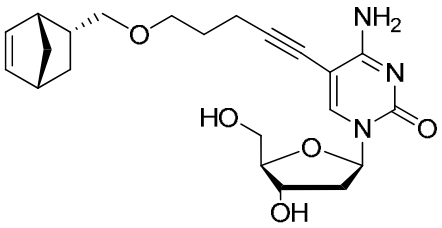
purified by flash chromatography (SiO_2, DCM/MeOH = 20:1 → 20:3, R_f (**dC^{Norb}**) = 0.23 (DCM/ MeOH = 10:1)).



dC^{Norb}

Yield: 107 mg, 262 µmol, 27%, R_f = 0.23 (DCM/MeOH = 10:1).

Habitus: White solid.

^{1}H-NMR (500 MHz, $CDCl_3$, 25 °C, TMS): 0.47 (ddd, *J* = 11.65, 4.44, 2.56 Hz, 1H, $H_{Norb\text{-}linker}$), 1.23 (d, *J* = 8.08 Hz, 1H, $H_{Norb\text{-}linker}$), 1.41 (ddd, *J* = 8.08, 3.94, 1.92 Hz, 1H, $H_{Norb\text{-}linker}$), 1.77-1.85 (m, 3H, $H_{Norb\text{-}linker}$), 2.27-2.36 (m, 2H, $H_{Norb\text{-}linker}$, $H_{2'}$), 2.44-2.49 (m, 1H, $H_{2'}$), 2.49 (t, *J* = 7.07 Hz, 2H, $H_{Norb\text{-}linker}$), 2.78 (bs, 1H, $H_{Norb\text{-}linker}$), 2.88 (bs, 2H, OH, $H_{Norb\text{-}linker}$), 2.95 (s, OH), 3.03 (t, *J* = 9.10 Hz, 1H, $H_{Norb\text{-}linker}$), 3.15 (dd, *J* = 9.10, 6.58 Hz, 1H, $H_{Norb\text{-}linker}$), 3.47 (dtd, *J* = 15.80, 9.77, 5.79 Hz, 2H, $H_{Norb\text{-}linker}$), 3.87-3.91 (m, 2H, $H_{5'}$), 4.01 (dd, *J* = 7.04, 3.03 Hz, 1H, $H_{4'}$), 4.53 (dd, *J* = 10.41, 5.77 Hz, 1H, $H_{3'}$), 5.91 (dd, *J* = 5.67, 2.81 Hz, 1H, $H_{Norb\text{-}linker}$), 6.00 (bs, 2H, NH_2), 6.08-6.13 (m, 2H, $H_{1'}$, $H_{Norb\text{-}linker}$), 8.09 (s, 1H, H_6).

$^{13}C\{^1H\}$-NMR (125 MHz, $CDCl_3$, 25 °C, TMS): δ =16.6, 28.7, 29.2, 38.7 ($C_{Norb\text{-}linker}$), 40.9 ($C_{2'}$), 42.2, 44.0, 49.4 ($C_{Norb\text{-}linker}$), 61.5 ($C_{5'}$), 69.4 ($C_{Norb\text{-}linker}$), 70.0 ($C_{3'}$), 71.2, 74.6 ($C_{Norb\text{-}linker}$), 87.4, 87.6 ($C_{1'}$, $C_{4'}$), 91.9 ($C_{Norb\text{-}linker}$), 96.5 (C_5), 132.3, 137.3 ($C_{Norb\text{-}linker}$), 144.3 (C_6), 154.5 (C_2), 164.7 (C_4).

MS (HR-ESI$^+$): m/z 438.2001 (calculated for $[C_{22}H_{29}N_3O_5+Na]^+$ 438.1999).

Synthesis of modified triphosphates

General procedure:

Under argon atmosphere, the desired nucleobase (120 µmol) was dissolved in freshly distilled trimethylphosphate (226 µL), cooled to 0°C and $POCl_3$ (11.8 µL, 120 µmol, freshly distilled) was added. The reaction mixture was then stirred for 1 h at 0 °C. Afterwards, a precooled solution of tributylammoniumpyrophosphate (249 mg, 453 µmol) in tributylamine (91 µL) and DMF (906 µL) was added at -15 °C (ice/NaCl-bath). After stirring at -15 °C for 1 h, water (0.5 mL,

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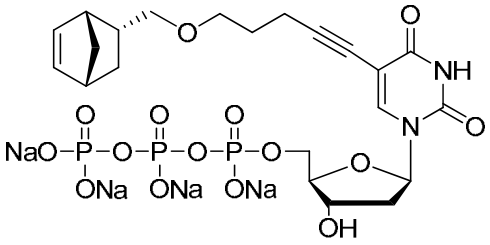
basified with triethylamine) was added and stirring continued for 5 min at RT. Solvents were removed under reduced pressure and the crude product coevaporated with water (3x). The residue was dissolved in water and after extraction with chloroform purified *via* column chromatography or HPLC. Fractions containing the nucleoside triphosphate were lyophilised and finally converted to the more stable Na-Salt *via* ion-exchange (Na^+-cycle).

5-(*endo*-Bicyclo[2.2.1]hept-5-ene-2-ylmethoxypent-1-ynyl)-2'-deoxyuridine-5'-triphosphate (dUTP^{Norb})



dUTP^{Norb}

Yield: 15.6 mg, 21 µmol, 18%

Column chromatography: C18-column, increase from 1% buffer B to 18% buffer B over 17 min, increase from 18% buffer B to 80% buffer B over 13 min, R_t (**dUTP^{Norb}**) = 23 min.

Habitus: Pale yellow solid.

^{1}H-NMR (500 MHz, D_2O, 25 °C, TMS): δ = 0.44 (dddd, *J* = 11.35, 6.90, 4.41, 2.59 Hz, 1H, $H_{Norb\text{-}linker}$), 1.22-1.24 (m, 1H, $H_{Norb\text{-}linker}$), 1.35-1.38 (m, 1H, $H_{Norb\text{-}linker}$), 1.76-1.82 (m, 1H, $H_{Norb\text{-}linker}$), 1.99 (quin, *J* = 6.75 Hz, 2H, $H_{Norb\text{-}linker}$), 2.23-2.32 (m, 1H, $H_{Norb\text{-}linker}$), 2.36-2.41 (m, 1H, $H_{2'}$), 2.62-2.67 (m, 1H, $H_{2'}$), 2.77-2.83 (m, 4H, $H_{Norb\text{-}linker}$), 3.10-3.22 (m, 2H, $H_{Norb\text{-}linker}$), 3.53-3.62 (m, 2H, $H_{Norb\text{-}linker}$), 4.28-4.36 (m, 3H, $H_{4'}$, $H_{5'}$), 4.64-4.67 (m, 1H, $H_{3'}$), 5.94-5.97 (m, 1H, $H_{Norb\text{-}linker}$), 6.20 (dd, *J* = 5.65, 3.04 Hz, 1H, H $H_{Norb\text{-}linker}$), 6.39 (t, *J* = 6.51 Hz, 1H, $H_{1'}$), 8.80 (s, 1H, H_6).

$^{13}C\{^1H\}$-NMR, APT (125 MHz, D_2O, 25 °C, TMS): δ = 15.6, 24.5, 28.4, 37.9 ($C_{Norb\text{-}linker}$), 40.0 ($C_{2'}$), 41.9, 43.6, 48.9 ($C_{Norb\text{-}linker}$), 64.6 ($C_{5'}$), 69.2 ($C_{Norb\text{-}linker}$), 69.3 ($C_{3'}$), 69.5, 74.3 ($C_{Norb\text{-}linker}$), 86.0 ($C_{4'}$), 88.0 ($C_{1'}$), 100.3 ($C_{Norb\text{-}linker}$), 109.8 (C_5), 132.2, 137.7 ($C_{Norb\text{-}linker}$), 156.0 (C_6), 160.0 (C_2), 171.4 (C_4).

$^{31}P\{^1H\}$-NMR (121 MHz, D_2O, 25 °C, phosphate buffer, δ = 2.35 ppm): δ = -6.66 (d, *J* = 20.1 Hz, P_γ), -10.84 (d, *J* = 20.1 Hz, P_α), -21.76 (t, *J* = 20.1 Hz, P_β).

MS (HR-ESI$^-$): m/z 655.0896 (calculated for $[C_{22}H_{31}N_2O_{15}P_3\text{-}H]^-$ 655.0854).

UV (H_2O): λ_{max} = 230 nm (ε = (10239 ± 355) $Lmol^{-1}cm^{-1}$), 296 nm (ε = (5717 ± 196) $Lmol^{-1}cm^{-1}$).

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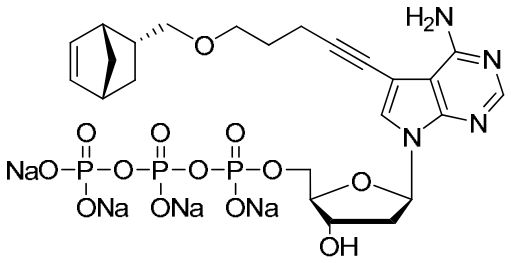
7-Deaza-7-(5-(*endo*-bicyclo[2.2.1]hept-5-ene-2-ylmethoxy)pent-1-ynyl)-2'-deoxyadenosine-5'-triphosphate (dATP^{Norb})



dATP^{Norb}

Yield: 12.5 mg, 16.3 µmol, 14%.

Purification: Reversed phase HPLC, gradient: Increase from 25% buffer B to 65 % buffer B over 15 min, R_t (**dATP^{Norb}**) = 13.3 min.

Habitus: White solid.

^{1}H-NMR (500 MHz, D_2O, 25 °C, TMS): δ = 0.46 (tdd, *J* = 11.45, 4.36, 2.21 Hz, 1H, $H_{Norb\text{-}linker}$), 1.16-1.20 (m, 1H, $H_{Norb\text{-}linker}$), 1.30-1.33 (m, 1H, $H_{Norb\text{-}linker}$), 1.78 (dddd, *J* = 11.45, 9.26, 3.94, 1.78 Hz, 1H, $H_{Norb\text{-}linker}$), 1.89 (qu, *J* = 6.75 Hz, 2H, $H_{Norb\text{-}linker}$), 2.26-2.39 (m, 1H, $H_{Norb\text{-}linker}$), 2.48 (ddd, *J* = 14.03, 6.23, 3.43 Hz, 1H, $H_{2'}$), 2.59 (t, *J* = 6.75 Hz, 2H, $H_{Norb\text{-}linker}$), 2.68 (ddd, *J* = 14.03, 7.73, 6.44 Hz, 1H, $H_{2'}$), 2.76 (bs, 1H, $H_{Norb\text{-}linker}$), 2.86 (bs, 1H, $H_{Norb\text{-}linker}$), 3.15 (dt, *J* = 9.84, 1.33 Hz, 1H, $H_{Norb\text{-}linker}$), 3.25 (dd, *J* = 9.84, 7.09 Hz, 1H, $H_{Norb\text{-}linker}$), 3.57-3.72 (m, 2H, $H_{Norb\text{-}linker}$), 4.10-4.22 (m, 2H, $H_{5'}$), 4.25 (dd, *J* = 7.42, 3.26 Hz, 1H, $H_{4'}$), 4.76 (td, *J* = 6.44, 3.26 Hz, 1H, $H_{3'}$), 5.94-5.97 (m, 1H, $H_{Norb\text{-}linker}$), 6.13 (dd, *J* = 5.44, 2.96 Hz, 1H, $H_{Norb\text{-}linker}$), 6.60 (dd, *J* = 7.73, 6.23 Hz, 1H, $H_{1'}$), 7.62 (s, 1H, H_8), 8.14 (s, 1H, H_2).

$^{13}C\{^1H\}$-NMR (125 MHz, D_2O, 25 °C, TMS): δ = 15.8, 27.3, 28.4, 37.9 ($C_{Norb\text{-}linker}$), 38.4 ($C_{2'}$), 41.9, 43.6, 48.8 ($C_{Norb\text{-}linker}$), 63.4 ($C_{5'}$, $J_{P\text{-}C}$ = 5.70 Hz), 69.3 ($C_{Norb\text{-}linker}$), 70.9, 73.0 ($C_{3'}$, $C_{Norb\text{-}linker}$), 74.4 ($C_{Norb\text{-}linker}$), 82.7 ($C_{1'}$), 85.1 ($C_{4'}$, $J_{P\text{-}C}$ = 8.81 Hz), 93.4 (C_7), 97.2 ($C_{Norb\text{-}linker}$), 103.0 (C_5), 125.2 (C_8), 132.2, 137.6 ($C_{Norb\text{-}linker}$), 148.5 (C_4), 152.2 (C_2), 157.5 (C_6).

$^{31}P\{^1H\}$-NMR (121 MHz, D_2O, 25 °C, phosphate buffer, δ = 2.35 ppm): δ = -6.49 (d, *J* = 19.7 Hz, P_γ), -10.73 (d, *J* = 19.7 Hz, P_α), -21.76 (t, *J* = 19.7 Hz, P_β).

MS (HR-ESI$^-$): m/z 677.1228 (calculated for $[C_{24}H_{32}N_4O_{13}P_3\text{-}H]^-$ 677.1184).

UV (H_2O): λ_{max} = 237 nm (ε = (12540 ± 400) $Lmol^{-1}cm^{-1}$), 280 nm (ε = (9020 ± 370) $Lmol^{-1}cm^{-1}$).

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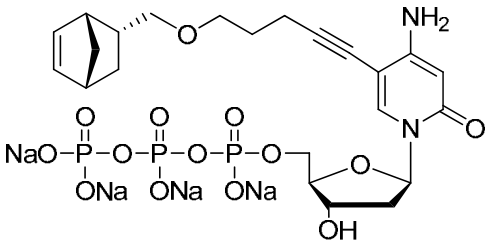
5-(5-(*endo*-Bicyclo[2.2.1]hept-5-ene-2-ylmethoxy)pent-1-ynyl)-2'-deoxycytosin-5'-triphosphate (dCTP$^{Norb)}$



dCTP^{Norb}

Yield: 19.4 mg, 26.1 µmol, 22%.

Purification: Reversed phase HPLC, gradient: Increase from 25% buffer B to 65 % buffer B over 15 min, R_t (**dCTP2**) = 11.7 min.

Habitus: White solid.

^{1}H-NMR (500 MHz, D_2O, 25 °C, TMS): δ = 0.49 (ddd, *J* = 11.49, 4.10, 2.78 Hz, 1H, $H_{Norb\text{-}linker}$), 1.26 (d, *J* = 8.08 Hz, 1H, $H_{Norb\text{-}linker}$), 1.35-1.40 (m, 1H, $H_{Norb\text{-}linker}$), 1.79-1.90 (m, 3H, $H_{Norb\text{-}linker}$), 2.27-2.39 (m, 2H, $H_{Norb\text{-}linker}$, $H_{2'}$), 2.44 (ddd, *J* = 14.26, 6.58, 4.28 Hz, 1H, $H_{2'}$), 2.56 (t, *J* = 7.00 Hz, 2H, $H_{Norb\text{-}linker}$), 2.81 (bs, 1H, $H_{Norb\text{-}linker}$), 2.89 (bs, 1H, $H_{Norb\text{-}linker}$), 3.17 (t, *J* = 9.63 Hz, 1H, $H_{Norb\text{-}linker}$), 3.26 (dd, *J* = 9.63, 6.73 Hz, 1H, $H_{Norb\text{-}linker}$), 3.56-3.72 (m, 2H, $H_{Norb\text{-}linker}$), 4.16-4.26 (m, 3H, $H_{4'}$, $H_{5'}$), 4.62 (td, *J* = 6.52, 3.44 Hz, 1H, $H_{3'}$), 6.00 (dd, *J* = 5.65, 2.77 Hz, 1H, $H_{Norb\text{-}linker}$), 6.20 (dd, *J* = 5.65, 2.87 Hz, 1H, $H_{Norb\text{-}linker}$), 6.28 (t, *J* = 6.58 Hz, 1H, $H_{1'}$), 8.05 (s, 1H, H_6).

$^{13}C\{^1H\}$-NMR (125 MHz, D_2O, 25 °C, TMS): δ = 18.4, 29.8, 31.0, 40.4 ($C_{Norb\text{-}linker}$), 41.5 ($C_{2'}$), 44.5, 46.2, 51.4 ($C_{Norb\text{-}linker}$), 61.5 ($C_{5'}$, $J_{P\text{-}C}$ = 5.68 Hz), 71.9 ($C_{Norb\text{-}linker}$), 72.7 ($C_{3'}$), 73.3, 76.9 ($C_{Norb\text{-}linker}$), 87.9 (C_4, $J_{P\text{-}C}$ = 8.81 Hz), 88.6 ($C_{1'}$), 95.9 ($C_{Norb\text{-}linker}$), 99.7 (C_5), 134.8, 140.3 ($C_{Norb\text{-}linker}$), 146.2(C_6), 158.6 (C_2), 167.8 (C_4).

$^{31}P\{^1H\}$-NMR (121 MHz, D_2O, 25 °C, phosphate buffer, δ = 2.35 ppm): δ = -6.47 (d, *J* = 19.9 Hz, P_γ), -10.82 (d, *J* = 19.9 Hz, P_α), -21.78 (t, *J* = 19.9 Hz, P_β).

MS (HR-ESI$^-$): m/z 654.1049 (calculated for $[C_{22}H_{31}N_3O_{14}P_3\text{-}H]^-$ 654.1024).

UV (H_2O): λ_{max} = 236 nm (ε = (7590 ± 390) $Lmol^{-1}cm^{-1}$), 297 nm (ε = (3970 ± 220) $Lmol^{-1}cm^{-1}$).

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Incorporation of dNTPs^{Norb} *via* primer extension and PCR

Primer extension:

1) Radioactive labeling

To 1 µM primer in PNK buffer A (50 mM Tris-HCl pH = 7.6, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine) was added 1 µM γ-^{32}P-ATP (final: 50 µCi/16.7µL, stock solution: 10 µCi/µL, Hartmann Analytics) and 0.1 U/µL polynucleotide kinase (Fermentas). The reaction mixture was incubated for 1 h at 37 °C and then purified over a 10% denaturing polyacrylamide gel using standard electrophoresis conditions (1XTBE buffer, 1 h 10 min @ 17 W). Primer bands were excised and eluted in 0.3 M Na-Acetate pH 5.5 overnight at room temperature. The eluted solution was isopropanol precipitated after addition of 5 µL glycogen (20µg/µL, Fermentas).

2) Primer extension

To 0.225 µM template in Phusion HF buffer and 0.15 µM radioactively labeled primer (γ-^{32}P-ATP, Hartmann Analytics) were added **dNTPs^{Norb}** and dNTPs (dATP, dGTP, dCTP, dUTP/dTTP, stock solution: 1 mM, Rapidozym, final concentration: 0.125 µM), respectively. After addition of 0.02 U/µL Phusion High Fidelity DNA polymerase (Thermo Fisher Scientific), the reaction mixture was incubated for 30 min at 60 °C. Then the reaction was stopped by addition of gel loading buffer and analysed *via* denaturing polyacrylamide gel electrophoresis.

Sequences (Primer region is in **bold**, templating sequence for elongation is in *italics*):

Primer: 5'-**GCAGTGAAGGCTGAGCTCC**-3' (IBA, Göttingen)
Template 1: 5'-*TCTAATACGATCTACTATA***GGAGCTCAGCCTTCACTGC**-3' (IBA, Göttingen)
Template 2: 5'-*TCTACTGCGCCTCGCTGTC***GGAGCTCAGCCTTCACTGC**-3' (IBA, Göttingen)
Template 3: 5'-*TCTAATGCGCCTCGCTGTC***GGAGCTCAGCCTTCACTGC**-3' (IBA, Göttingen)
Template 4: 5'-*TCTAATACGCCTCGCTGTC***GGAGCTCAGCCTTCACTGC**-3' (IBA, Göttingen)
Template 5: 5'-*TCTAATACGACTCGCTGTC***GGAGCTCAGCCTTCACTGC**-3' (IBA, Göttingen)
Template 6: 5'-*TCTAATACGACTCACTGTC***GGAGCTCAGCCTTCACTGC**-3' (IBA, Göttingen)
Template 7: 5'-*TCTAATACGACTCACTATC***GGAGCTCAGCCTTCACTGC**-3' (IBA, Göttingen)
Template 8: 5'-*ACTGAATGCGACTCGTACG***GGAGCTCAGCCTTCACTGC**-3' (Biomers, Ulm)

PCR

70mer template:

Synthesised triphosphate **dUTP^{Norb}** was enzymatically incorporated *via* PCR using *KOD-XL* DNA polymerase. For PCR reaction, standard protocols were followed using 22 ng template, 0.5 µM of each forward and reverse primer, 0.2 mM of each dNTP and 0.05 U/µl *KOD-XL* DNA

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polymerase (Merck) in a final volume of 50 µL. For removing primers, the PCR products were purified using a Qiagen PCR purification kit.

Step		Temperature [°C]	Time [min]
1	Denaturation	94	0.5
2	Denaturation	94	0.5
3	Annealing	54	0.5
4	Elongation	72	1
5	15 times to step 2		
6	Final elongation	74	10

Sequences:

3‘-Primer: 5‘-CGATGATAAGCTGTCAAACATG-3‘
5‘-Primer: 5‘-GCATTAGTGTATCAACAAGCTG-3‘
Template: (primer binding sites shown in **bold**)
5’-**GCATTAGTGTATCAACAAGCTG**GGGATCCTAGAAGCTTATCGAATTCT**CATGTTTGACAGCTTATCATCG**-3’

The inhibition of archaebacterial DNA polymerases [3, 4] by dU-modified template strands causes lower amounts of PCR product in case of dUTP and **dUTP^{Norb}**, respectively. Importantly, no difference in efficiency is detectable comparing dUTP and norbornene-modified **dUTP^{Norb}** (figure S1, lane 5 and 6). The absence of any band in the control lanes, where no template (figure S1, lane 2) or neither dUTP, dTTP nor **dUTP^{Norb}** were added (figure S1, lane 3), verifies the successful amplification of the template employing **dUTP^{Norb}**.

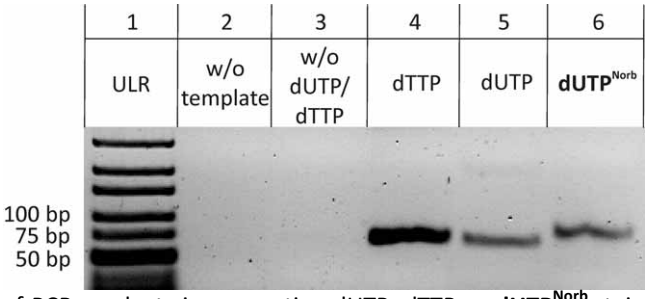

Figure S1. 3% Agarose gel of PCR products incorporating dUTP, dTTP or **dUTP^{Norb}** stained with ethidium bromide. ULR: Ultra low range base pair ladder (Fermentas).

429mer template:

Synthesised triphosphates **dUTP^{Norb}**, **dCTP^{Norb}** and **dATP^{Norb}** were enzymatically incorporated *via* PCR using *KOD-XL* DNA polymerase. For PCR reaction, standard protocols were followed using

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77 ng template, 0.5 µM of each forward and reverse primer, 0.2 mM of each dNTP and 0.05 U/µl *KOD-XL* DNA polymerase (Merck) in a final volume of 100 µL. For removing primers, the PCR products were purified using a Qiagen PCR purification kit.

Step		Temperature [°C]	Time [min]
1	Denaturation	94	0.5
2	Denaturation	94	0.5
3	Annealing	54	0.5
4	Elongation	72	1
5	20 times to step 2		
6	Final elongation	74	10

Sequences:

3‘-Primer: 5‘-CGATGATAAGCTGTCAAACATG-3‘
5‘-Primer: 5‘-GCATTAGTGTATCAACAAGCTG-3‘
Template (*LysC* in pDG1661): [1] (primer binding sites shown in **bold**)
5‘-**GCATTAGTGTATCAACAAGCTG**GGATCCAAGAGCGCTTTGTCCACAATAAAAAAAGCAATGAGAG
GAATACTCTCATTGCTTATCAATTAATCATCATAAATGATTATGAACAACGAGATAGCCCTCCAAGAAAAT
GATTTCTTGACAGCCTTACATTTATTCAATGCACGGCCAGCAAATAATACCGATGGGGTTTTATTTGCTTC
GGCGACGCTCCCCTTTCAGCCTTTTTCACAGAATCCATCTTTCTCCAAAGGCATACTCTTGAAGTTCGCACC
TCTATCTTCACCATATAACACGATCTAACTATGAAATTAGTTCACATTTTATCAGATCTTATTTAAAAGGAC
AACATTATTTTTGCAATTTGTCGTAAAGGAGCTGAGCCGCCGCTGCGGAGAATTCT**CATGTTTGACAGCT
TATCATCG**-3’

Sequencing of PCR products

70mer:

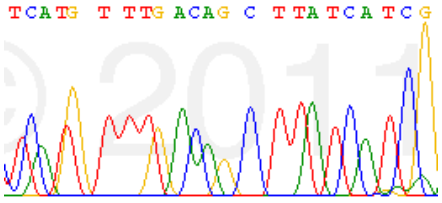
20 ng of purified PCR product were mixed with 20 pmol 5‘-primer in a final volume of 7 µl and submitted for sequencing (Extended Hot Shot Sequencing, Seqlab).

a) Sequence of PCR template

47bp-TCATG TTTG ACAGC TTA TCATCG

b) PCR product of a PCR reaction using an unmodified template and natural dNTPs

[1] Plasmid pDG1661KH1 was prepared by K. Höfer and *LysC* (= template) hence obtained *via* PCR.



c) PCR product of a PCR reaction using an unmodified template, dUTP^{Norb}, dCTP, dATP and dGTP

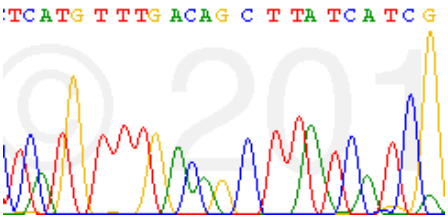


Figure S2. Representative section of sequencing results using dUTP (b) or **dUTP^{Norb}** (c) for PCR.

429mer:

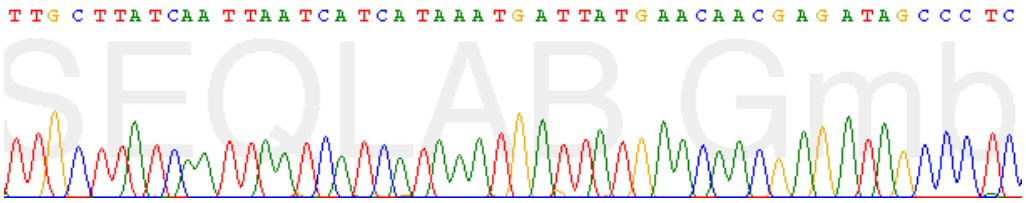
For sequencing, the modified ds429mer PCR products were purified using a Qiagen PCR purification kit and used as template for PCR reactions employing natural dNTPs. For PCR reaction, standard protocols were followed using 38 ng template, 0.5 µM of each forward and reverse primer, 0.2 mM of each dNTP and 0.05 U/µl *KOD-XL* DNA polymerase (Merck) in a final volume of 100 µL. The obtained unmodified PCR products were purified using a Qiagen PCR purification kit.

100 ng of purified PCR product were mixed with 20 pmol 3'-primer in a final volume of 7 µl and submitted for sequencing (Extended Hot Shot Sequencing, Seqlab).

a) Sequence of PCR template

75bp-TTG C TTA TCAA TTAA TCA TCA TAAA TG AT TA TGAA CAAC GAG A TAG CCC TC-303bp

b) PCR product of a PCR reaction using an unmodified template and natural dNTPs



c) PCR product of a PCR reaction using a modified template (obtained by PCR using **dUTP^{Norb}**, **dATP^{Norb}**, **dCTP^{Norb}**, **dGTP**) and natural dNTPs

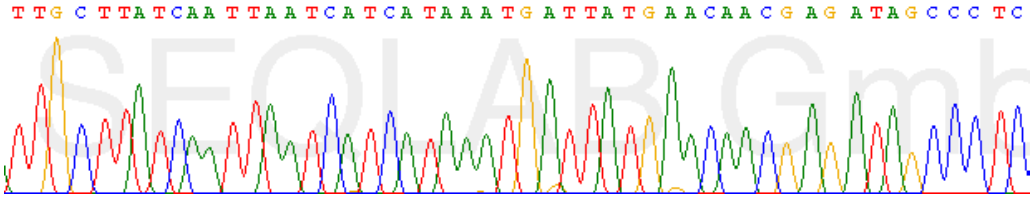
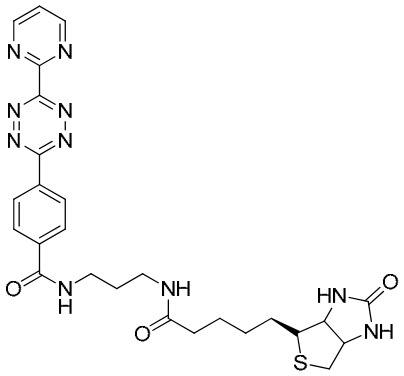


Figure S3. Representative section of sequencing results using unmodified (b) or modified templates (c) for PCR.

DARinv on PEX and PCR products

Tetrazine used:



biotin-tetrazine

PEX products:

For DARinv, norbornene-modified PEX reaction mixtures were purified over a polyacrylamide gel (10%, 1X TBE buffer, run for 1 h 30 min @ RT) and PEX product bands excised and eluted in 0.3 M Na-acetate pH 5.5 overnight at room temperature. The eluted solution was isopropanol precipitated (after addition of glycogen) and then dissolved in neutral water (Millipore water, MilliQ). For DARinv, PEX products (1 µM) were incubated with a 10-fold excess of biotin-tetrazine in water for 60 min at RT. After incubation, 1 eq of streptavidin (with respect to biotin, New England Biolabs) as well as gel loading buffer were added to the reaction mixture and then loaded on a gel. The DARinv product formation was analysed on a 12% denaturing polyacrylamide gel under standard electrophoresis conditions.

PCR products:

PCR products (3 µL of a 0.4 µM aqueous solution, final concentration: 0.3 µM) were mixed with biotin-tetrazine (1 µL of a 550 µM stock solution in DMSO) and incubated for 1 h at RT. After addition of gel loading buffer product formation was analysed on a 2% agarose gel.

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To confirm product formation, a second DARinv was performed and after incubation for 1 h at RT 1 eq of streptavidin was added to the reaction mixture. After addition of gel loading buffer, samples were loaded onto a 2% agarose gel and the gel run under standard electrophoresis conditions (Fig. S3).

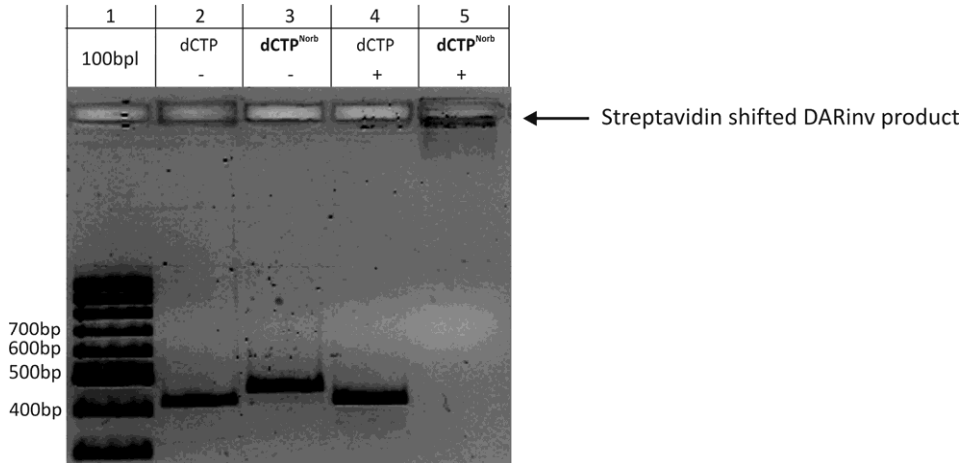

Figure S4. 2% agarose gel (run for 35 min at 150 V) of DARinv on norbornene-modified PCR products (KOD-XL DNA polymerase) stained with ethidium bromide. All samples were incubated for 1 h at RT and treated with 1 eq of streptavidin before loading. Lane 1: 100 base pair ladder; lane 2: Unmodified PCR product; lane 3: Norbornene-modified PCR product (**dCTP^{Norb}**); lane 4: Unmodified PCR product incubated with biotin-tetrazine; lane 5: Norbornene-modified PCR product (**dCTP^{Norb}**) incubated with biotin-tetrazine.

References:

1. K. Gutsmiedl, C. T. Wirges, V. Ehmke and T. Carell, *Org. Lett.*, 2009, **11**, 2405-2408.
2. F. Seela and M. Zulauf, *Synthesis*, 1996, 726-730.

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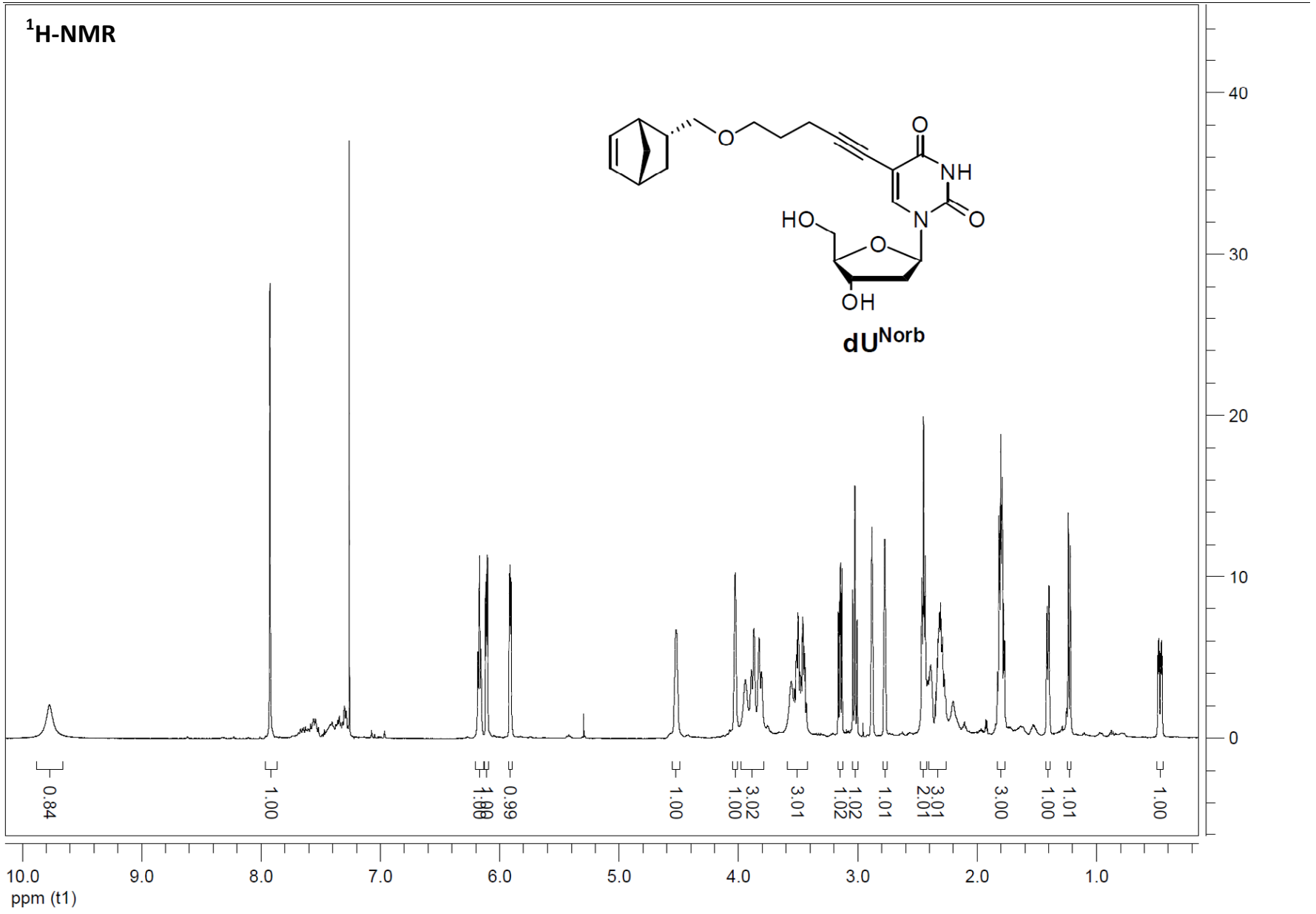

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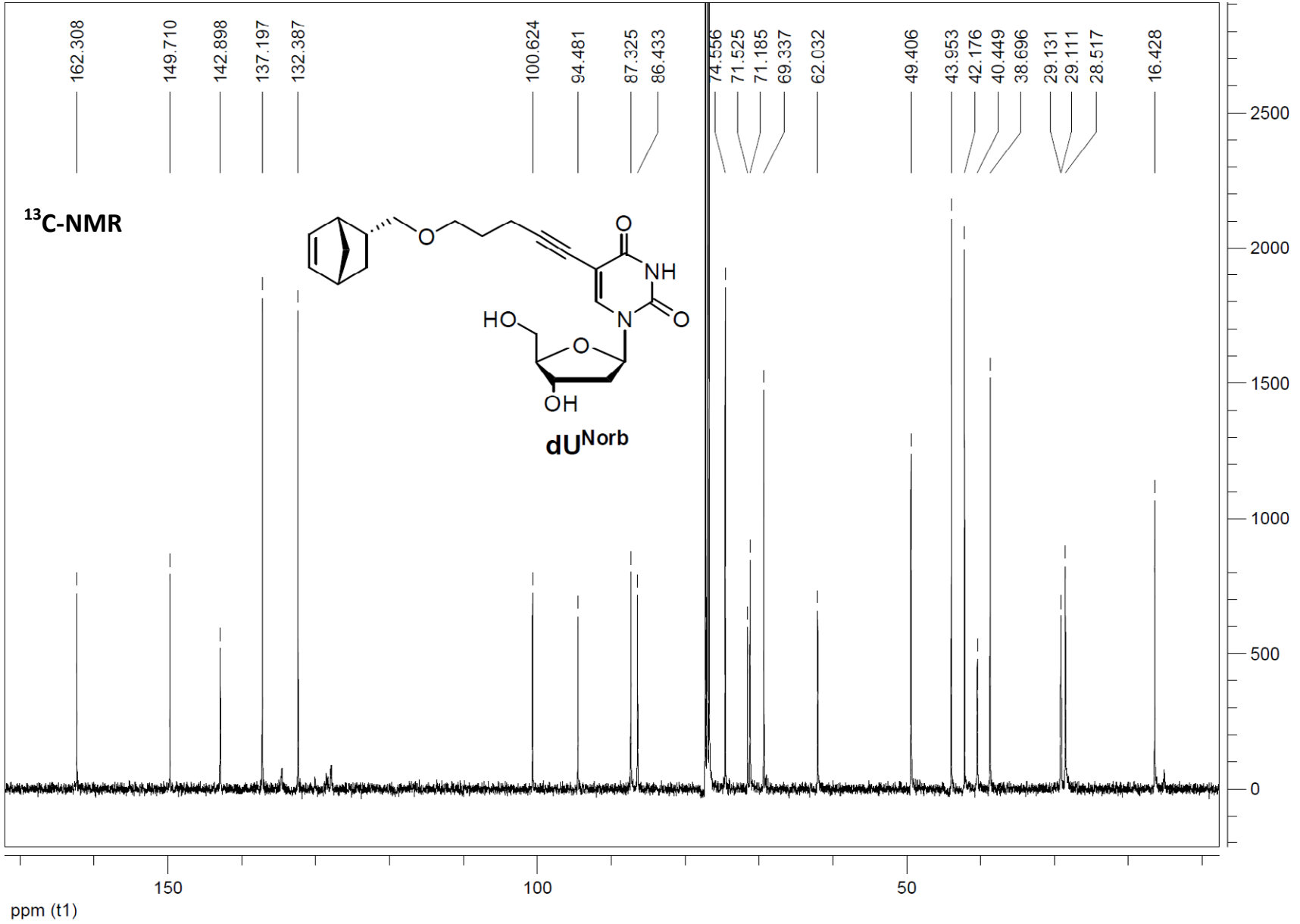

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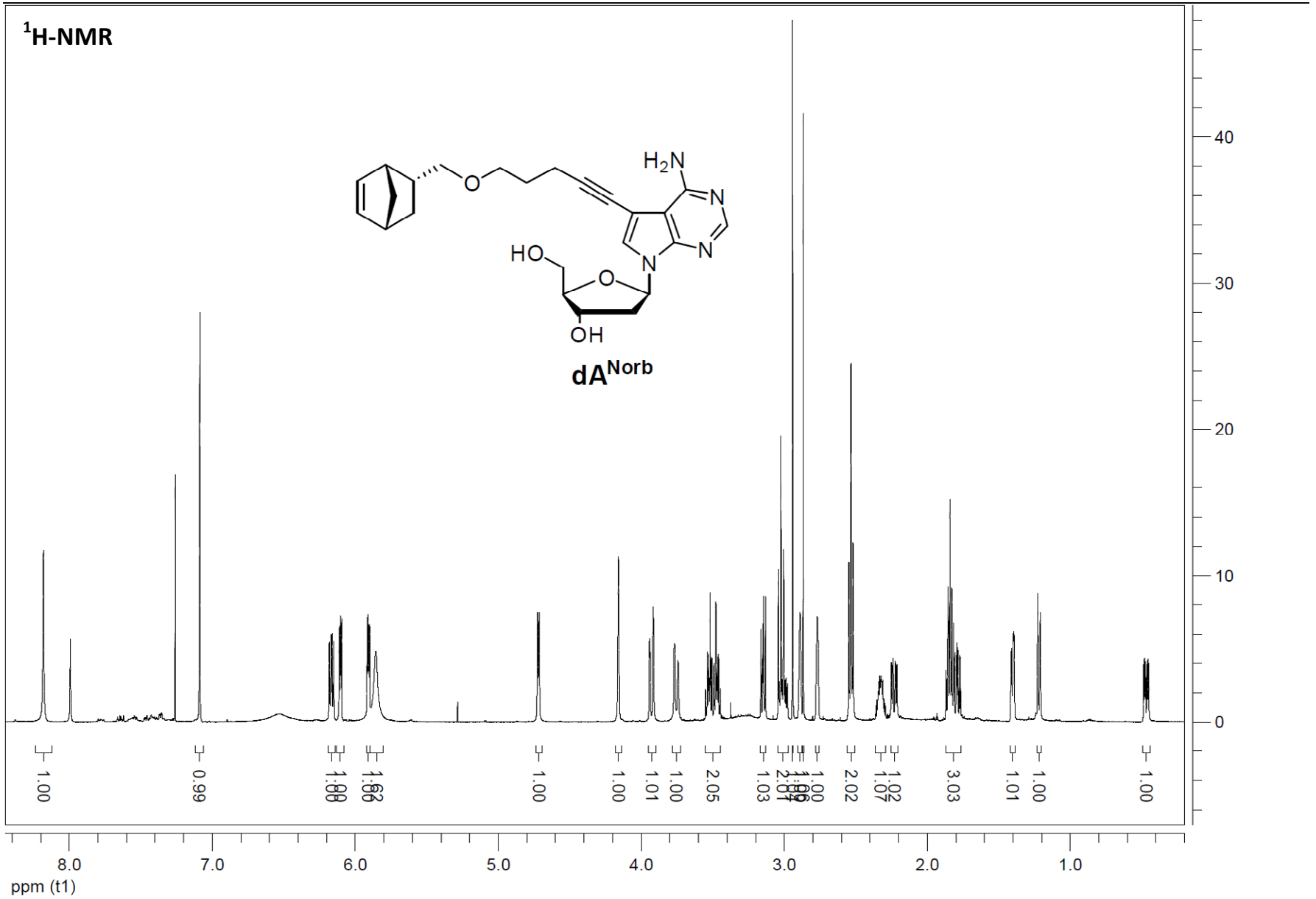

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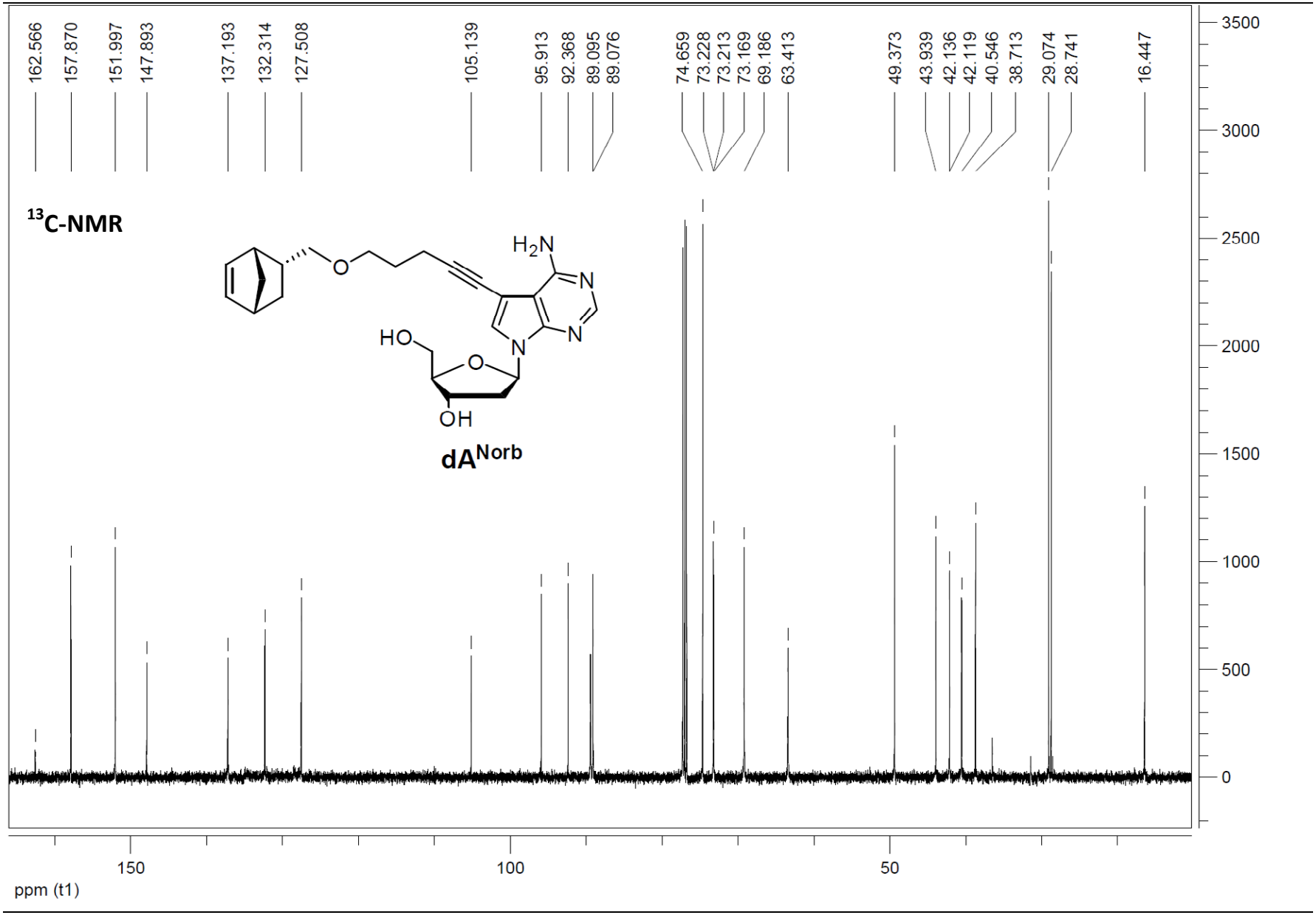

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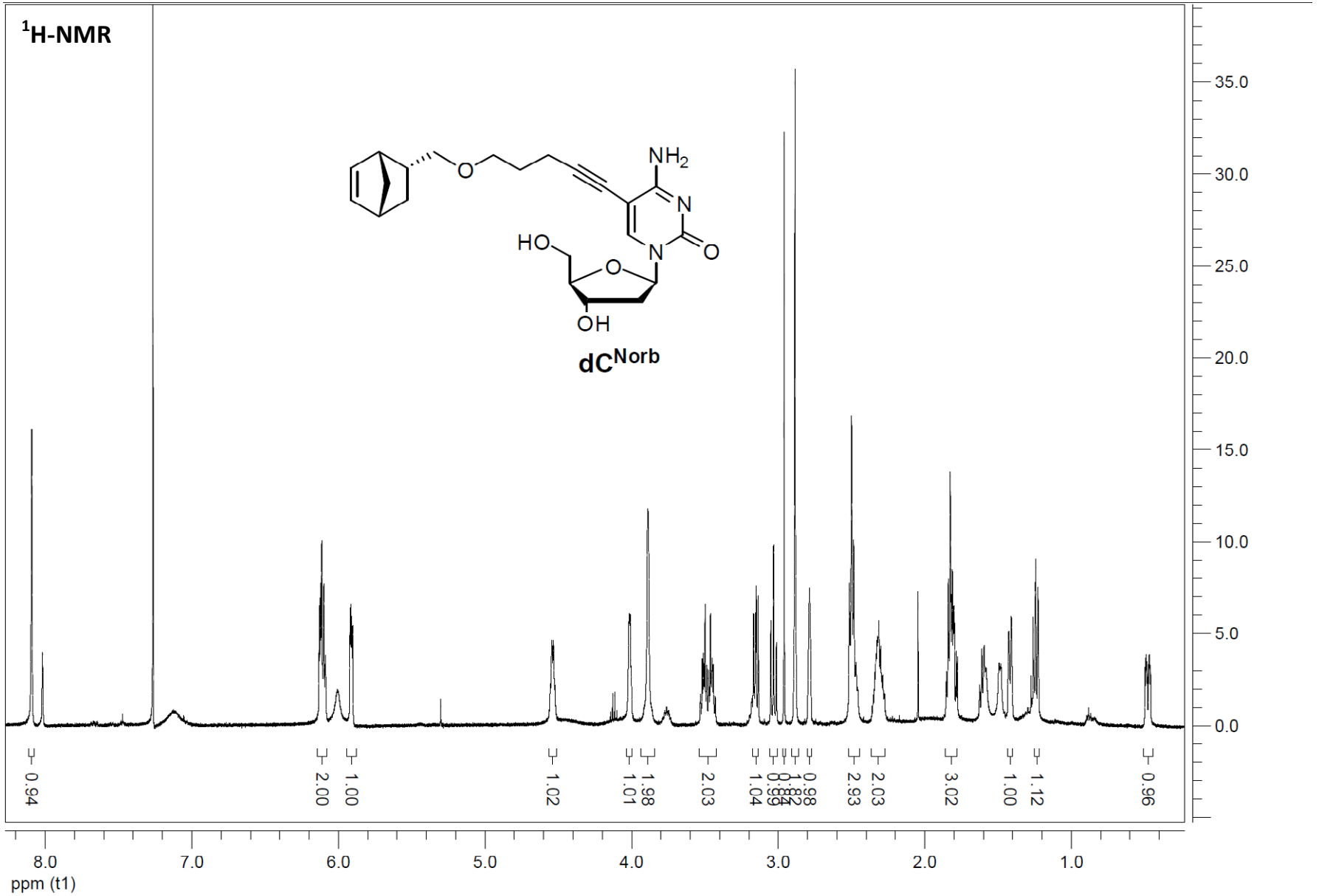

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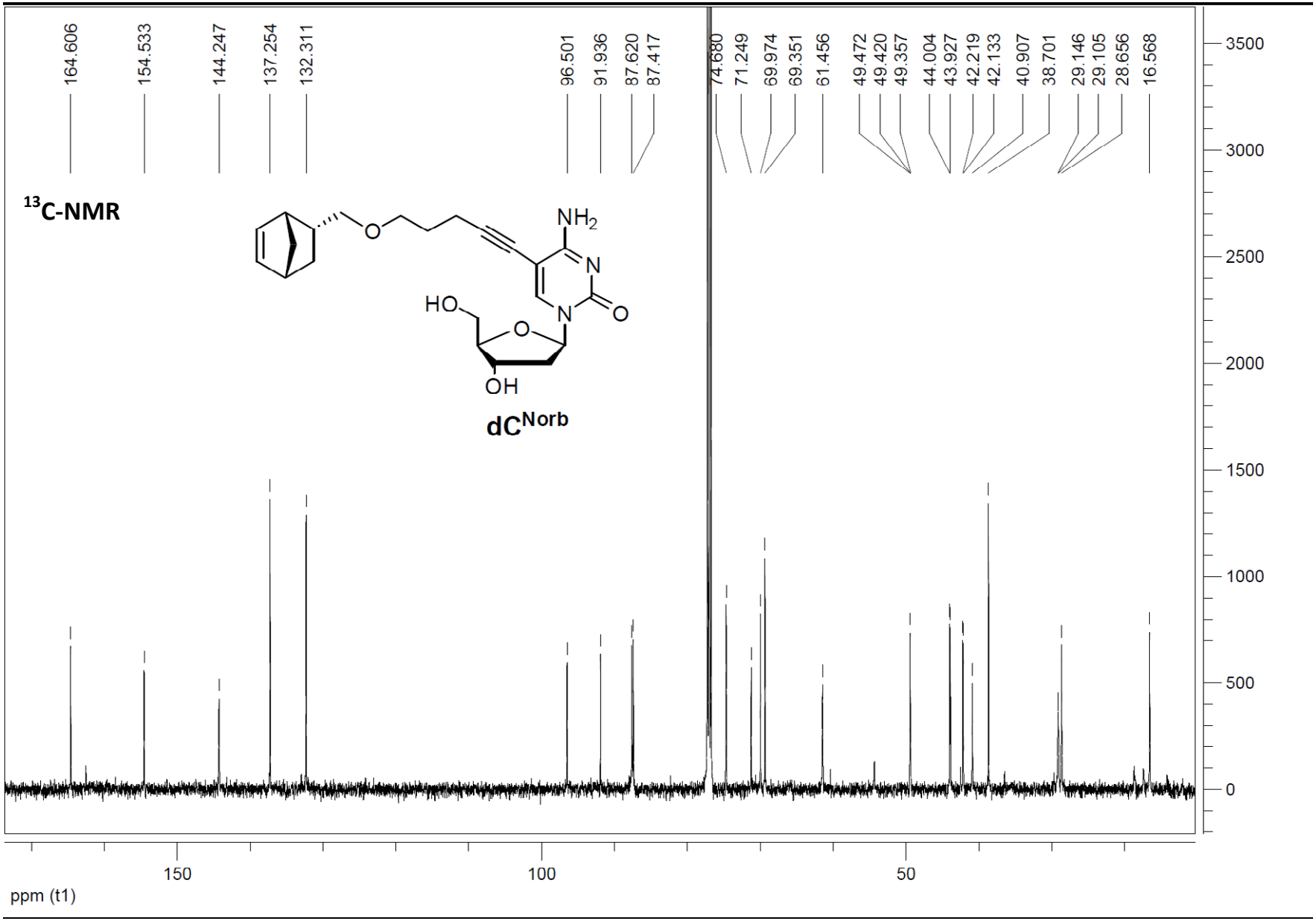

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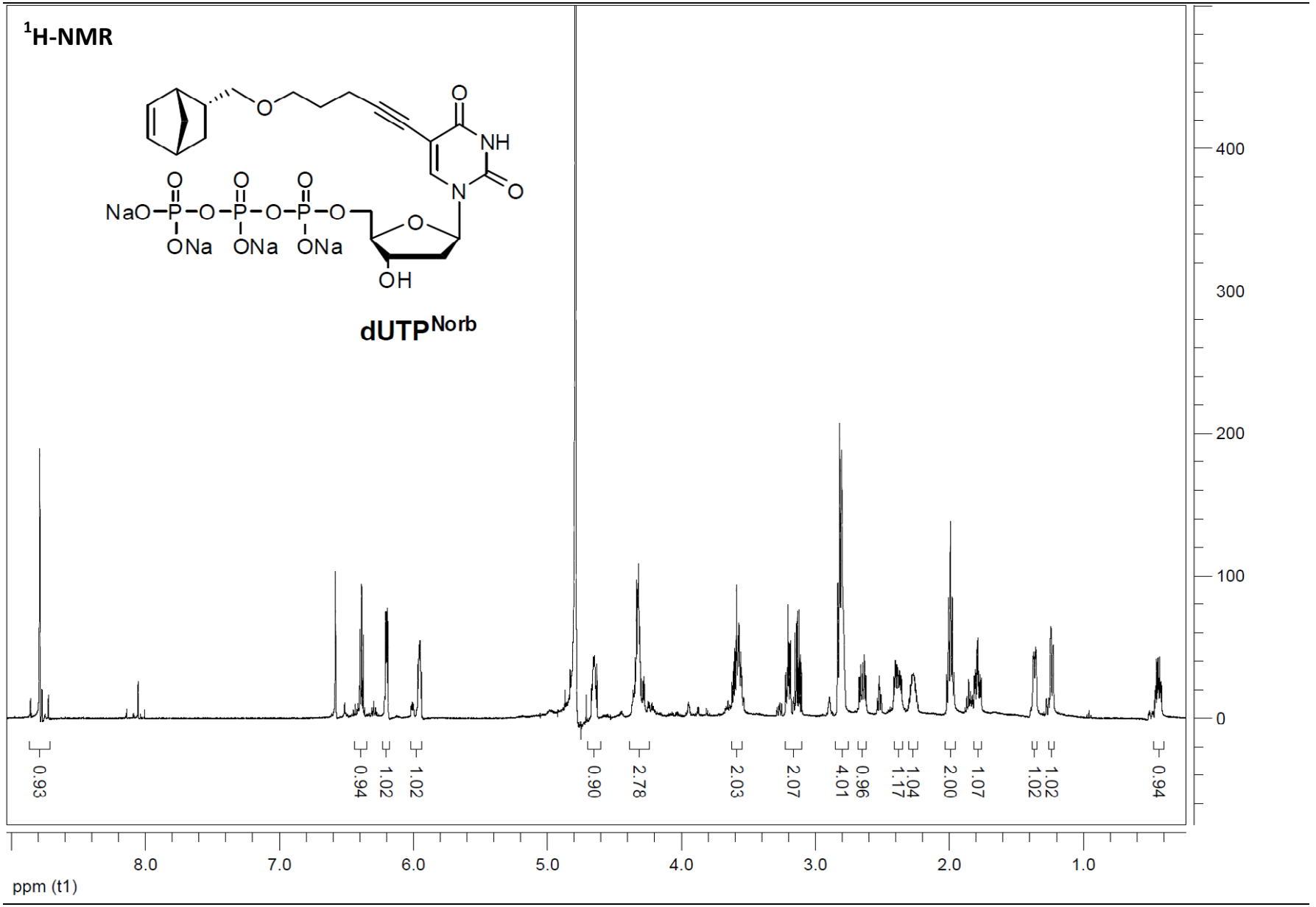

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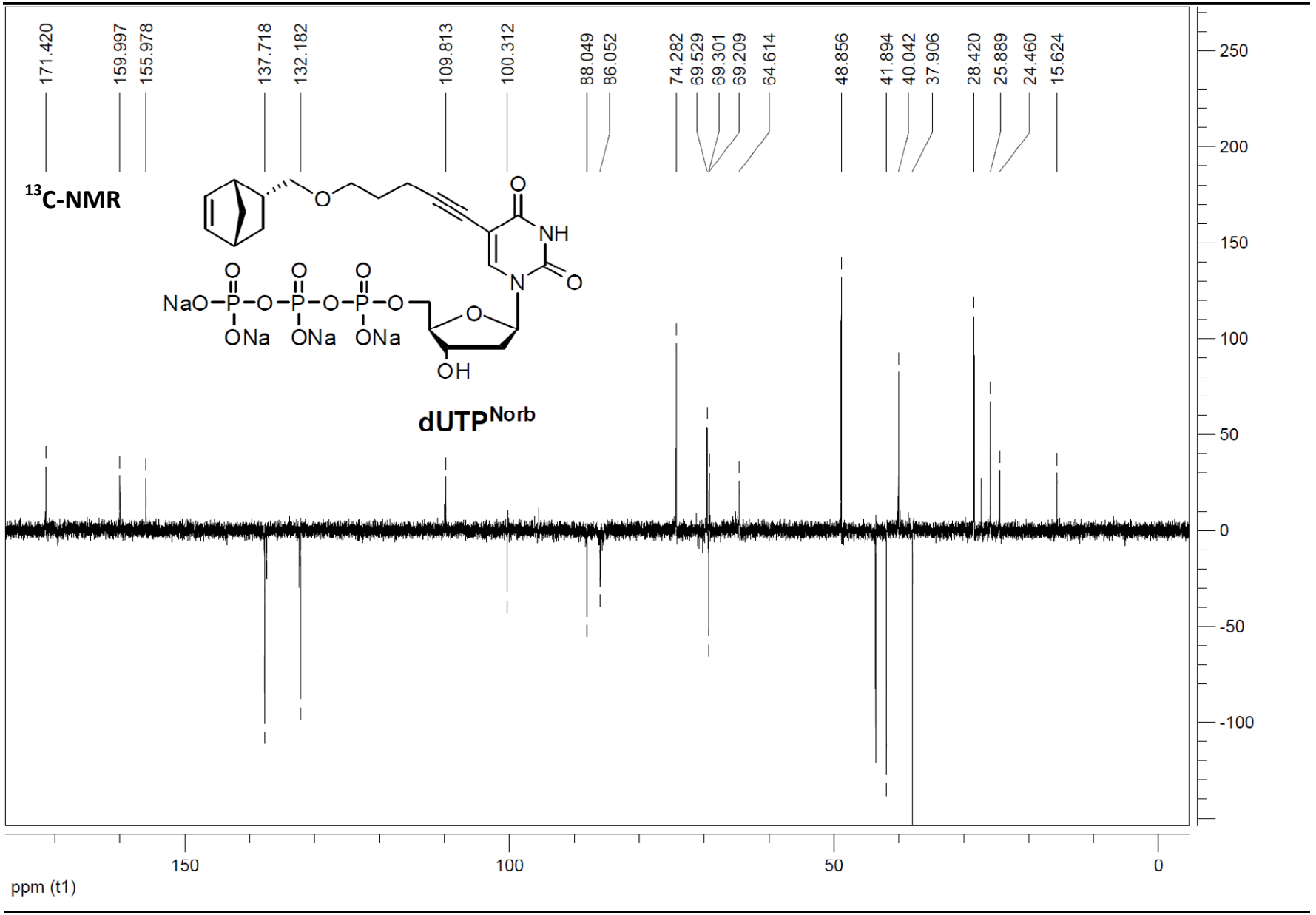

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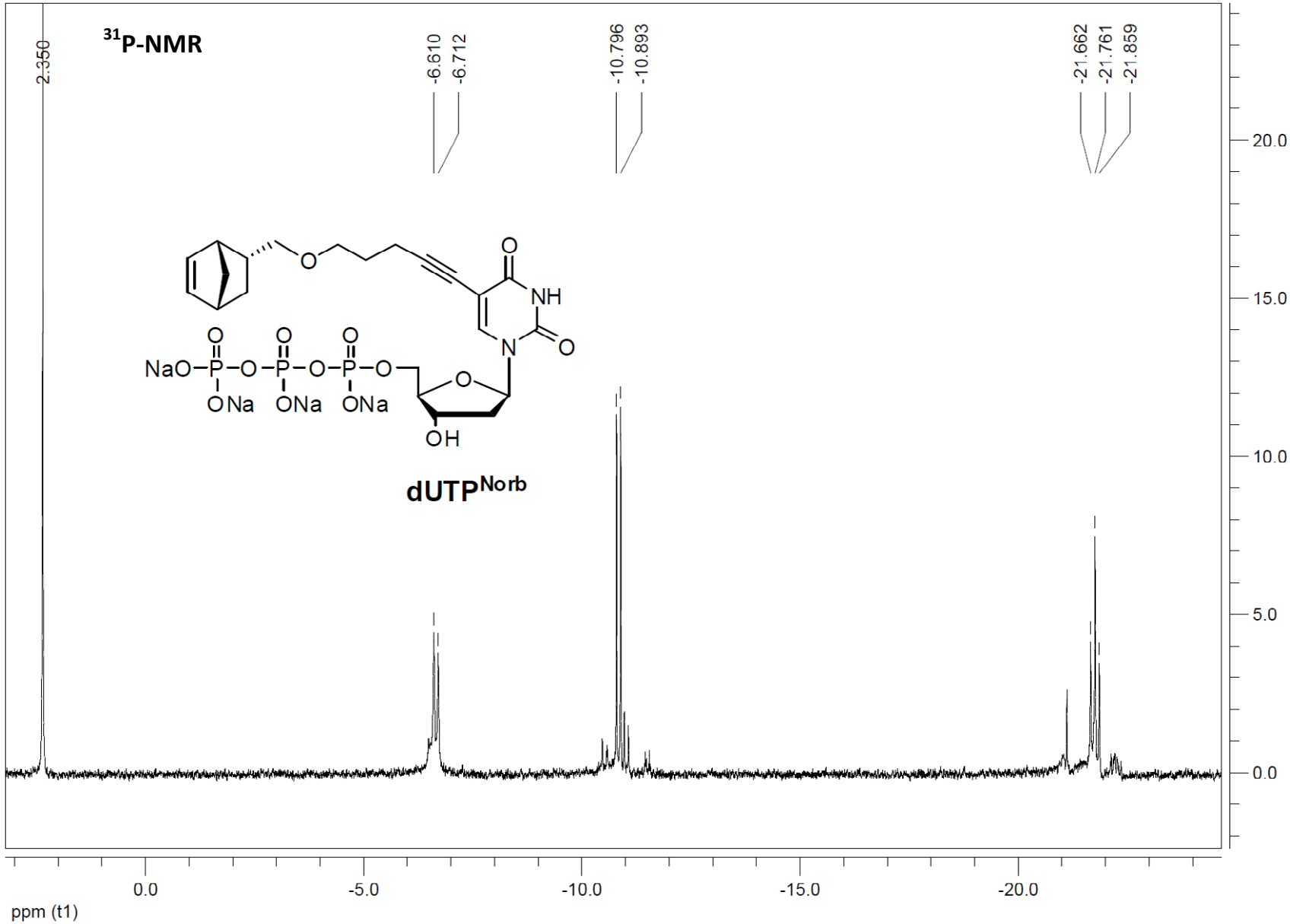

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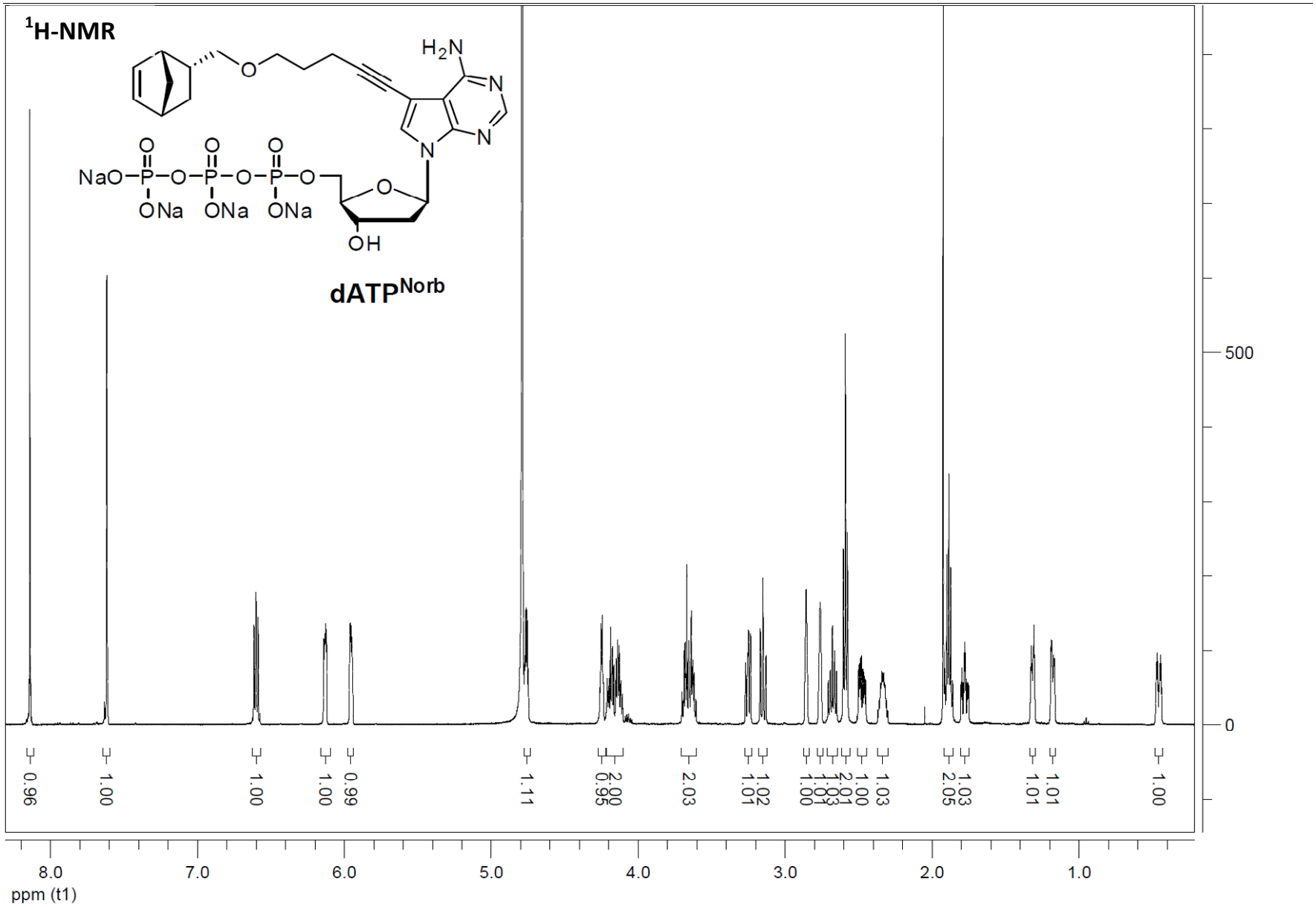

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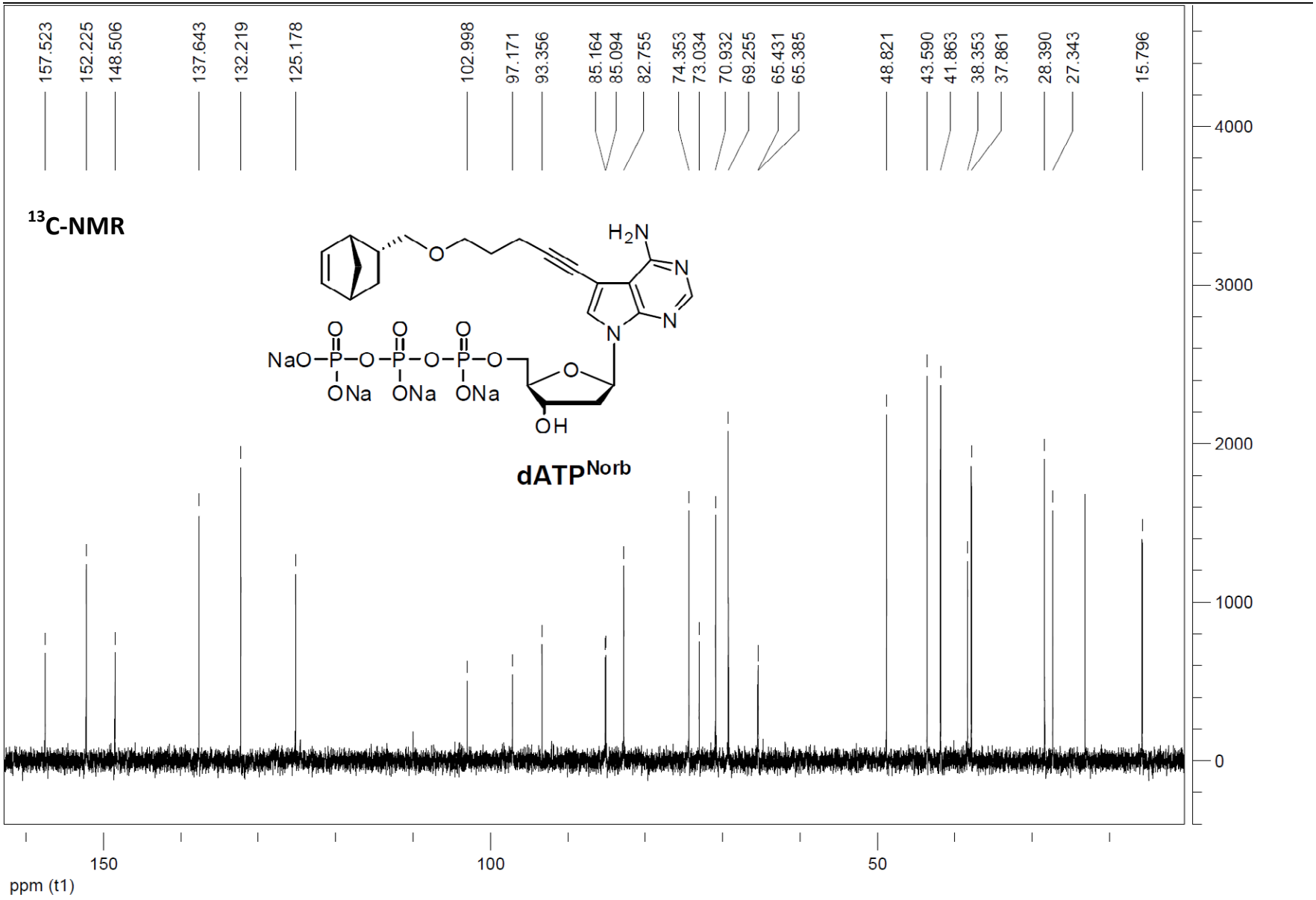

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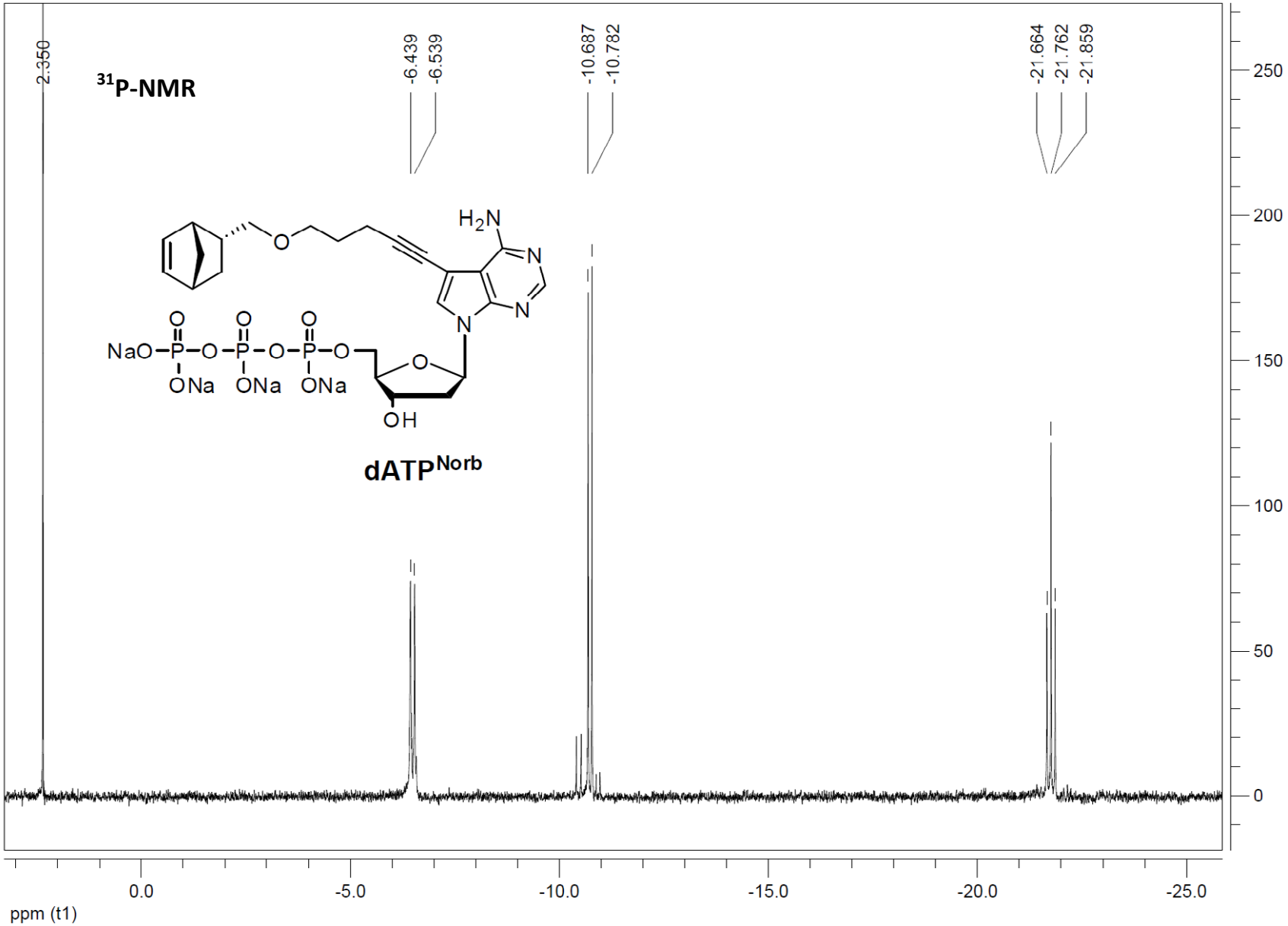

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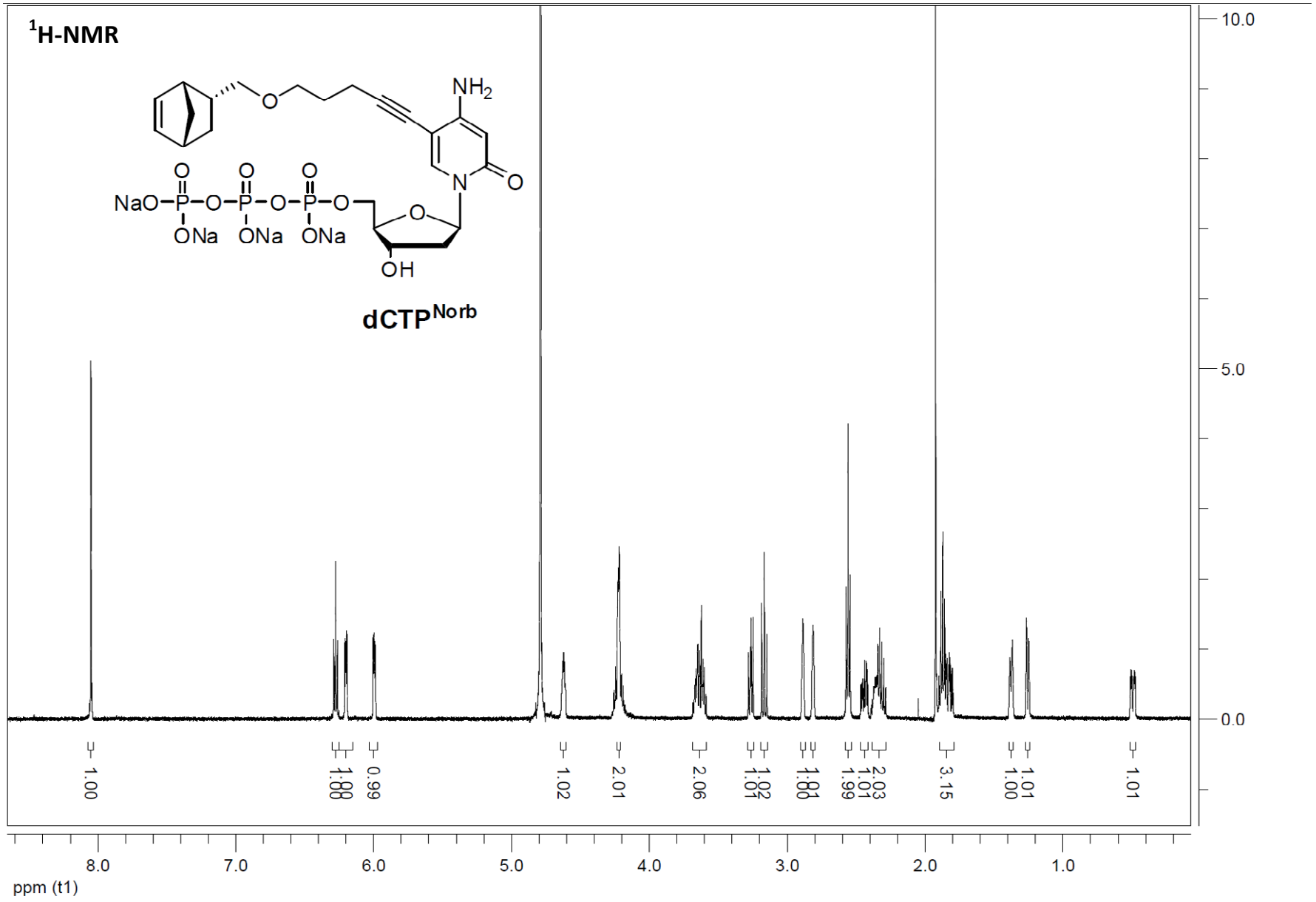

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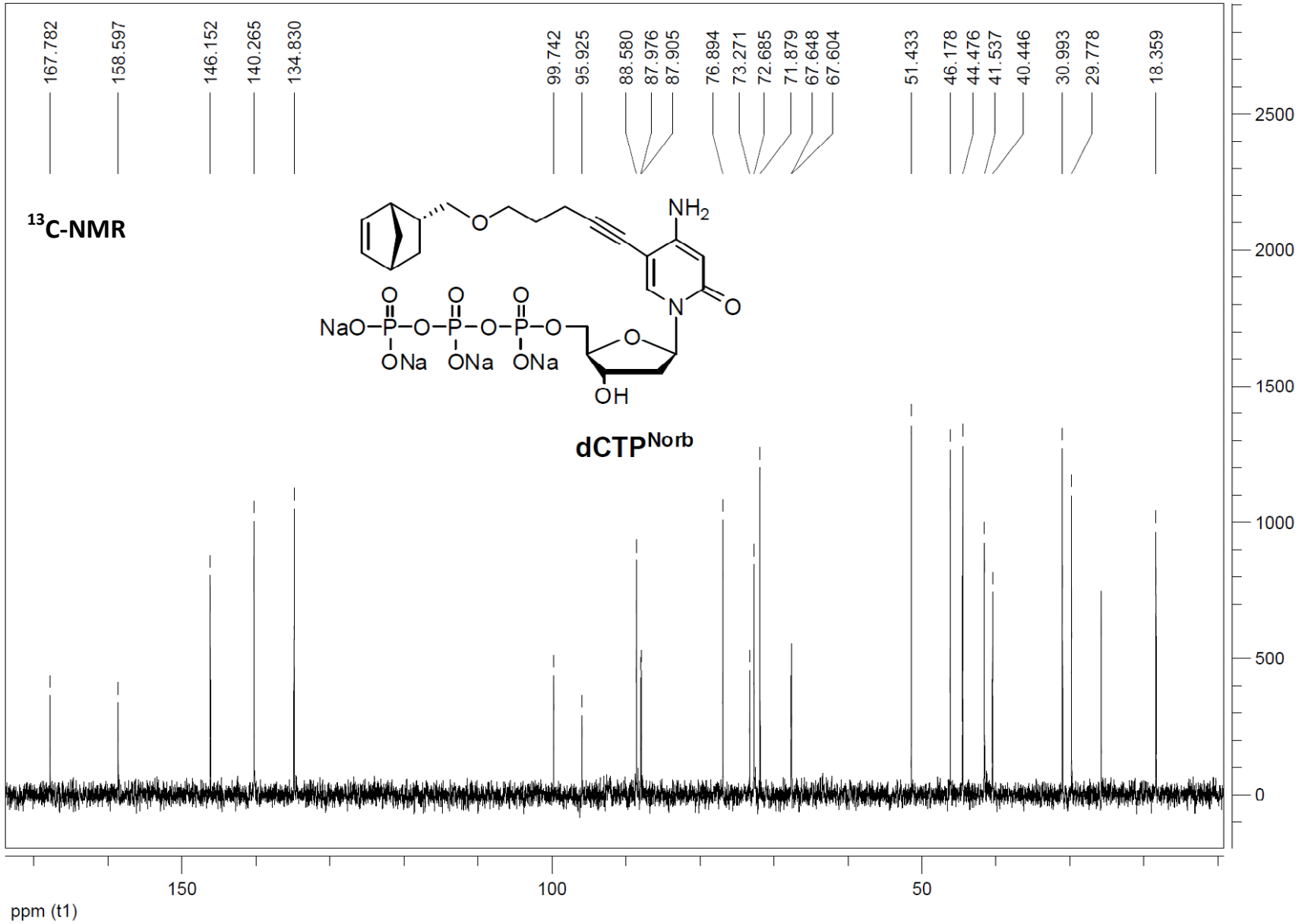

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