ELECTRONIC SUPPLEMENTARY INFORMATION (ESI)

FOR

Interrogation of the Active Site of OMP Decarboxylase from *Escherichia coli* with a Substrate Analogue Bearing an Anionic Group at C6

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General. All starting materials and reagents were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Tetrahydrofuran (THF) was distilled from sodium metal in the presence of benzophenone under argon. Dichloromethane (DCM) was distilled from calcium hydride under argon. Petrol refers to petroleum ether (b.p. 40 – 60 °C) and ether refers to diethyl ether unless stated otherwise. All solvents were purchased from Fisher Scientific (Ottawa, ON, Canada). Trimethyl phosphite was freshly distilled before use. Acetonitrile was distilled from phosphorus pentoxide and then from potassium carbonate and stored over molecular sieves (4 Å). Flash column chromatography was conducted using silica gel (60 Å , 70-230 mesh) purchased from Rose Scientific Ltd. (Edmonton, AB, Canada). ^{1}H-, ^{13}C-, and ^{31}P-NMR spectra were recorded on a Brüker Avance 500 MHz spectrometer. For ^{1}H-NMR in $CDCl_3$, chemical shifts (δ) are reported in ppm relative to the solvent residual peak (δ 7.26). For ^{1}H-NMR in MeOD, chemical shifts are reported in ppm relative to tetramethylsilane (δ 0.0, internal standard). For ^{1}H-NMR in D_2O, chemical shifts are reported in ppm relative to the solvent residual peak (δ 4.79). For ^{13}C-NMR in $CDCl_3$, the chemical shifts are reported in ppm relative to $CDCl_3$ (δ 77.36 for the central peak). All ^{31}P-NMR chemical shifts are reported relative to 85% H_3PO_4 in D_2O (δ 0.0, external standard). NMR data are reported as follows: app., apparent; br, broad; s, singlet; d, doublet; t, triplet; q, quartet; dd, double doublet, qt, quintet; and m, multiplet. Low resolution (LR) and high resolution (HR) electrospray ionization (ESI) mass spectra (MS) were obtained using a Bruker microTOF mass spectrometer operating in either the positive or negative ion mode as indicated in the experimental procedures below. High resolution (HR) electron impact ionization (EI) mass spectra were obtained using a CEC 21-110B mass spectrometer operated at a mass resolution of 8000 (10% valley) by computer controlled peak matching to appropriate perfluoro kerosene (PFK) ions. Elemental analyses of the compounds were performed by Canadian Microanalytical Service Ltd. (Delta, BC, Canada).

2′,3′-*O*-Isopropylideneuridine. To a solution of uridine (4.88 g, 20 mmol) and a catalytic amount of *p*-toluenesulfonic acid ($TsOH \cdot H_2O$, 0.380 g, 2.0 mmol) in acetone (120 mL), 2,2-

dimethoxypropane (2.65 mL, 2.24 g, 21.8 mmol) was added at 0 °C. The suspension was slowly heated to a gentle reflux which was maintained for 1 h. The solution was then cooled to room temperature and treated with sodium bicarbonate (0.3036 g, 3.6 mmol) and stirred at room temperature for an additional 0.5 h. The solid was filtered and the filtrate was adsorbed on silica gel and purified by flash chromatography (gradient of 5–10% methanol in DCM) to give 5.69 g (100%) of the title compound as a white crystalline solid. R_f = 0.27 (10% MeOH in DCM); mp 165-167 °C (lit. 163-164 °C)[1]; ^{1}H-NMR (MeOD, 500 MHz) δ (ppm): 7.82 (1H, d, $J_{6,5}$ = 8.05 Hz, 6-C*H*), 5.86 (1H, d, $J_{1',2'}$ = 2.7 Hz, 1′-C*H*), 5.67 (1H, d, $J_{5,6}$ = 8.04 Hz, 5-C*H*), 4.19 (1H, app. t, $J_{2',3'}$ = 3.70 Hz, 2′-C*H*), 3.54 (3H, m, 5′-CH_2 & 4′-C*H*), 3.30 (1H, m, 3′-C*H*), 1.54 & 1.34 (3H each, s, 2 × CH_3); ^{13}C-NMR (MeOD, 500 MHz) δ (ppm): 164.8 (4-*C*O), 150.7 (2-*C*O), 142.4 (6-*C*H), 113.7 (*C*(CH$_3$)$_2$), 101.3 (5-*C*H), 92.7 (1′-*C*H), 87.0 (2′-*C*H), 84.4 (4′-*C*H), 80.8 (3′-*C*H), 61.7 (5′-*C*H$_2$), 26.1 (*C*H$_3$) & 24.1 (*C*H$_3$); LRMS (ESI$^+$, MeOH) *m/z*: 307.1 [(M+Na)$^+$, 100%], 591.2 [(2M+Na)$^+$, 60%], 875.3 [(3M+Na)$^+$, 30%]; HRMS (ESI$^+$, MeOH) *m/z*: Found 307.0898; calculated 307.0901 for $C_{12}H_{16}N_2NaO_6$ [M+Na]$^+$.

5′-*O*-*tert*-Butyldimethylsilyl-2′,3′-*O*-isopropylideneuridine (6a). The title compound was prepared according to the method described by Fujihashi *et al.*[2] A solution of 2′,3′-*O*-isopropylideneuridine (5.12 g, 18.0 mmol) in anhydrous DCM was treated with imidazole (2.45 g, 36.0 mmol) and TBSCl (3.12 mL, 2.72 g, 18.0 mmol) at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred for 1.5 h. When the reaction was complete (TLC), it was diluted with DCM and the organic layer was washed with water and brine and then dried over $MgSO_4$. The solvent was evaporated *in vacuo* and the crude product was purified by flash chromatography (gradient of 30–50% EtOAc in petrol) to give 7.0 g (99%) of the TBS ether **6a** as a white solid. R_f = 0.33 (40% EtOAc in hexanes); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ (ppm): 9.08 (1H, br s, N*H*), 7.71 (1H, d, $J_{6,5}$ = 8.12 Hz, 6-C*H*), 6.01 (1H, d, $J_{1',2'}$ = 2.76 Hz, 1′-C*H*), 5.71 (1H, dd, $J_{5,6}$ = 8.1 Hz, 5-C*H*), 4.79 (1H, dd, $J_{2',3'}$ = 6.15 Hz, 2′-C*H*), 4.72 (1H, dd, $J_{3',2'}$ = 6.15 Hz, 3′-C*H*), 4.34 (1H, app. d, $J_{4',3',5'}$ = 2.65 Hz, 4′-C*H*), 3.95 (1H, app. q, $J_{5'a,5'b,4'}$ = 2.41 &

11.5 Hz, 5′-C$H_{2(a)}$), 3.82 (1H, app.q, $J_{5'b,5'a,4'}$ = 3.0 & 11.5 Hz, 5′-C$H_{2(b)}$), 1.61 & 1.38 (3H each, s, 2 × -C(CH_3)$_2$), 0.93 (9H, s, SiBu-*t*), 0.1 (6H, s, Si-CH_3); ^{13}C-NMR (CDCl$_3$, 500 MHz) δ (ppm): 163.3 (4-*C*O), 150.1 (2-*C*O), 140.6 (6-*C*H), 114.2 (-*C*(CH$_3$)$_2$), 102.2 (5-*C*H), 91.9 (1′-*C*H), 86.7 (2′-*C*H), 85.4 (4′-*C*H), 80.3 (3′-*C*H), 63.4 (5′-*C*H$_2$), 27.3 & 25.9 (-C(*C*H$_3$)$_2$), 25.4 (-C(*C*H$_3$)$_3$), 18.4 (-*C*(CH$_3$)$_3$), –5.4 and –5.1 (-Si-*C*H$_3$); LRMS (ESI$^+$, MeOH) *m/z*: 818.8 [(2M+Na)$^+$, 100%], 421.1 [(M+Na)$^+$, 35%].

5′-*O*-*tert*-Butyldimethylsilyl-2′,3′-*O*-isopropylidine-6-diethylphosphonouridine (7a). The title compound was prepared by following the literature procedure reported by Honjo *et al.*[3] with some minor modifications. To a solution of diisopropylamine (0.35 mL, 0.252 g, 2.5 mmol) in THF, 1.45 mL of *n*-butyllithium (1.8 M solution in hexanes) was added in a dropwise manner at –78 °C. The mixture was maintained with stirring at –78 °C for 1 h under argon. To this, a solution of the TBS protected compound **6a** (0.398 g, 1.0 mmol) in THF was then added dropwise at –78 °C over a period of 5-10 min and the resultant mixture was maintained at –78 °C for 1 h. Then, diethyl chlorophosphate (0.43 mL, 0.52 g, 3.0 mmol) was added dropwise at –78 °C over a period of 10 min and the reaction was maintained at this temperature for 2 h. When the reaction was complete (TLC), aqueous saturated ammonium chloride was added slowly to quench the reaction. The aqueous layer was then extracted with EtOAc. The combined organic layer was then washed well with water and brine and then dried over MgSO$_4$. The solvent was evaporated *in vacuo* and the crude product was adsorbed on silica gel and purified by flash chromatography (gradient of 15–50% EtOAc in petrol) to give 0.180 g (33%) of compound **7a** as colourless viscous liquid. ^{1}H-NMR (CDCl$_3$, 500 MHz) δ (ppm): 9.82 (1H, br s, -N*H*), 6.44 (1H, d, $J_{1',2'}$ = 4.03 Hz, 1′-C*H*), 6.14 (1H, s, 5-C*H*), 5.16 (1H, app. d, $J_{2',3'}$ = 6.5 Hz, 2′-C*H*), 4.76 (1H, app. t, $J_{3',4'}$ = 4.92 & 6.02 Hz, 3′-C*H*), 4.25 (4H, m, 2 × -OCH_2CH$_3$), 4.09 (1H, app. q, $J_{4',3',5'}$ = 5.2 & 11.6 Hz, 4′-C*H*), 3.79 (2H, m, 5′-CH_2), 1.51 & 1.31 (3H each, s, -C(CH_3)$_2$), 1.39 (6 H, t, $J_{H\text{-}H}$ = 7.04 Hz, 2 × -OCH$_2$CH_3), 0.85 (9H, s, SiBu-*t*), 0.01 (6H, s, Si-CH_3); ^{13}C-NMR (CDCl$_3$, 500 MHz) δ (ppm): 162.3 (d, 4-*C*O), 150.4 (d, 2-*C*O), 146.1 (d, 6-*C*PO), 114. 0 (s, -*C*(CH$_3$)$_2$), 112.4

(d, 5-*C*H), 94.9 (s, 1′-*C*H), 89.5 (s, 2′-*C*H), 84.5 (s, 4′-*C*H), 82.4 (s, 3′-*C*H), 64.8-64.1 (app. qt., 2 × -O*C*H_2CH$_3$ & 5′-*C*H_2), 27.5 & 26.2 (s, -C(*C*H_3)$_2$), 25.7 (s, -C(*C*H_3)$_3$), 18.7 (s, -*C*(CH$_3$)$_3$), 16.4 (app. q, 2 × -OCH$_2$*C*H$_3$), -4.9 (d, 2 × -Si-*C*H$_3$); LRMS (ESI$^+$, MeOH) *m/z*: 1090.8 [(2M+Na)$^+$, 55%], 557.2 [(M+Na)$^+$, 100%]; HRMS (EI) *m/z*: Found 534.2178; calculated 534.2162 for $C_{22}H_{39}N_2O_9PSi$ [M$^+$].

5′-*O*-(Tetrahydro-2-furanyl)-2′,3′-*O*-isopropylideneuridine (6b). The title compound was prepared following the method reported by Honjo *et al*.[3] 2,3-Dihydrofuran (2.9 mL, 2.7 g, 38.35 mmol) and PPTS (0.503 g, 2 mmol) were added to a solution of 2′,3′-*O*-isopropylideneuridine (5.68 g, 20 mmol) in anhydrous DCM. After standing at room temperature for 1 h, the reaction went to completion (TLC). The mixture was then concentrated *in vacuo* and purified by flash chromatography (gradient of 2–5% methanol in DCM) to give 7.0 g (100%) of a viscous oil which formed a colorless, hygroscopic solid upon standing. R_f = 0.4 (10% MeOH in DCM) ; ^{1}H-NMR ($CDCl_3$, 500 MHz) δ (ppm): 9.54 (1H, br s, N*H*), 7.57 (1H, d, $J_{6,5}$ = 7.6 Hz, 6-C*H*), 7.55 (1H, d, $J_{6,5}$ = 7.6 Hz, 6-C*H*), 5.85 (1H, d, $J_{1',2'}$ = 2.20 Hz, 1′-C*H*), 5.67 (1H, d, $J_{5,6}$ = 8.04 Hz, 5-C*H*), 5.10 (1H, app. q, 2′-C*H*), 4.75-4.71 (2H, m, 3′-C*H*, 1′′-C*H*), 4.41-4.34 (1H, m, 4′-C*H*), 3.97-3.51 (5H, m, 5′-CH_2 & 4′′-CH_2), 1.97-184 (6H, m, 2′′, 3′′ & 4′′-CH_2), 1.56 & 1.33 (3H each, s, 2 × CH_3); ^{13}C-NMR ($CDCl_3$, 500 MHz) δ (ppm): 163.6 (4-*C*O), 150.2 (d, 2-*C*O), 141.0 (d, 6-*C*H), 114.1 (d, *C*(CH$_3$)$_2$), 104.0 (d, 1′′-*C*H), 102.0 (d, 5-*C*H), 93.0 (d, 1′-*C*H), 85.5 (d, 2′-*C*H), 85.0 (d, 3′-*C*H), 81.0 (d, 4′-*C*H), 67.3 (d, 4′′-*C*H_2), 67.0 (d, 5′-*C*H_2), 32.4 (d, 2′′-*C*H_2), 27.2 & 25.3 (2 × d, 2 × *C*H$_3$) and 23.4 (d, 3′′-*C*H_2); LRMS (ESI$^+$, MeOH) *m/z*: 754.1 [2(M+Na)$^+$, 35%], 731.0 [(2M+Na)$^+$, 55%], 377.2 [(M+Na)$^+$, 100%].

Diethyl 2′,3′-*O*-isopropylidinuridine-6-phosphonate, 8: Step 1, synthesis of 7b. This compound was prepared according to the procedure reported by Honjo *et al*.[3] with some minor modifications. To a solution of diisopropylamine (3.7 mL, 2.67 g, 26.0 mmol) in THF, cooled to –78 °C and under argon, *n*-butyllithium (14.5 mL of 1.8 M solution in hexane, 26.0 mmol) was

added dropwise at –78 °C. The mixture was then stirred at –78 °C for 1 h. A solution of compound **8** (3.55 g, 10.0 mmol) in THF was then added dropwise to the mixture at –78 °C. The mixture was stirred for 1 h and then diethyl chlorophosphate (4.75 ml, 5.7 g, 33 mmol) was added dropwise at –78 °C when the colour of the solution changed from yellow to dark brown. The solution was maintained at this temperature for 1 h and after completion of the reaction (TLC), the reaction was quenched by the addition of aqueous saturated solution of ammonium chloride. The aqueous layer was then extracted with ethyl acetate, the combined organic layer was then washed with water and the solvent evaporated *in vacuo* to give ~5.0 g of yellowish-brown viscous liquid which was taken for the deprotection step without any further purification.

Step 2, synthesis of 8. The product from the above step was dissolved in EtOH (20 mL), and PPTS (0.26 g, 1 mmol) was added. After standing at room temperature for 0.5 h to 0.75 h (TLC), the solvent in the reaction mixture was evaporated *in vacuo*. The resultant crude product was adsorbed on silica gel and purified by flash chromatography (gradient of 60–100% ethyl acetate in petrol) to give 3.93 g of **8** as a pale yellow viscous oil. Yield – 70%; R_f = 0.32 (10% MeOH in DCM) ; ^{1}H-NMR ($CDCl_3$, 500 MHz) δ (ppm): 10.50 (1H, br s, N*H*), 6.44 (1H, d, J_{HCCP} = 13.1 Hz, 5-C*H*), 6.10 (1H, d, $J_{1',2'}$ =2.1 Hz, 1′-C*H*), 5.23 (1H, q, $J_{2',3'}$ = 6.6 Hz, 2′-C*H*), 5.01 (1H, q, $J_{3',4'}$ = 6.5 Hz, 3′-C*H*), 4.25 (5H, m, 5′-O*H* & 2 × CH_2CH$_3$), 4.10 (1H, m, 4′-C*H*), 3.85 (1H, q, $J_{5'a,4'}$ = 3.0 Hz & $J_{5'a,5'b}$ = 12.07 Hz, 5′a-CH_2), 3.75 (1H, q, $J_{5'b,4'}$ = 3.9 Hz, 5′b-CH_2), 1.53 & 1.32 (3H each, s, 2 × CH_3), 1.38 (6H, sestet, 2 × CH$_2$CH_3); ^{13}C-NMR ($CDCl_3$, 500 MHz) δ (ppm): 171.23 (4-*C*O), 161.8 (2-*C*O), 151.2 (6-*C*),114.2 (*C*(CH$_3$)$_2$), 112.6 (5-*C*H), 94.0 (1′-*C*H), 87.2 (2′-*C*H), 83.7 (4′-*C*H), 80.4 (3′-*C*H), 64.5 and 62.4 (2 × -*C*H$_2$CH$_3$), 60.4 (5′-*C*H$_2$), 27.1 and 25.2 (2 x *C*H$_3$), 16.1 & 14.1 (2 × -CH$_2$*C*H$_3$); ^{31}P-NMR ($CDCl_3$, 500 MHz) δ (ppm): 5.60 (app. septet); LRMS (ESI$^+$, MeOH) *m/z*: 907.1 [(2M+3Na)$^+$, 52%], 886 [2(M+Na)$^+$, 30%], 862.8 [(2M+Na)$^+$, 23%], 443.1 [(M+Na)$^+$, 100%]; HRMS (ESI$^+$, MeOH) *m/z*: Found 443.1192; calculated 443.1190 for $C_{16}H_{25}N_2NaO_9P$ [M+Na]$^+$.

Diethyl *2′,3′-O*-isopropylidine-6-diethylphosphonouridine-5′-dimethyl phosphate, 9. The title compound was prepared using a method similar to that described by Oza *et al*.[4] with minor

modifications. Carbon tetrabromide (CBr_4) (2.92 g, 8.8 mmol) and compound **8** (1.65 g, 4.0 mmol) were added to an argon-purged flask equipped with a magnetic stirring bar. After addition of anhydrous pyridine (12 mL) to the reaction mixture, the resulting solution was cooled to 0 °C. Freshly distilled trimethyl phosphite (TMP) (1.2 mL, 12 mmol) was then added dropwise to the mixture. (Note: A yellow-orange precipitate was formed). The mixture was maintained at 0 °C and the reaction was complete within 0.5 h (TLC). After dilution with chloroform, pyridine was evaporated *in vacuo* from the reaction mixture. The process was repeated three more times by adding EtOH (15-20 mL) to the reaction mixture. The crude product was then adsorbed onto silica gel and purified by flash chromatography (gradient of 80–100% EtOAc in petrol) to give 1.6 g of compound **9** as colourless viscous oil. Yield – 76%; R_f = 0.35 (10% MeOH in DCM) ; ^{1}H-NMR ($CDCl_3$, 500 MHz) δ (ppm): 10.21 (1H, br s, N*H*), 6.39 (1H, d, J_{HCCP} = 14.1 Hz, 5-C*H*), 6.18 (1H, d, $J_{1',2'}$ = 2.3 Hz, 1′-C*H*), 5.15 (1H, q, $J_{2',3'}$ = 6.5 Hz, 2′-C*H*), 4.85 (1H, q, $J_{3',4'}$ = 6.5 Hz, 3′-C*H*), 4.23 (7H, m, 5′-CH_2, 4′-C*H* & 2 × -OCH_2CH$_3$), 3.71 (6H, dd, J_{OPOCH} = 11.2 Hz, 2 × -OCH_3), 1.49 & 1.29 (3H each, s, 2 × -CH_3), 1.37 (6H, t, *J* = 7.0 Hz, 2 × -CH$_2$CH_3); ^{13}C-NMR ($CDCl_3$, 500 MHz) δ (ppm): 162.0 (d, 4-*C*O), 150.4 (d, 2-*C*O), 145.2 (d, 6-*C*), 113.9 (d, -*C*(CH$_3$)$_2$), 112.2 (d, 5-*C*H), 94.7 (d, 1′-*C*H), 87.2 (d, 2′-*C*H), 84.6 (3′-*C*H), 82.1 (4′-*C*H), 67.5 (d, 5′-*C*H$_2$), 64.7 & 64. 5 (dd, 2 × -O*C*H$_2$CH$_3$), 54.4 (app. t, 2 × -O*C*H$_3$), 26.9 & 25.2 (2 × -*C*H$_3$), 16.0 (app. t, 2 × -OCH$_2$*C*H$_3$); ^{31}P-NMR ($CDCl_3$, 500 MHz) δ (ppm): 5.62 (app. septet, -O*P*O) and 1.02 (m, 6-C*P*O); LRMS (ESI$^+$, MeOH) *m/z*: 1078.7 [(2M+Na)$^+$, 20%], 551.1 [(M+Na)$^+$, 100%]; HRMS (ESI$^+$, MeOH) *m/z*: Found 551.1158; calculated 551.1166 for $C_{18}H_{30}N_2NaO_{12}P_2$ [M+Na]$^+$.

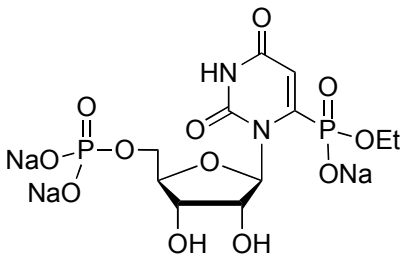
6-phosphono uridine-5′-phosphate (10). To a solution of compound **9** (0.264 g, 0.5 mmol) in anhydrous acetonitrile under argon, bromotrimethylsilane (1.15 g, 0.97 mL, 7.5 mmol) was added in a dropwise manner using a syringe at 0 °C. After completion of the addition, the mixture was allowed to warm to room temperature and was maintained at room tempertaure for 24 h (TLC; phosphonate/phosphate detected using a sulfosalicylic acid-iron (III) chloride spray

reagent[5]). The solvent was then evaporated *in vacuo* followed by the addition of methanol which was subsequently evaporated. This process was repeated at least three more times to give a pale yellow solid which was purified by anion exchange chromatography (DEAE Sephadex A-25, Cl^--form) using a linear gradient of triethylammonium bicarbonate (TEAB, 0.1 – 0.5 M) buffer (pH 7.5 – 8.0) to give three UV (265 nm) active fractions that were lyophilized to constant weight to give the title compound (last fraction) along with monoethyl phosphonate (**11**) (second fraction) and other unidentified minor impurities (first fraction) as their triethylammonium salts. Compound **10** was converted to its sodium salt by passing it through a cation exchange resin (BioRad AG-1X8, Na^+-form) to yield the final product (0.051 g, 25%).

Compound **10**: ^{1}H-NMR (D_2O, 500 MHz) δ (ppm): 6.53 (1H, app. d, $J_{1',2'}$ = 2.2 Hz, 1′-C*H*), 6.26 (1H, d, J_{HCCP} = 10.66 Hz, 5-C*H*), 4.69 (1H, app. q, $J_{2',3'}$ = 6.53 Hz, 2′-C*H*), 4.29 (1H, app. t, $J_{3',4'}$ = 7.3 & 7.0 Hz, 3′-C*H*), 4.12-3.97 (3H, m, 5′-CH_2 & 4′-C*H*); ^{13}C-NMR (D_2O, 500 MHz) δ (ppm): 166.7 (d, 2-*C*O), 159.3 (d, 6-*C*), 106.3 (d, 5-*C*H), 94.7 (d, 1′-*C*H), 81.8 (d, 4′-*C*H), 72.0 (s, 2′-*C*H), 69.6 (s, 3′-*C*H), 64.9 (d, 5′-*C*H_2); ^{31}P-NMR (D_2O, 500 MHz) δ (ppm): 0.93 (app. t, $J_{5'P}$ = 5.8 & 5.69 Hz, 5′-O*P*O) and –0.85 (app. d, J_{6P} = 10.54 Hz, 6-C*P*O); LRMS (ESI^-, H_2O) *m/z*: 402.9 (M^-, 80%), 190.9 ((M-base)$^-$, 100%); HRMS (ESI^-, H_2O) *m/z*: Found 402.9940; calculated 402.9949 for $C_9H_{13}N_2O_{12}P_2$ [M^-]; Elemental Analysis (%): Found C, 20.22; H, 3.31; N, 5.12; and P, 11.84. Calculated C, 20.24; H, 3.06; N, 5.24; P, 11.60; Na, 14.63; and O, 45.23 for $C_9H_{16.2}N_2Na_{3.4}O_{15.1}P_2$ (containing 3.4 Na and 3.1 H_2O).

Compound **11**: Yield – 0.010 g, 5%; ^{1}H-NMR (D_2O, 500 MHz) δ (ppm): 6.37 (1H, d, J_{HCCP} = 12.0 Hz, 5-C*H*), 6.26 (1H, d, $J_{1',2'}$ = 2.3 Hz, 1′-C*H*), 2′-C*H* was hidden under the solvent peak but was identified using 2D-COSY and HMQC spectra, 4.52 (1H, app. t, $J_{3',4'}$ = 7.3 Hz, 3′-C*H*), 4.15 (1H, m, 4′-C*H*), 4.07 (4H, m, 5′-CH_2 & -OCH_2CH$_3$), 1.35 (3H, t, *J* = 7.1 Hz, -OCH$_2$CH_3); ^{13}C-NMR (D_2O, 500 MHz) δ (ppm): 165.2 (d, 4-*C*O), 151.6 (app. t, 2-*C*O & 6-*C*), 109.3 (d, 5-*C*H), 95.0 (d, 1′-*C*H), 81.8 (d, 4′-*C*H), 70.63 (s, 2′-*C*H), 69.3 (s, 3′-*C*H), 64.4 (d, 5′-*C*H_2), 62.9 (d, –O*C*H_2CH$_3$), 15.7 (d, –OCH$_2$*C*H$_3$); ^{31}P-NMR (D_2O, 500 MHz) δ (ppm): 1.83 (app. t, $J_{5'OPO}$ = 5.5 & 4.9 Hz, 5′-O*P*O) and 0.68 (m, 6-C*P*O); LRMS (ESI^-, H_2O) *m/z*: 884.9 [(2M+Na)$^-$, 23%], 862.2

[$2M^-$, 57%] 431.1 [M^-, 100%]; HRMS (ESI^-, H_2O) *m/z*: Found 431.0259; calculated 431.0262 for $C_{11}H_{17}N_2O_{12}P_2$ [M^-].



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Sub-cloning of the open reading frame encoding *E. coli* ODCase (*Ec*ODCase). *E. coli* DH5α cells were used for cloning and BL21 (DE3) cells were used for protein expression. Plasmids were isolated from 5-mL overnight cultures using a QIAprep Miniprep Kit (Qiagen, Mississauga, ON, Canada) and following the directions of the manufacturer. All cultures were grown in LB (Luria-Bertani) broth supplemented with ampicillin (100 μg/mL) or on LB agar plates containing ampicillin (100 μg/mL) unless otherwise noted.

The open reading frame (ORF) from the *pyrF* gene (SWISS-PROT accession number P08244) coding for *E. coli* ODCase was PCR-amplified from *E. coli* chromosomal DNA using the following synthetic deoxyoligonucleotide primers: 5′-GGTCTGCATATGACGTTAACTGCTT-CATC-3′ (forward) and 5′-GTTGGAATGGATCCTCATGCACTCCGCTG-3′ (reverse). The forward and reverse primers included *Nde*I and *Bam*HI recognition sites (underlined), respectively, for insertion of the doubly digested PCR product into the multiple cloning site of the pET15b expression vector (Novagen Inc., Madison, WI, USA), generating the pET15b-*Ec*ODCase plasmid. This plasmid encodes ODCase as a fusion protein bearing an N-terminal hexahistidine tag (His_6-tag) with an intervening thrombin cleavage site.

Template DNA was obtained from *E. coli* genomic DNA isolated from the DH5α strain. An overnight culture (1.5 mL) of *E. coli* DH5α cells was re-suspended in Tris-EDTA (TE) buffer (600 mL; 10 mM Tris-Cl, 0.1 mM EDTA, pH 8.0) containing proteinase K (100 μL/mL)

and SDS (0.5%) and incubated for 1 h at 37 °C. The salt concentration was increased to 0.7 M with 2 M NaCl. Protein was precipitated by adding a solution of hexadecyltrimethylammonium bromide (CTAB) and sodium chloride (102 mL; 10% CTAB, 0.7 M NaCl) and incubating for 10 min at 65 °C. The solution was extracted twice with chloroform/isoamyl alcohol (24:1) and the DNA in the aqueous layer was precipitated by adding 0.6 volumes of isopropanol. The DNA pellet was washed with 70% ethanol and re-suspended in TE (50 mL; 10 mM Tris-Cl, 1 mM EDTA, pH 8.0).

The ORF was amplified using *Pfu*Turbo DNA polymerase (Stratagene, La Jolla, CA, USA) and the following polymerase chain reaction (PCR) protocol: 1 min at 95 °C; 29 cycles of 1 min at 95 °C, 2 min at 50 °C, and 4 min at 72 °C; and a final extension of 4 min at 72 °C. The PCR product was purified using a QIAquick PCR Purification Kit (Qiagen). The pET-15b plasmid and the PCR-amplified ORF were each doubly digested with the restriction enzymes *Nde*1 and *Bam*HI (New England Biolabs, Ipswich, MA, USA). *Bam*HI was added after digestion with *Nde*1 and the concentration of NaCl in the digestion was increased to 150 mM with 2 M, sterile NaCl. The pET15b plasmid was also treated with alkaline phosphatase (New England Biolabs) for 1 h after the double digestion. The digested plasmid was then purified using a QIAquick PCR Purification Kit (Qiagen). The ORF was ligated into the doubly digested, phosphatase-treated pET15b plasmid overnight at 15 °C using T4 DNA ligase (Invitrogen, Burlington, ON, Canada) in a ratio of 90 fmol insert to 30 fmol vector. The ligation reaction (20 μL) was stopped by adding 0.5 M EDTA (1 μL) and then diluted 5-fold. This diluted ligation mixture (3 μL) was used to transform 100 μL of chemically competent *E. coli* DH5α cells. The transformation mixture was left on ice for 45 min, heat shocked for 3 min at 42 °C, and placed back on ice for 5 min before plating. Colonies were selected and examined for the ODCase ORF insert by digesting the recovered plasmids with *Nde*1, and, when necessary, by double digesting with *Bam*HI and *Nde*1. The cut plasmids were analyzed using agarose gel (1%) electrophoresis. Insertion of the correct sequence was verified by commercial DNA sequencing (Center for Functional Microbial Genomics and Host Defence, Halifax, NS, Canada). Competent *E. coli*

BL21 (DE3) cells were transformed with the pET15b-*Ec*ODCase vector as previously described for the DH5α strain.

Production and purification of *Ec*ODCase. Starter cultures of the *E. coli* BL21 (DE3) cells containing the pET15b-*Ec*ODCase vector were grown overnight at 37 °C in LB broth (2 mL) containing ampicillin (100 μg/mL) and used to inoculate LB broth (1.0 L) containing ampicillin. Protein expression was induced by the addition of isopropyl-β-thiogalactopyranoside (IPTG, 0.1 mM) to the culture when the OD_{600} reached a value between 0.5-0.7. The cultures were grown for an additional 5-7 h and then the cells were harvested by centrifugation (4000 × *g*, 15 min, 4 °C) and washed with binding buffer (5 mM imidazole, 250 mM NaCl, 20 mM Tris-Cl, 10% v/v glycerol, 5 mM UMP, pH 7.9). The cell pellets were frozen with liquid nitrogen and stored at –20 °C. To purify the enzyme, the cell pellets were re-suspended in binding buffer and lysed using sonication (Branson Sonifier 250; 5 × 10 s burst, duty cycle 5, constant output). The cell lysate was clarified by centrifugation (31 000 × *g*, 20 min, 4 °C), filtered through a 0.45 μm filter, and applied to a Ni^{+}-charged His·bind resin (Novagen). Following treatment with binding buffer (25 mL) and wash buffer (15 mL; 60 mM imidazole, 0.5 M NaCl, 20 mM Tris-Cl, pH 7.9), the His_6-tagged ODCase was eluted with elution buffer (8 mL; 5 mM UMP, 100 mM EDTA, 300 mM NaCl, 20 mM Tris base, 10% v/v glycerol, pH 7.9). The enzyme was then dialyzed against 3 × 500 mL of storage buffer (10% v/v glycerol, 30 mM Tris-Cl, 250 mM NaCl, 2 mM DTT, pH 8.0) overnight. The purified protein (8.0 mL) was stored at –80 °C. The typical yield of protein was 2.2 mg from 1.0 L of culture (**Figure 1S**).

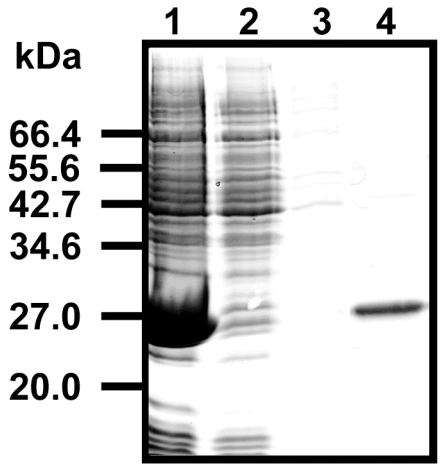

Figure 1S. SDS-PAGE (12%) analysis of *E. coli* ODCase. Lane 1, clarified cell lysate; lane 2, column flow through; lane 3, wash buffer; and lane 4, ODCase obtained after the elution buffer wash.

Enzyme assay. The decarboxylation of OMP to UMP catalyzed by ODCase was monitored spectrophotometrically at 279 nm ($\Delta\varepsilon_{279} = 2.4 \pm 0.2$ mM^{-1} cm^{-1})[6] using a Hewlett-Packard UV-Vis 8453 spectrophotometer. Assays were conducted in a quartz cuvette with a 10 mm light path at 25 °C. Reactions (1 mL total volume) were initiated by addition of ODCase (20 μL, to give a final total enzyme concentration equal to 11.0 ± 0.7 nM) to a solution of OMP (10, 20, 30, 50, 75 and 100 μM) in assay buffer (30 mM Tris-Cl, 250 mM NaCl, pH 8.0). Initial velocity data were fit to equation 1 using non-linear regression analysis and the GraphPad Prism software (v 4.00, GraphPad Software, San Diego, California). The Michaelis-Menten plot obtained from the data and the corresponding values for the kinetic parameters are shown in **Figure 2S**.

We found that in the presence of the 6-phosphonate analogue and at low concentrations of OMP, it was difficult to obtain reliable initial velocities using the spectrophotometric assay. To overcome this problem, we utilized a complete progress curve method similar to that reported by Porter *et al.*[6] for determination of the steady-state kinetic parameters of yeast ODCase. The

progress curves (**Figure 3S**) for the decarboxylation of OMP (2.0 μM) were followed for 5 min in the presence of the 6-phosphonate inhibitor **10** (0, 10, 20, 30, 40, 50, and 60 μM) under conditions where [S] < K_m / 7 (equation 2). The data were then fit to the first-order decay equation (3) to give apparent V_{max}/K_m values which were then re-plotted in their reciprocal form to determine the inhibition constant K_i value using equation 4 (having verified that the inhibition mode was competitive as shown in **Figure 2**).

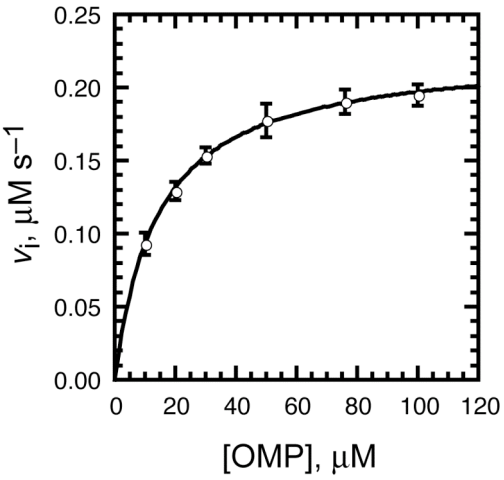

Figure 2S. Representative Michaelis-Menten plot (with no inhibitor) showing the dependence of initial velocities (v_i) on the concentration of substrate (OMP). The corresponding steady-state kinetic parameters obtained from fitting the data to equation 1 are K_m = 14 ± 1 mM, V_{max} = 0.225 ± 0.005 μM s^{-1}. The assay was conducted with $[E]_T$ = 11.0 ± 0.7 nM, yielding k_{cat} = 20 ± 1 s^{-1} and k_{cat}/K_m = (1.44 ± 0.15) × 10^6 M^{-1} s^{-1}.

$$v_i = -\frac{d[S]}{dt} = -\frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

$$v_i = -\frac{d[S]}{dt} = -\frac{V_{max}[S]}{K_m} \qquad \text{for } [S] << K_m \quad (2)$$

$$[P]_t = [P]_\infty \left(1 - e^{-\frac{V_{max} t}{K_m}}\right) \quad (3)$$

$$\left(\frac{K_m}{V_{max}}\right)^{app} = \frac{K_m}{V_{max}} + \frac{K_m}{V_{max}}\frac{[I]}{K_i} \quad (4)$$

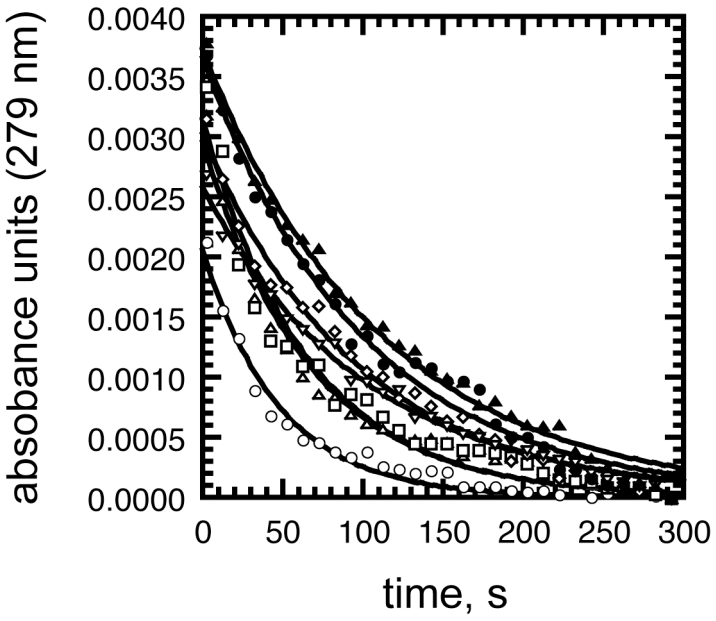

Figure 3S. Representative product curves obtained in the presence of varying concentrations of compound **10**. The data shown were fit to equation 3 to obtain the values of V_{max}/K_m (see Figure 2). The concentrations of **10** were 0 μM (○), 10 μM (△), 20 μM (□), 30 μM (▽), 40 μM (◇), 50 μM (●), and 60 μM (▲).

Reversed-phase HPLC. For HPLC analyses, a Waters 510 pump and 610 controller were used for solvent delivery. Injections were made using a Rheodyne 7725i sample injector fitted with a 200-μL injection loop. Analytes were detected by monitoring the absorbance at 260 nm using a Waters 486 absorbance detector. A Restek column (Ultra IBD, 5.0 μm, 250 × 4.6 mm) was used to separate nucleotides after quenching of the reaction as described below. ODCase was assayed by detecting uridine monophosphate (UMP) formed after the enzymatic decarboxylation of orotidine monophosphate (OMP). The assay mixture (1.0 mL) contained 5, 10, 20, or 40 μM of OMP in assay buffer (Tris-Cl buffer, 30 mM, pH 8.0, containing 240 mM NaCl), 13.3 μL of enzyme (final $[E]_T$ = 11 nm), and compound **10** (100 μM) when applicable. After incubating OMP (and/or compound **10**) without the enzyme at 25 °C for 2 min, the reaction was initiated by the addition of the enzyme and was immediately divided into 4 × 230 μL aliquots. The reaction

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was quenched by the addition of 84 μL of an ice-cold solution of 1.5 M $HClO_4$ after 2, 5, 9, and 14 min, and cooled on ice for 0.5 h. The pH of the reaction mixture was then adjusted to ~7.0 by the addition of an ice-cold solution of 6.0 M KOH and the reaction mixture was cooled on ice for an additional 0.5 h. The potassium perchlorate that precipitated from the reaction mixture was removed by centrifugation (14 000 × g, 10 min). The supernatant was then filtered through a 0.25 μm filter (Chromspec, ON, Canada) and analyzed immediately or stored at –20 °C (for a maximum of 2 d) prior to HPLC analysis. A sample of 200 μL of the reaction mixture was loaded onto the column and the components (unreacted OMP, UMP and/or inhibitor **10**) in the reaction mixture were separated by isocratic elution with ammonium acetate buffer (20 mM, pH 5.8) at a flow rate of 1.0 mL/min. Retention times (t_r) of OMP, UMP, and the inhibitor **10** are as shown in **Figures 4S** and **5S**. The amount of UMP produced from the enzymatic reaction was measured by integrating the area under the peak using PeakSimple (ver 3.29). Analyses of the data were then performed using GraphPad Prism (v 4.00, GraphPad Software, San Diego, California).

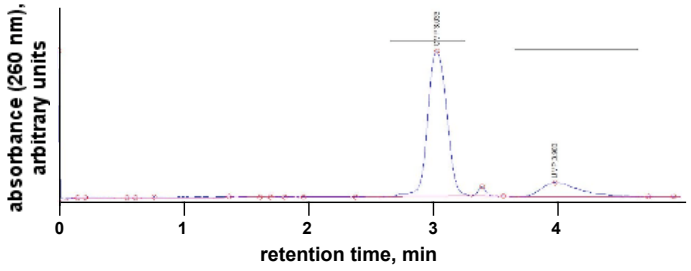

Figure 4S: Typical elution profile for a reaction mixture (40 μM OMP; t_r = 3.03 min) and the corresponding amount of UMP (t_r = 3.98 min) formed during the enzymatic reaction in the absence of inhibitor. The reaction was quenched after 14 min.

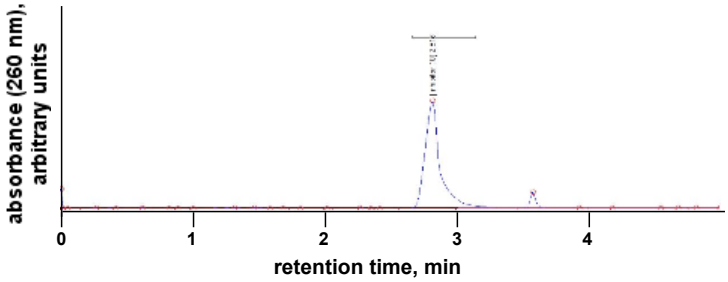

Figure 5S: Elution profile of inhibitor **10** (t_r = 2.82 min) after treatment with *E. coli* ODCase (11 nM) using the experimental conditions described above. The small peak at t_r = 3.58 min is an artifact that arises when nucleotides (OMP, UMP, or **10**) are chromatographed with the elution system. Compound **10** is not a substrate for the enzyme.

Table 1S. Reported K_i and pK_a values for inhibitors mentioned in the text.

inhibitor[a]	K_i (μM)	ODCase source[b]	pK_a[c]	references
OMP (substrate)	0.7 (K_m) 14 (K_m)	*Sc* *Ec*	2.4[d]	7,8
pyrazofurin 5′-MP	0.005	*Sc*	6.6[e]	9
6-azauridine 5′-MP	0.064	*Sc*	7.0[f]	10,11
xanthosine 5′-MP	0.41, 130, 43	*Sc*, *Mt*, *Pf*	–	7,12
6-thiocarboxamido-UMP	3.5×10^{-3}	*Sc*	–	7,13
barbituric acid ribofuranoside 5′-MP (BMP)	8.8×10^{-6}	*Sc*	~2.8 - 4.5	11,14
6-$CH_2NH_3^+$-UMP (MAUMP$^+$)	≥ 15	*Sc*′	6.6	15
6-CH_2NH_2-UMP (MAUMP)	3.3	*Sc*′	–	15
5,6-dihydro-OMP-6-SO_3^-	20.8	*Sc*′	~–1.7[g]	14
5,6-dihydro-UMP-6-SO_3^-	29.2	*Sc*′	~–1.7[g]	14
5,6-dihydro-OMP	0.25	*Sc*′	3.1[h]	7,16
6-NH_2-UMP	0.84, 2.1	*Mt*, *Pf*	–	17
6-PO_3H_2-UMP (**10**)	25.8	*Ec*	~6.9[i]	this work

[a]Abbreviations used: MP, 5′-monophosphate; U, uridine; O, orotidine; [b]Organisms:, *Ec*, *Escherichia coli*; *Mt*, *Methanobacterium thermoautotrophicum*; *Pf*, *Plasmodium falciparum*; *Sc*, *Saccharomyces cerevisiae*; *Sc*′ (C155S mutant of *Sc* ODCase); [c]Values are for ionizations of the indicated groups on the nucleotide base; [d]pK_a value for 6-COOH of orotic acid; [e]Although this value is often quoted as the pK_a of the 4-OH on the base, it is not clear that the authors in ref. 9 are referring to this group or how the value was obtained; [f]pK_a value for the triazine ring; [g]pK_a value for ethylsulfonic acid[18]; [h]pK_a value for 6-COOH of 5,6-dihydroorotic acid; [i]pK_a value for ionization of 6-PO_3H^- estimated using second pK_a for phenylphosphonic acid adjusted to the ionic strength of the assay (0.4 M) at 25 °C.[19]

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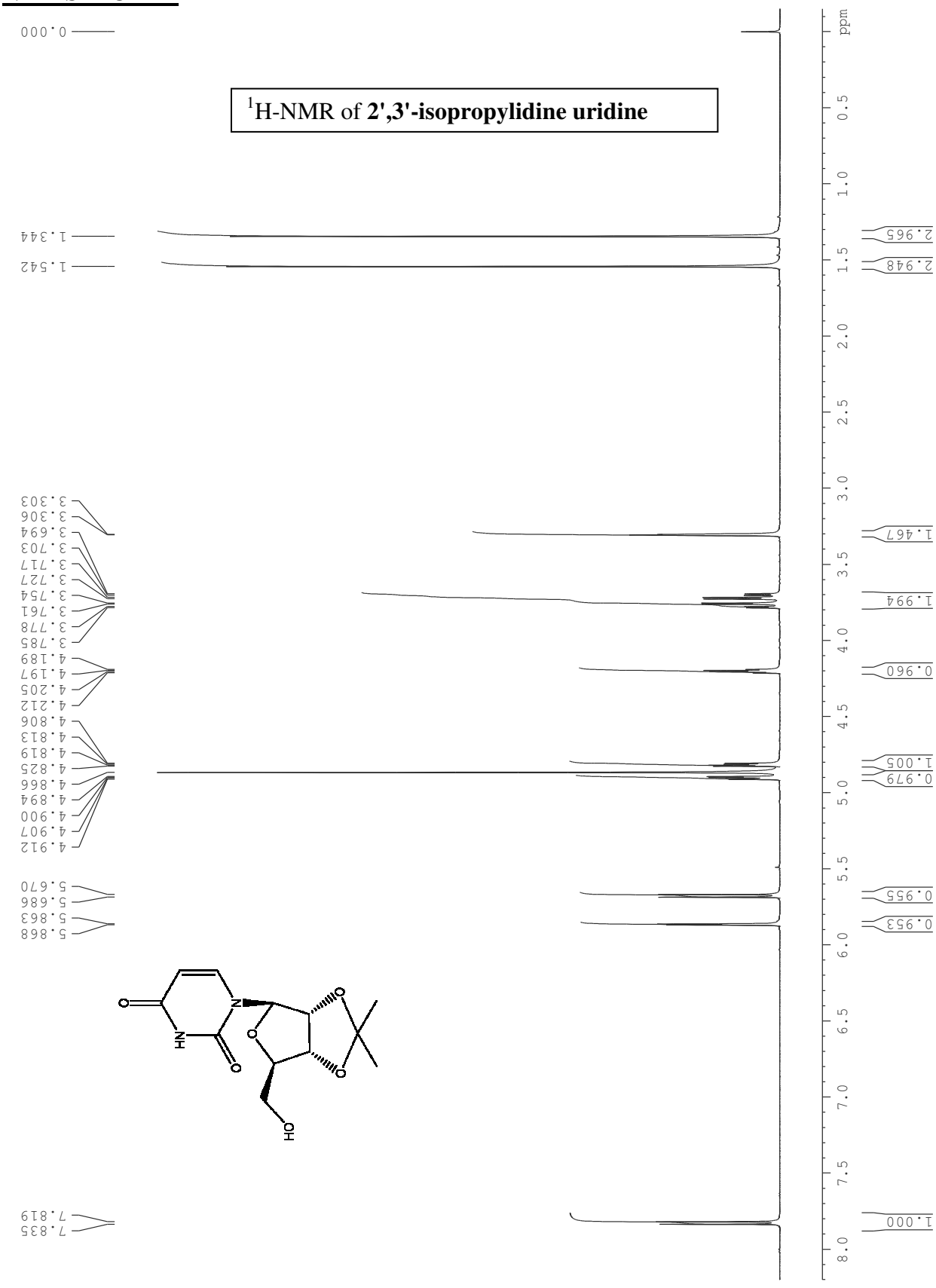
NMR SPECTRA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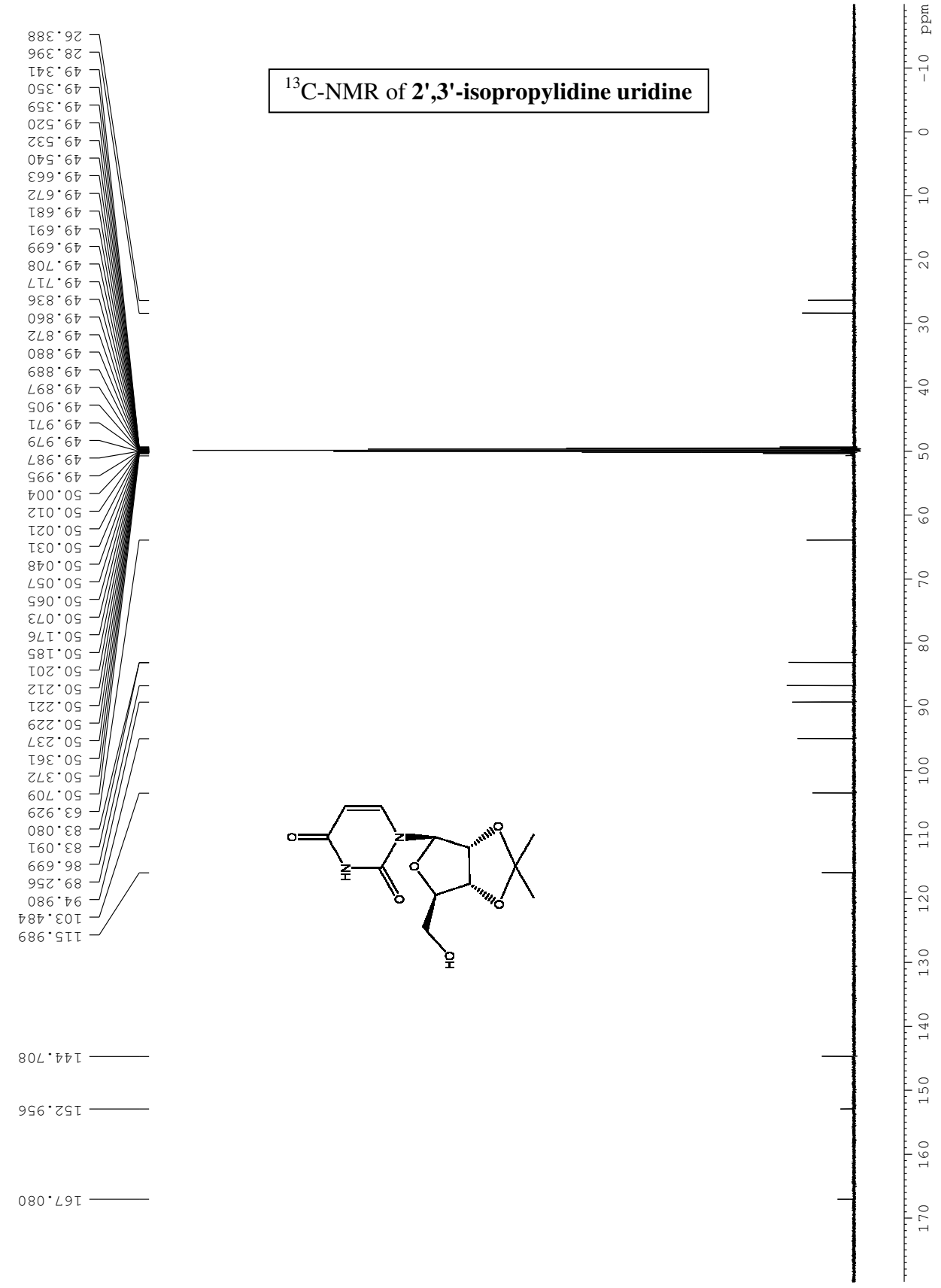
1.000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49.699
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50.021
50.031
50.048
50.057
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50.221
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50.372
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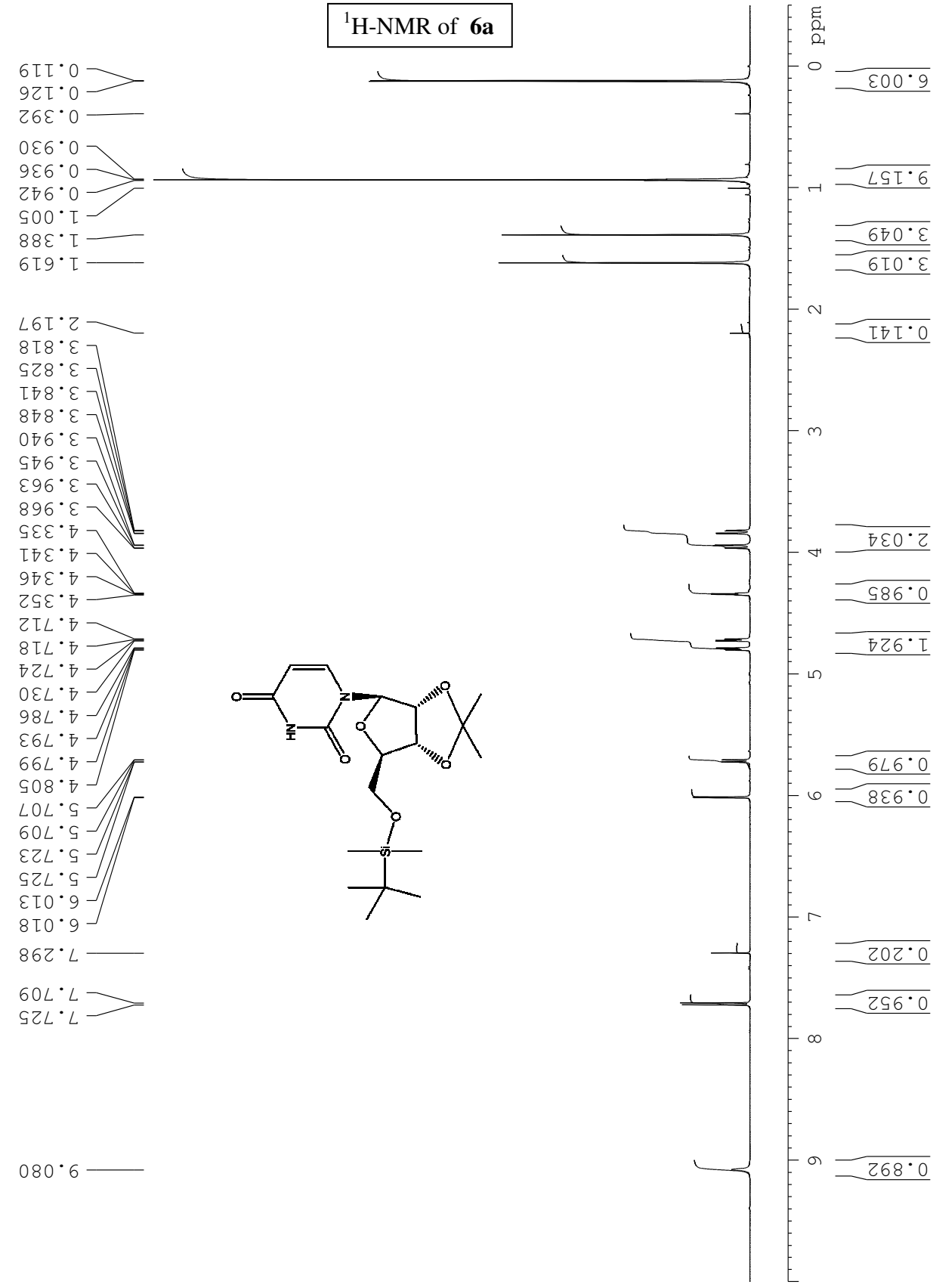

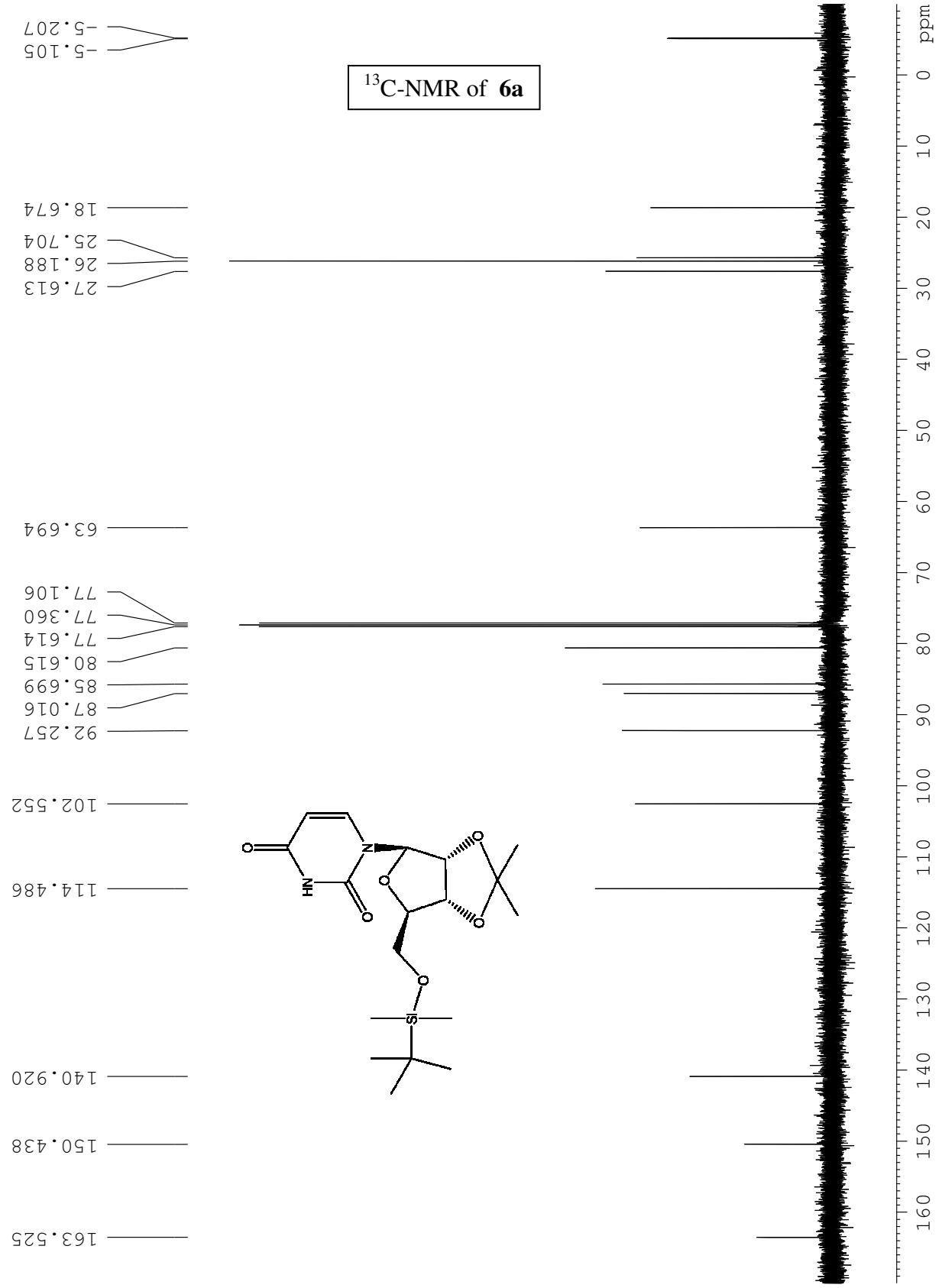
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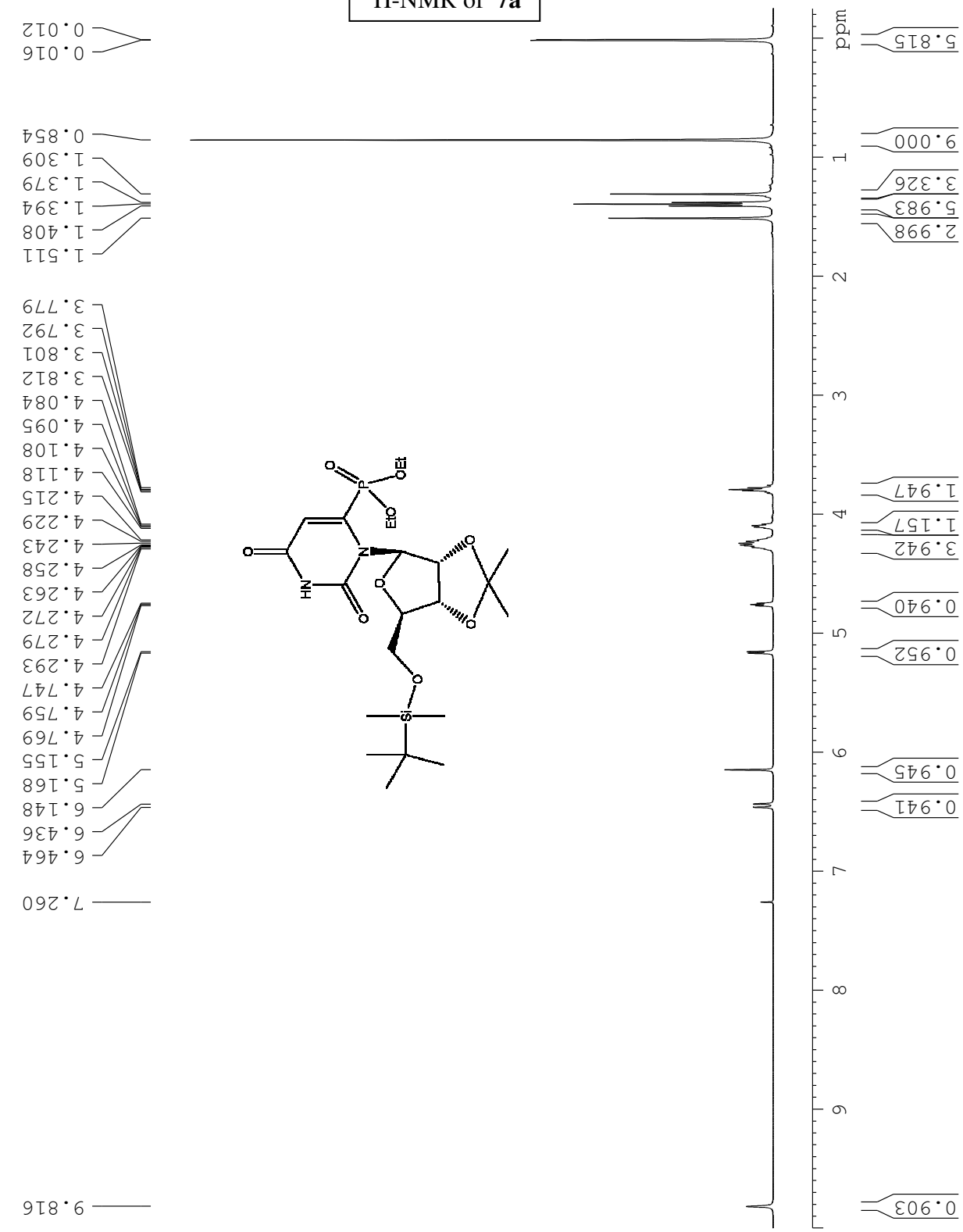
^{1}H-NMR of **7a**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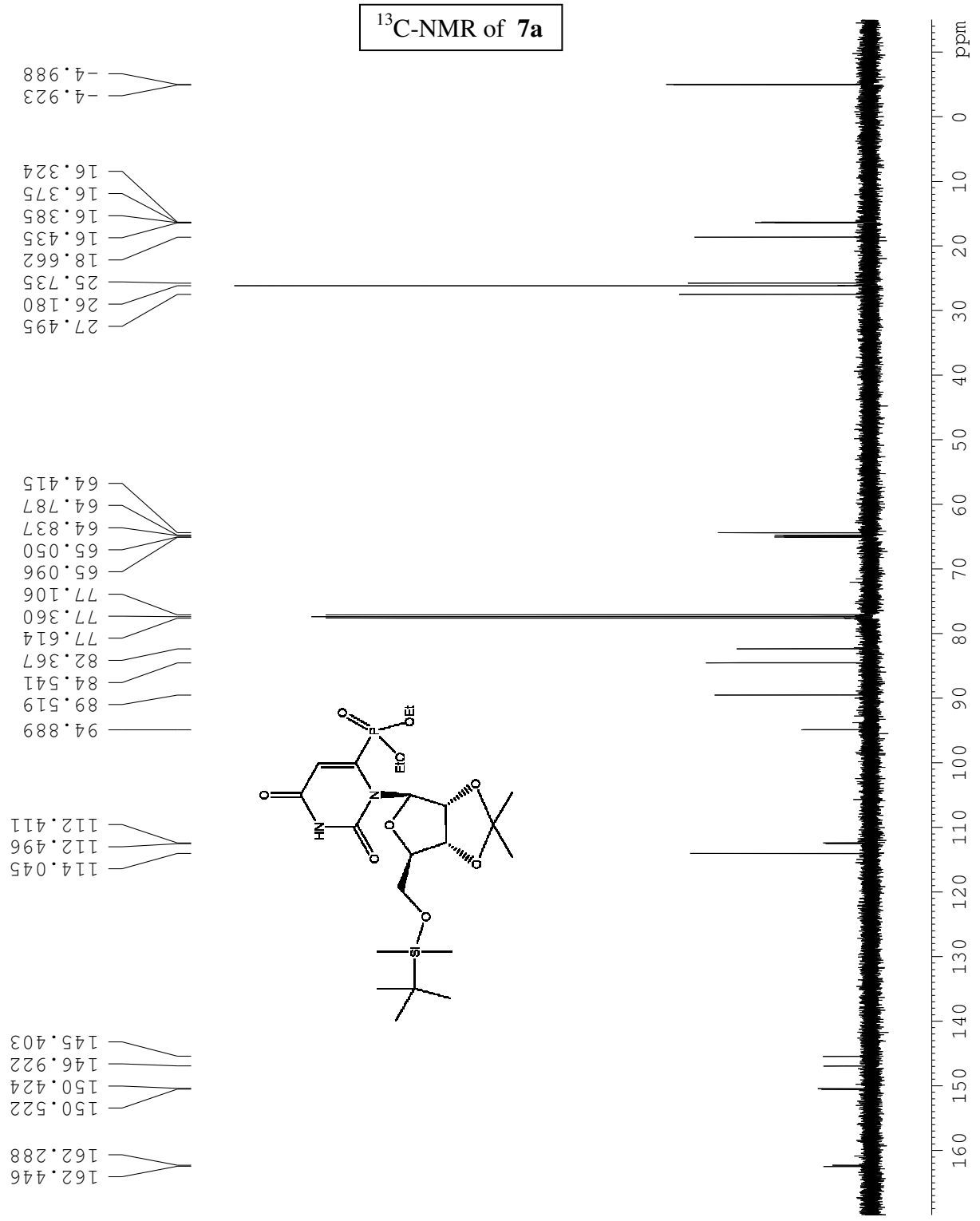
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65.050
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77.614
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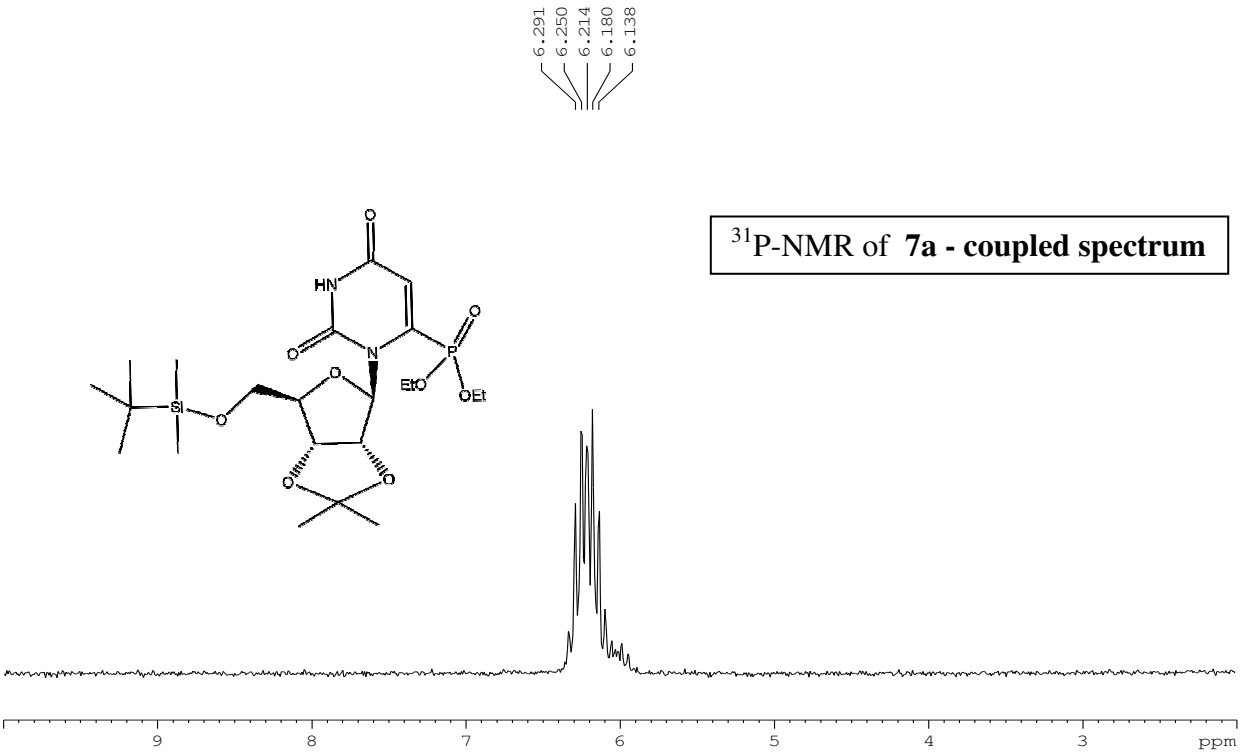
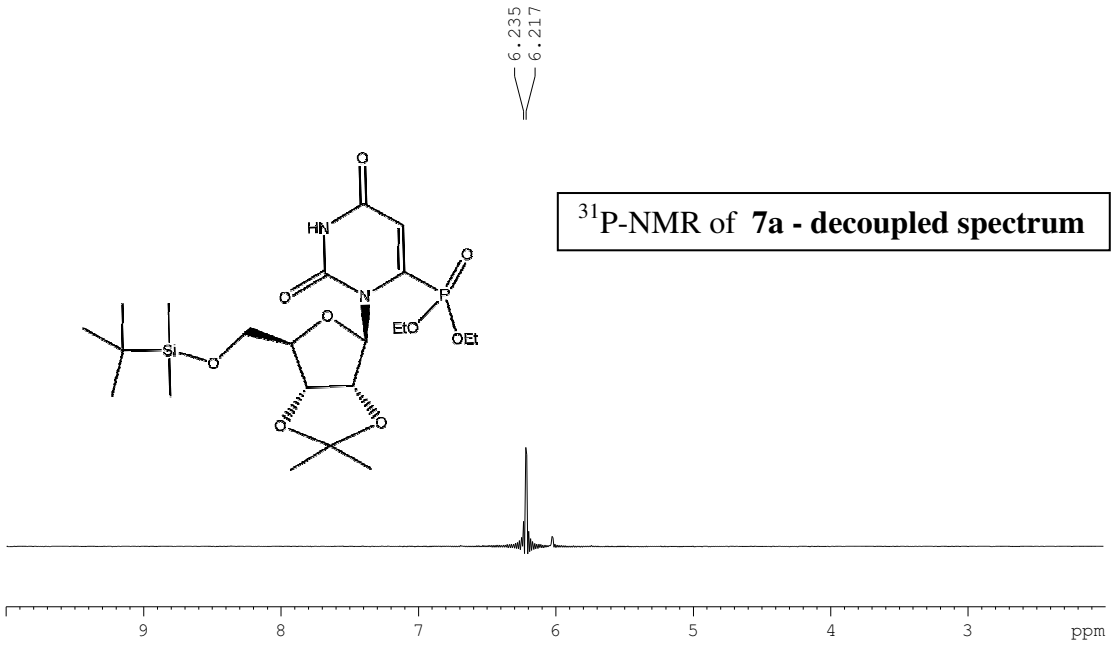
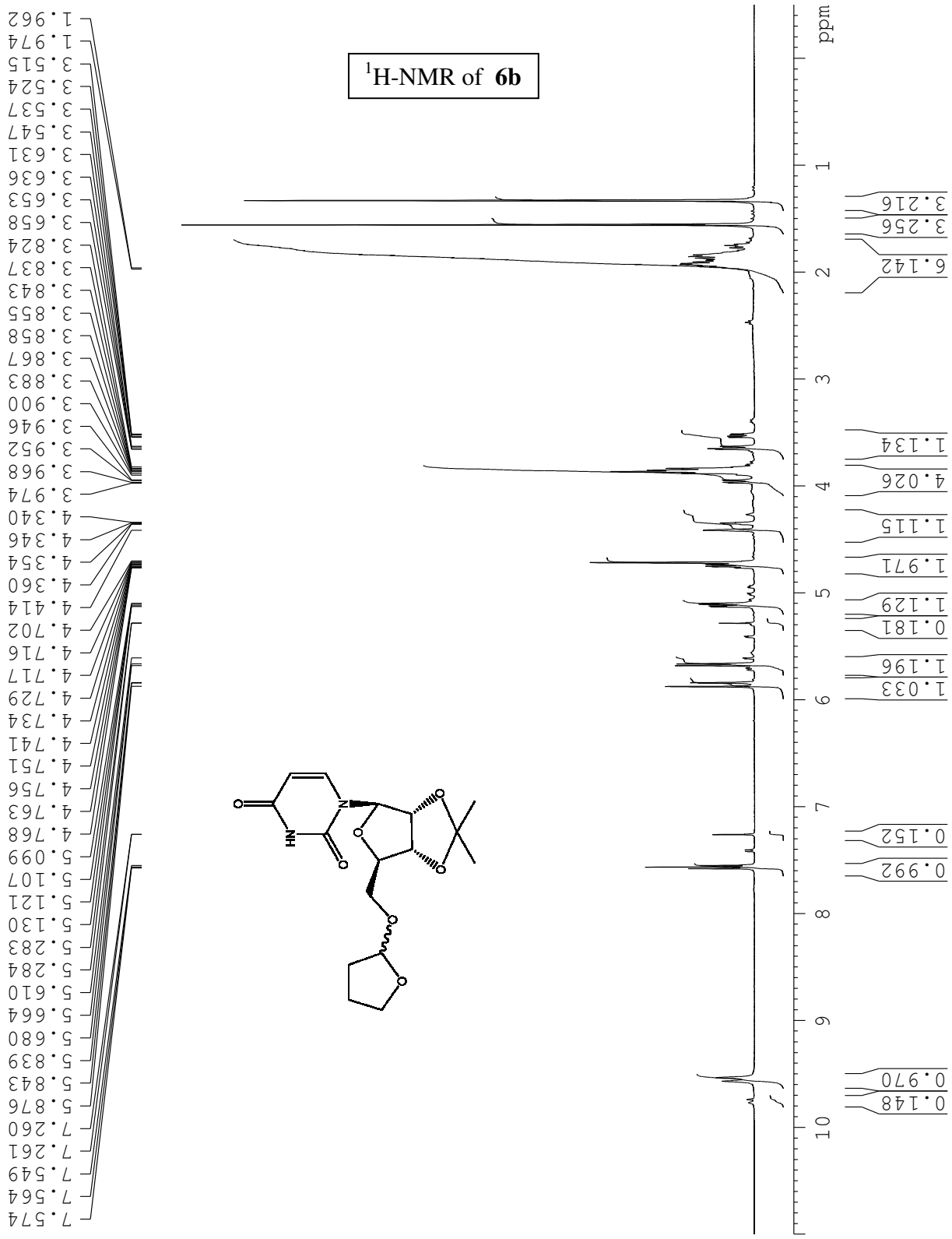


^{31}P-NMR of **7a - coupled spectrum**



^{31}P-NMR of **7a - decoupled spectrum**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0.181
1.196
1.033
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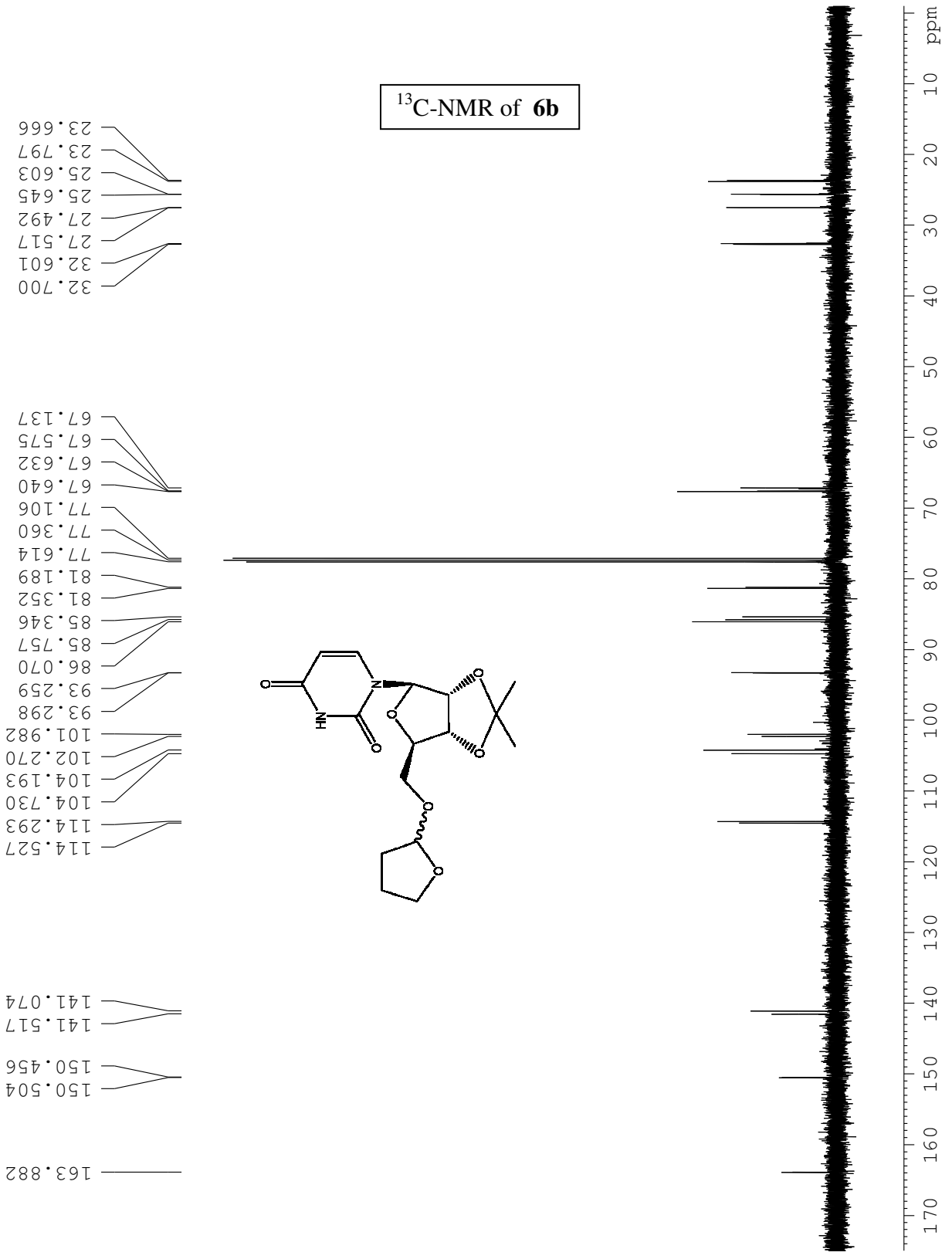
0.992
0.970
0.148
1.962 1.974 3.515 3.524 3.537 3.547 3.631 3.636 3.653 3.658 3.824 3.837 3.843 3.855 3.858 3.867 3.883 3.900 3.946 3.952 3.968 3.974 4.340 4.346 4.354 4.360 4.414 4.702 4.716 4.717 4.729 4.734 4.741 4.751 4.756 4.763 4.768 5.099 5.107 5.121 5.130 5.283 5.284 5.610 5.664 5.680 5.839 5.843 5.876 7.260 7.261 7.549 7.564 7.574


13C-NMR of 6b
23.666
23.797
25.603
25.645
27.492
27.517
32.601
32.700
67.137
67.575
67.632
67.640
77.106
77.360
77.614
81.189
81.352
85.346
85.757
86.070
93.259
93.298
101.982
102.270
104.193
104.730
114.293
114.527
141.074
141.517
150.456
150.504
163.882
ppm

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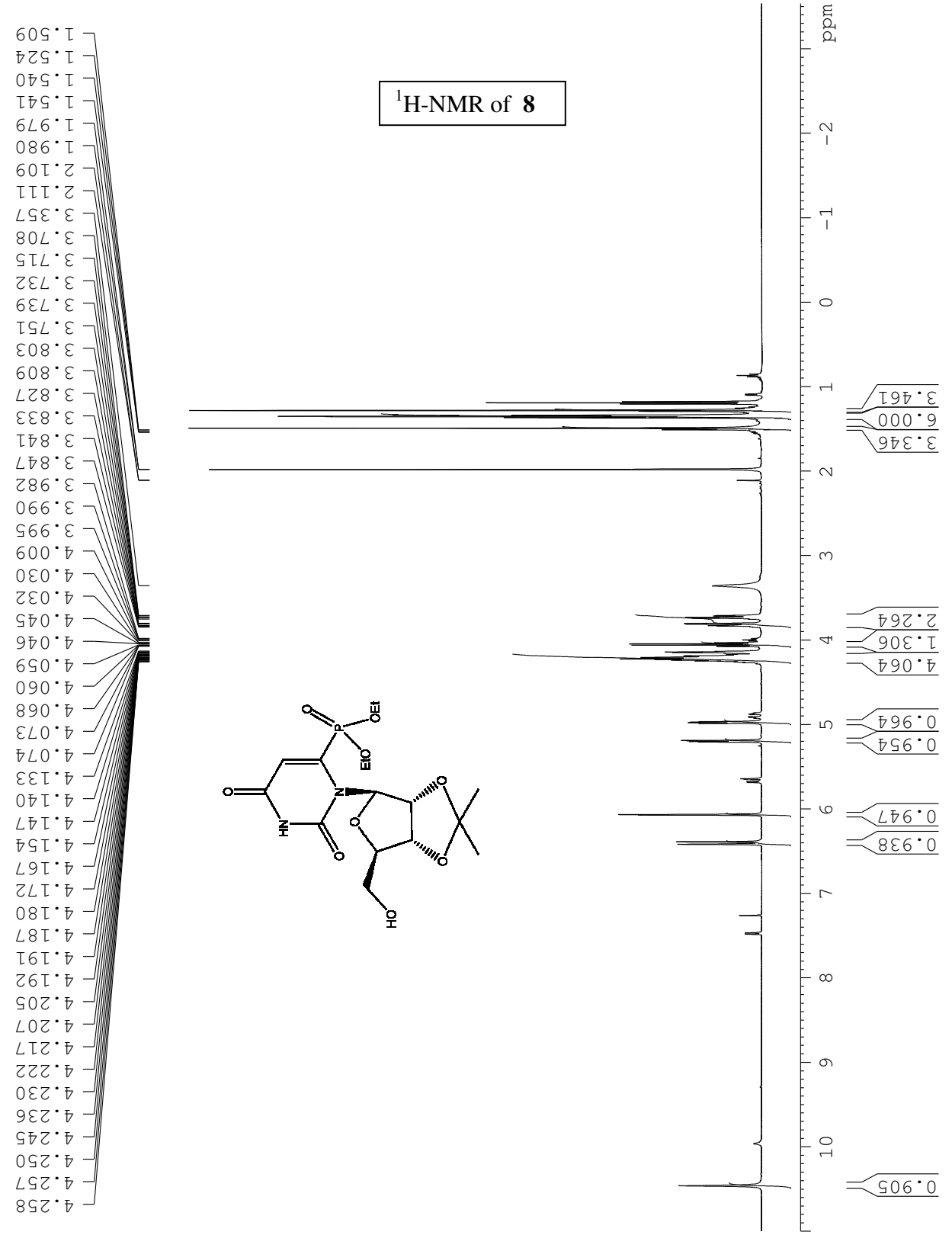

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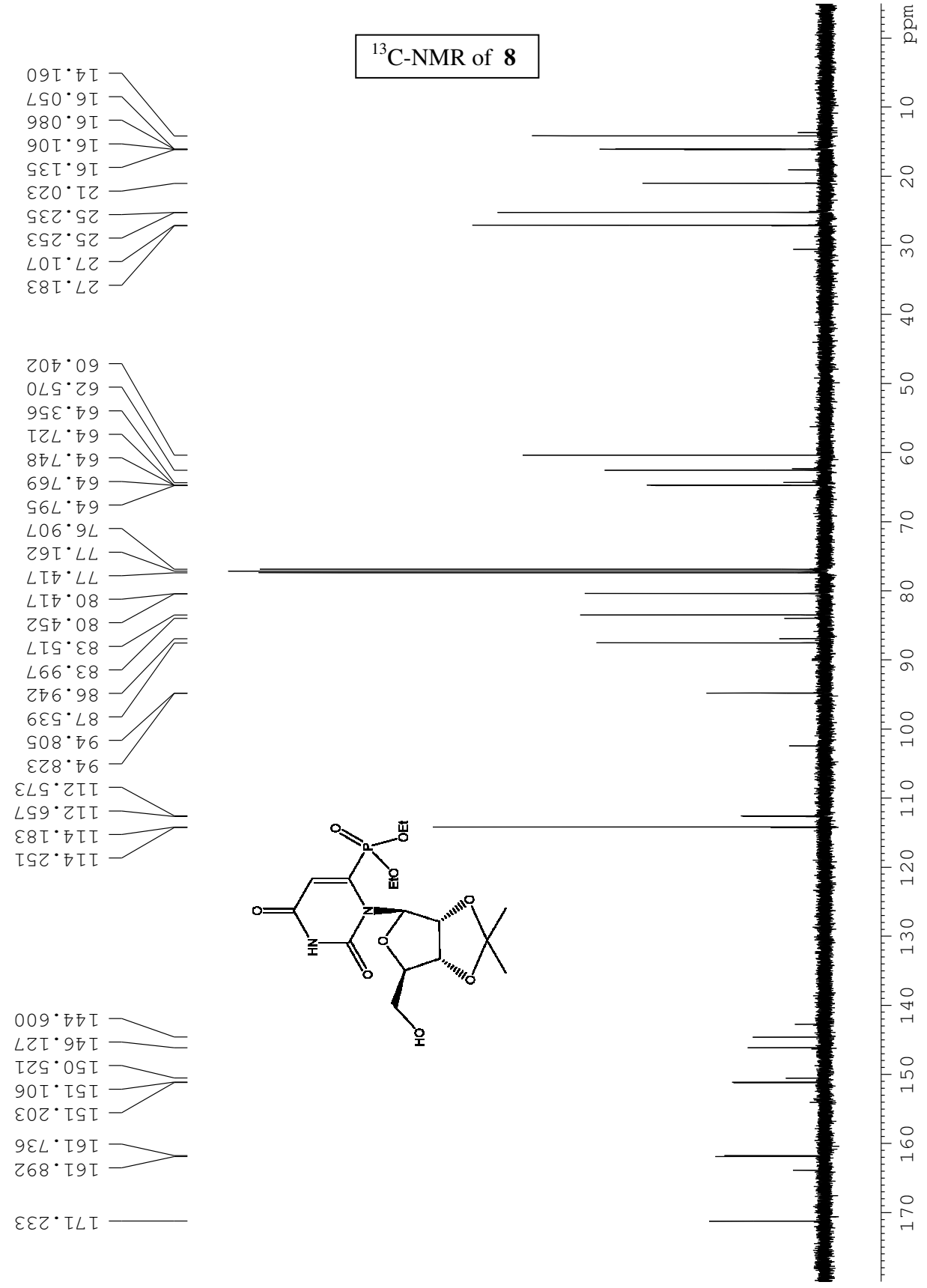

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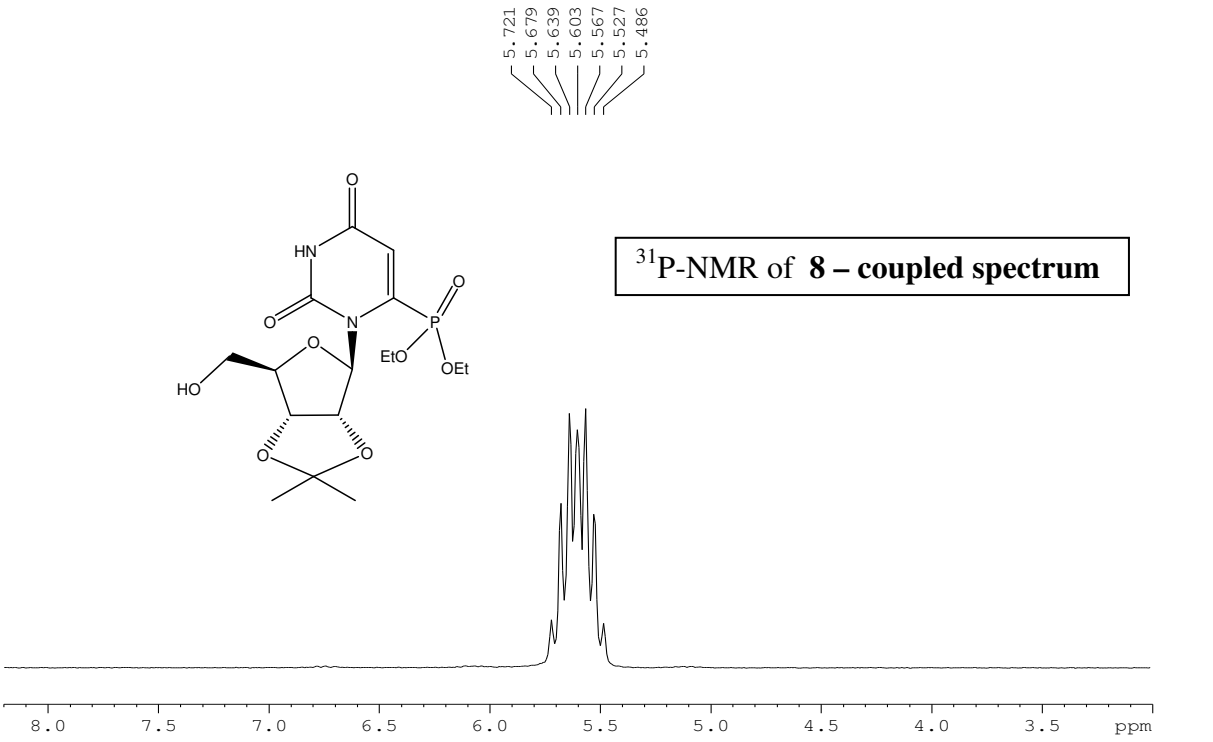
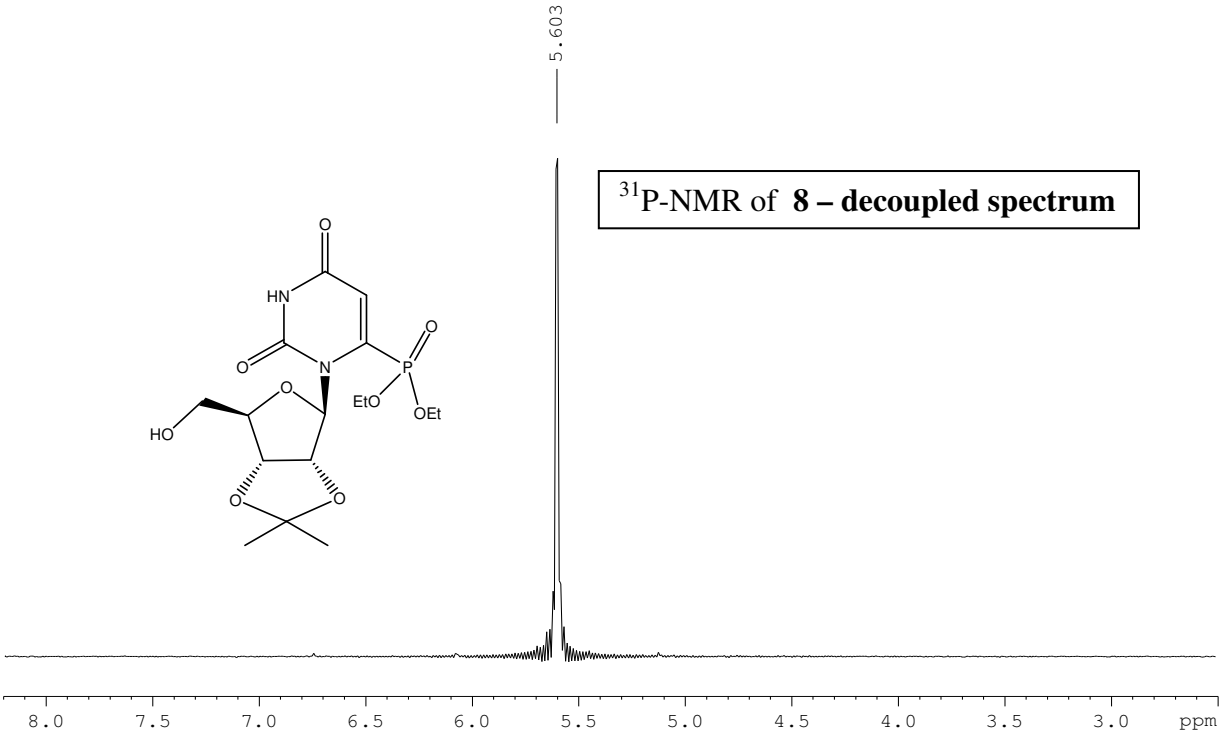
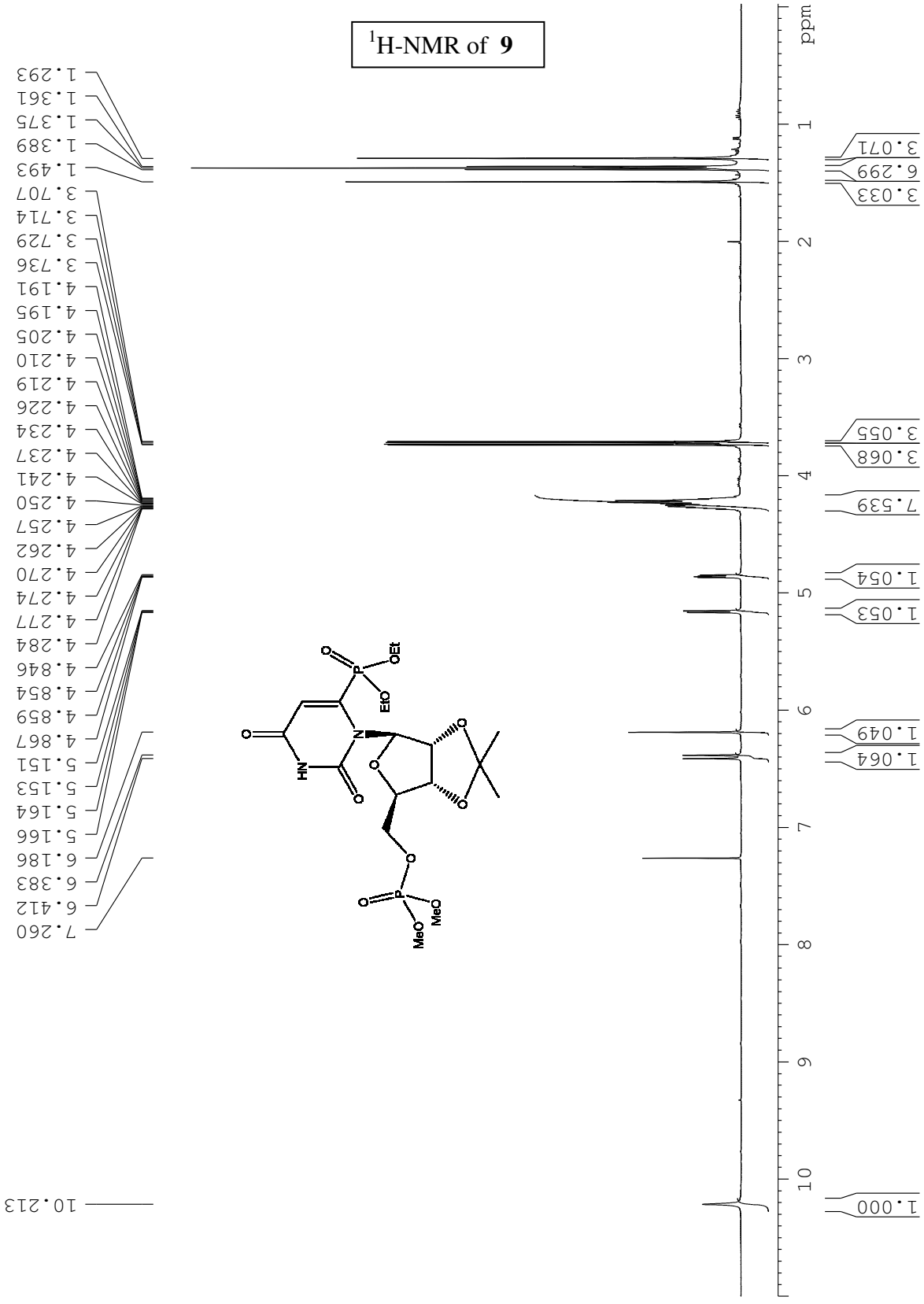
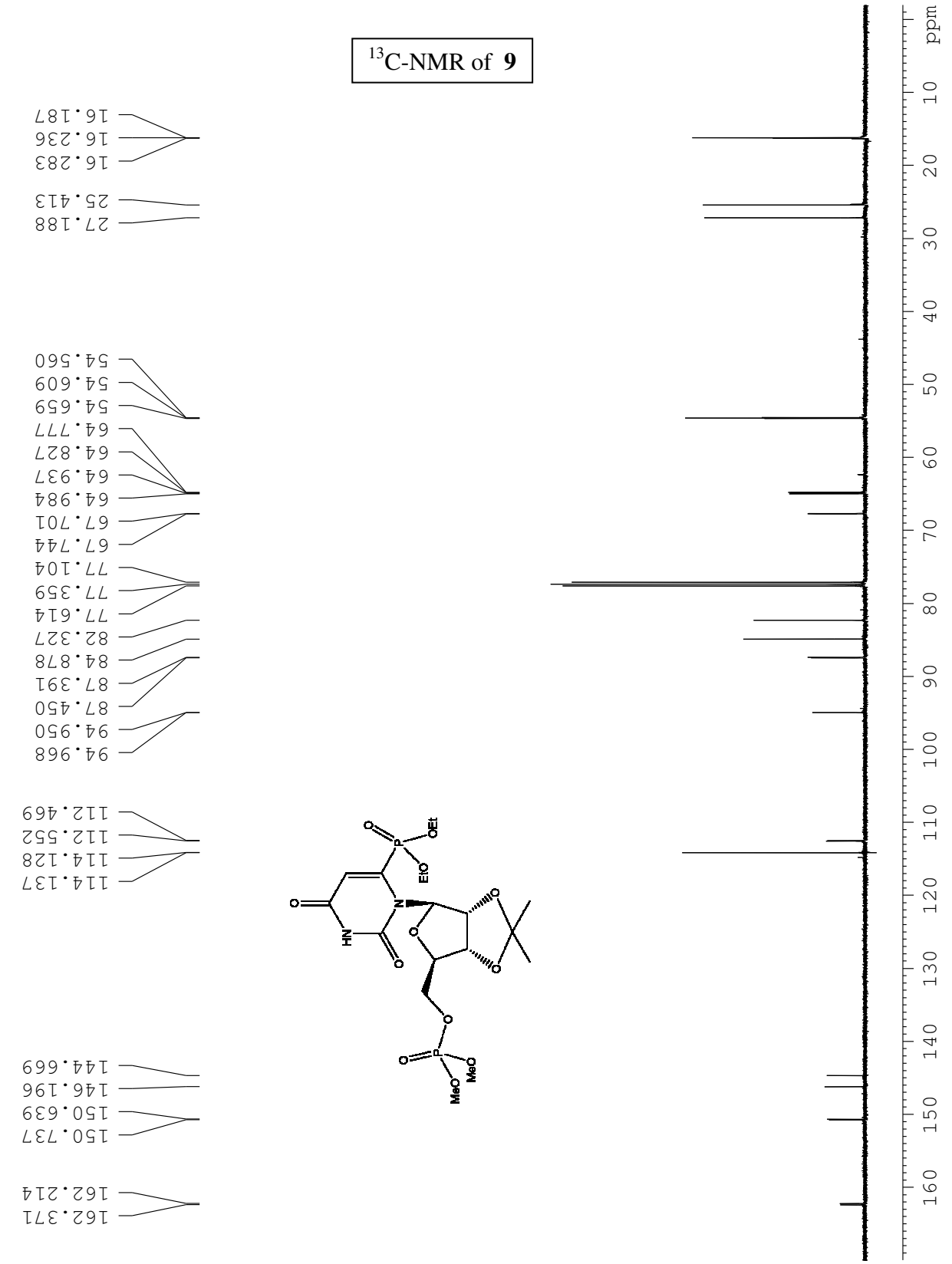


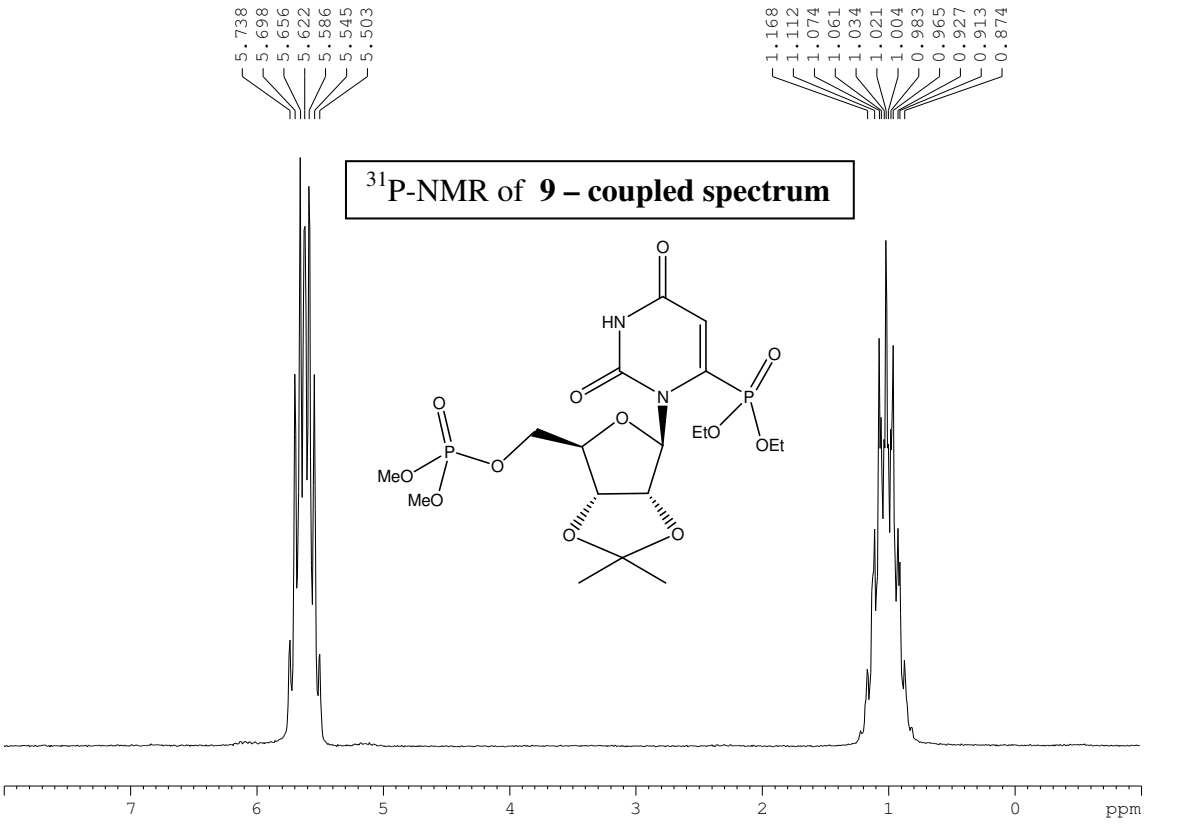
^{31}P-NMR of **8 – coupled spectrum**

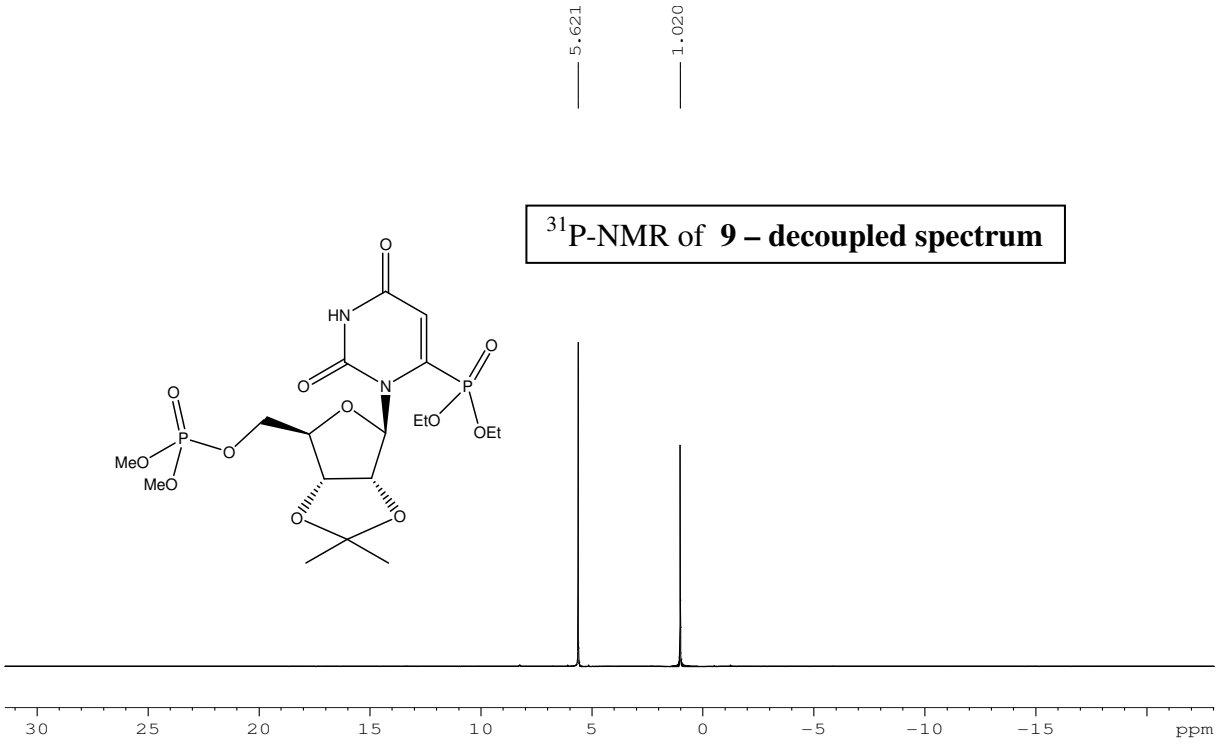


^{31}P-NMR of **8 – decoupled spectrum**


^{1}H-NMR of **9**
ppm
1.293
1.361
1.375
1.389
1.493
3.707
3.714
3.729
3.736
4.191
4.195
4.205
4.210
4.219
4.226
4.234
4.237
4.241
4.250
4.257
4.262
4.270
4.274
4.277
4.284
4.846
4.854
4.859
4.867
5.151
5.153
5.164
5.166
6.186
6.383
6.412
7.260
10.213
3.071
6.299
3.033
3.055
3.068
7.539
1.054
1.053
1.049
1.064
1.000
EtO
OEt
MeO
MeO
HN
N
O
P


13C-NMR of 9
16.187
16.236
16.283
25.413
27.188
54.560
54.609
54.659
64.777
64.827
64.937
64.984
67.701
67.744
77.104
77.359
77.614
82.327
84.878
87.391
87.450
94.950
94.968
112.469
112.552
114.128
114.137
144.669
146.196
150.639
150.737
162.214
162.371
ppm
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
EtO
OEt
MeO
MeO
HN
N
O
P


5.738
5.698
5.656
5.622
5.586
5.545
5.503
1.168
1.112
1.074
1.061
1.034
1.021
1.004
0.983
0.965
0.927
0.913
0.874
31P-NMR of 9 – coupled spectrum
HN
O
N
P
EtO
OEt
MeO
MeO
7
6
5
4
3
2
1
0
ppm


5.621
1.020
31P-NMR of 9 – decoupled spectrum
HN
O
N
P
EtO
OEt
MeO
MeO
30
25
20
15
10
5
0
−5
−10
−15
ppm

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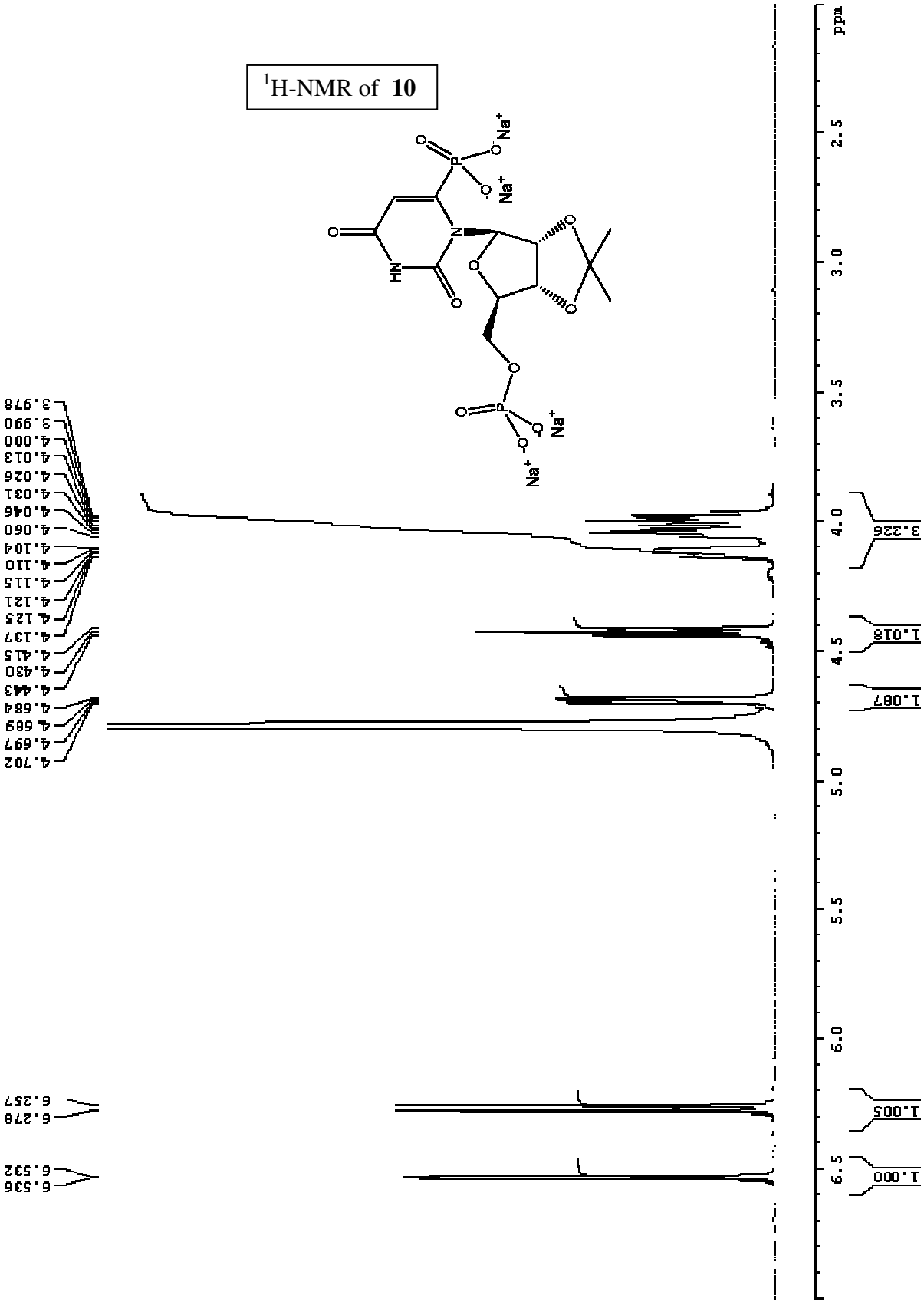

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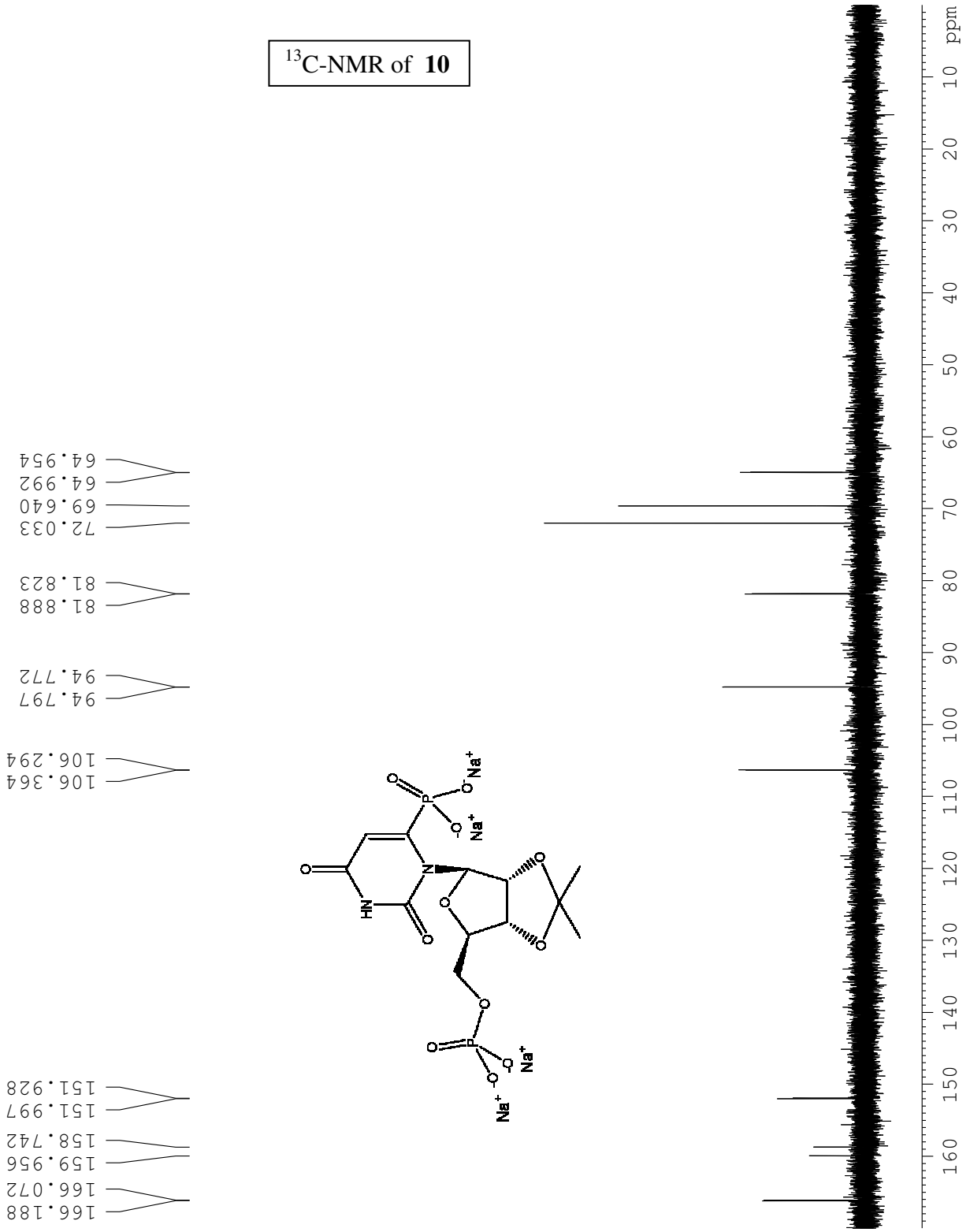
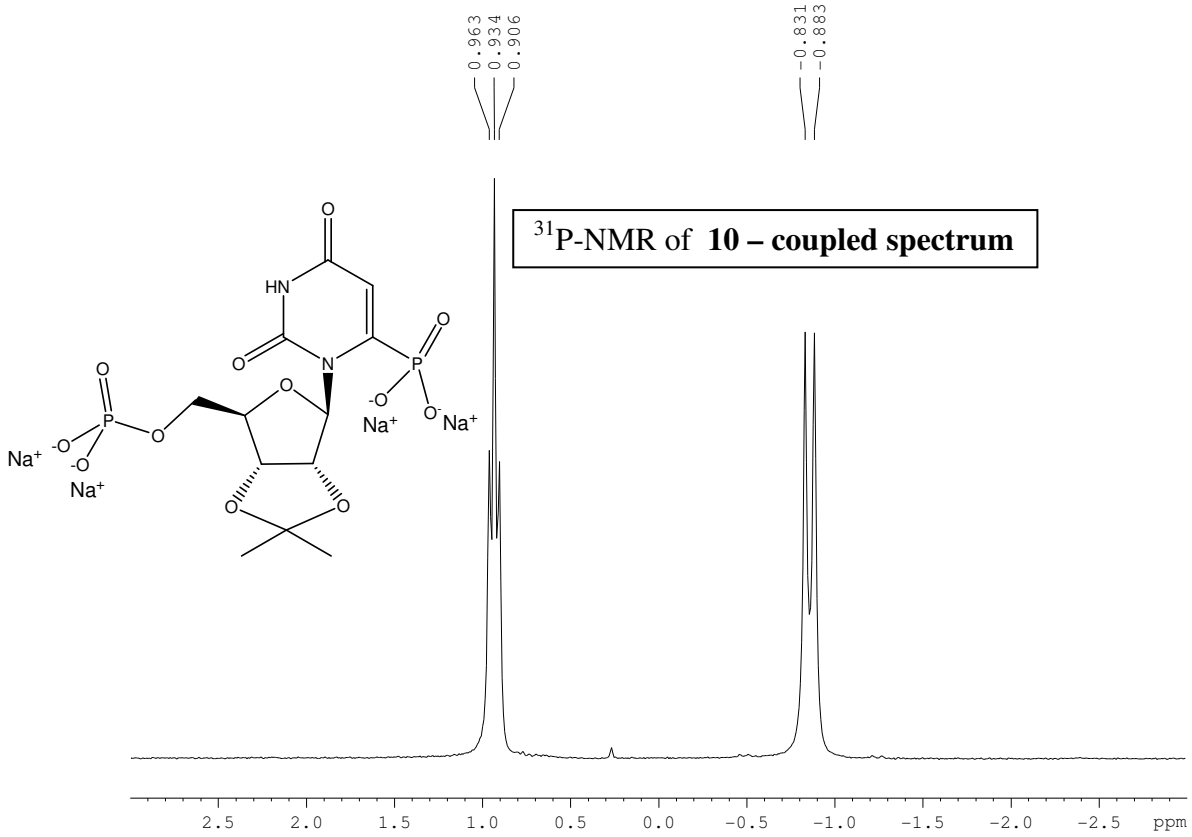
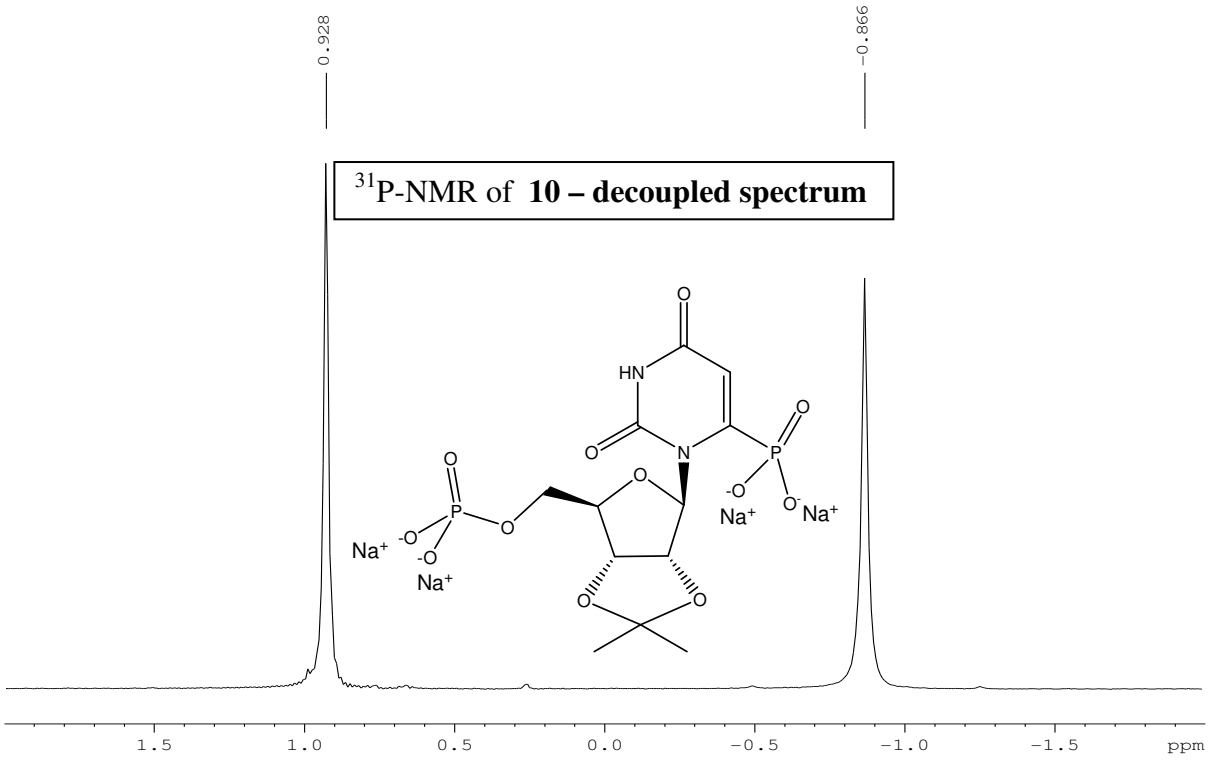
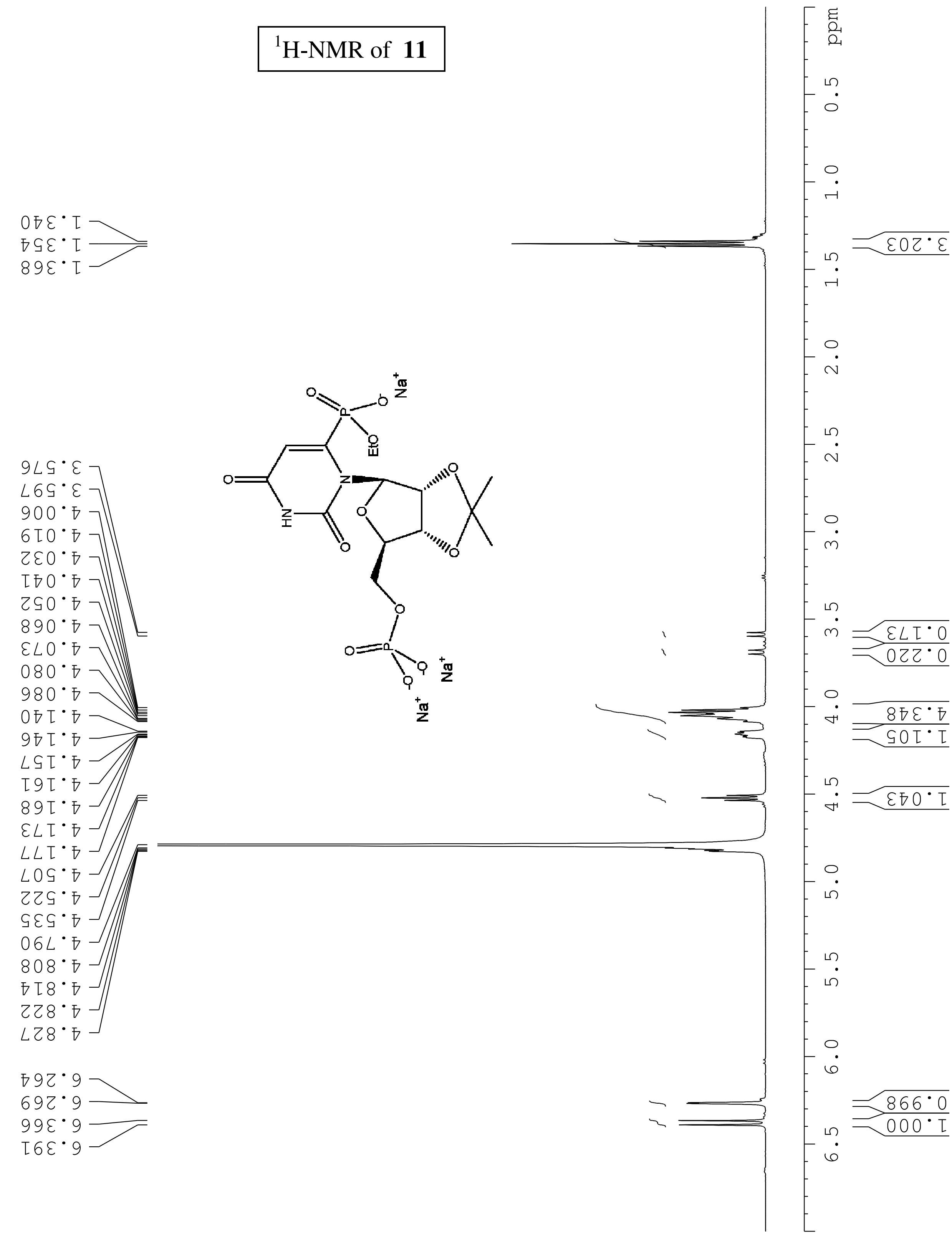
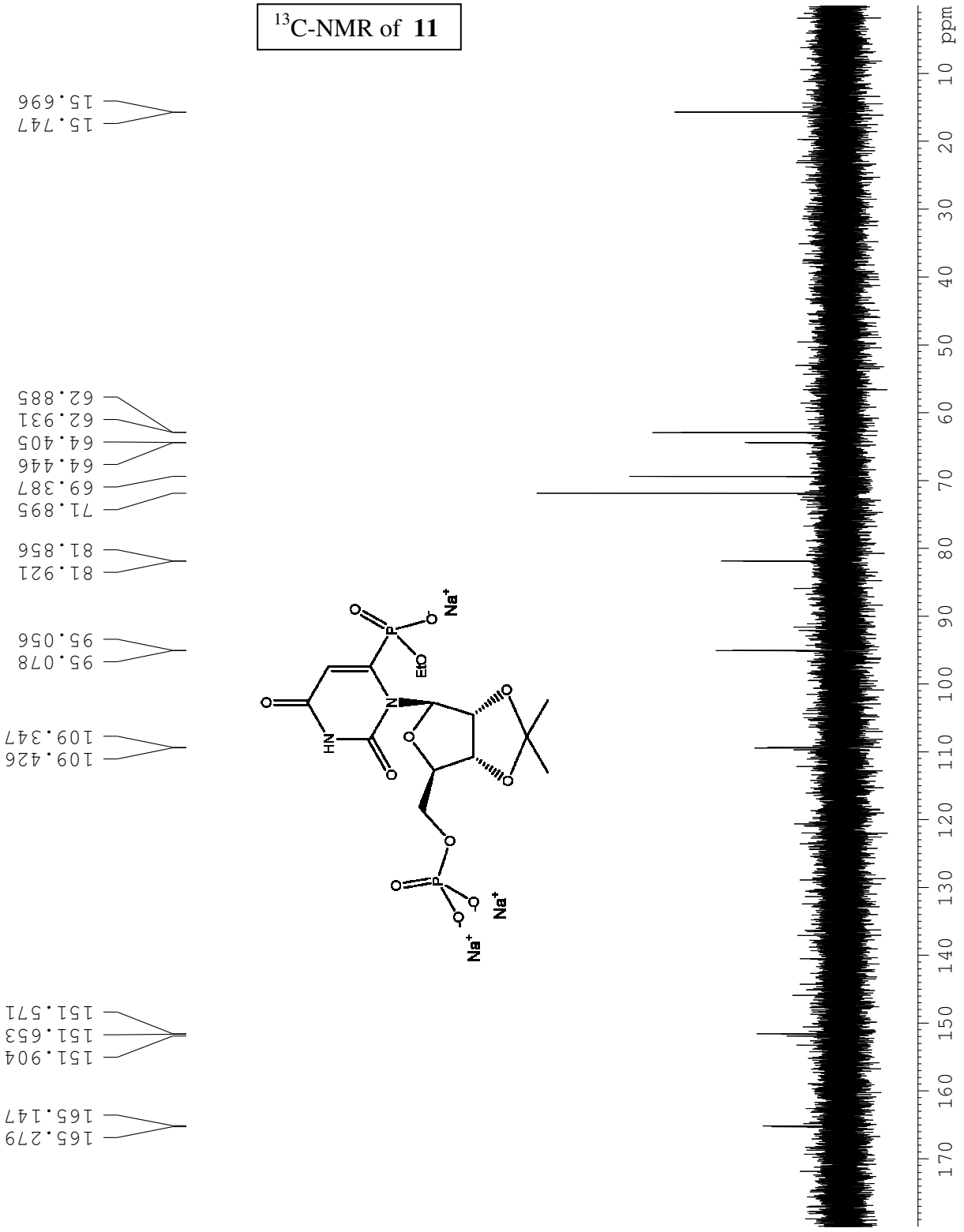
^{13}C-NMR of **10**

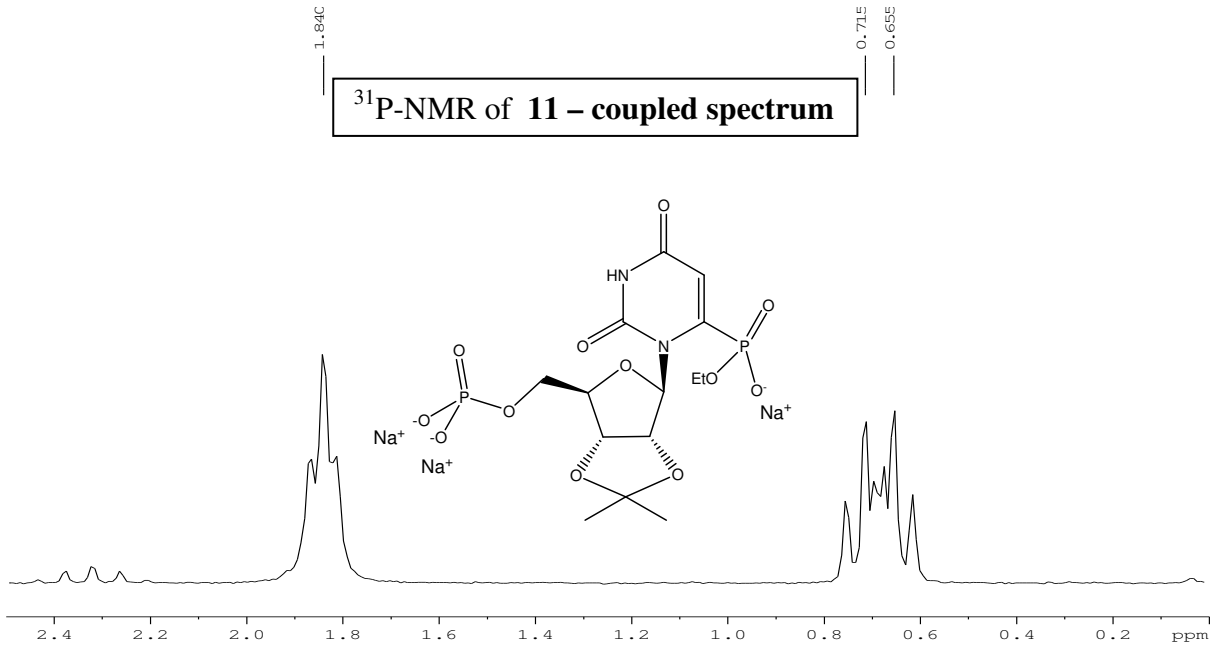


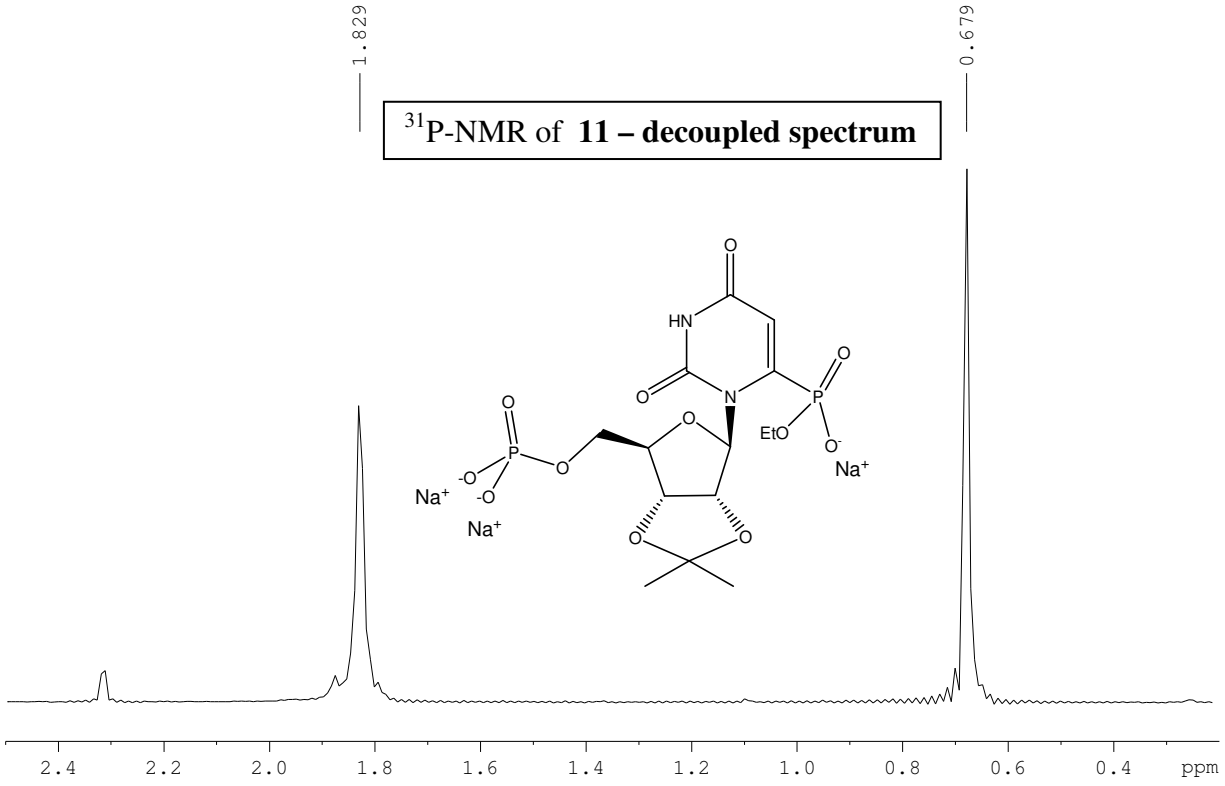

0.963
0.934
0.906
-0.831
-0.883
31P-NMR of 10 – coupled spectrum
HN
O
N
P
Na+
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
ppm

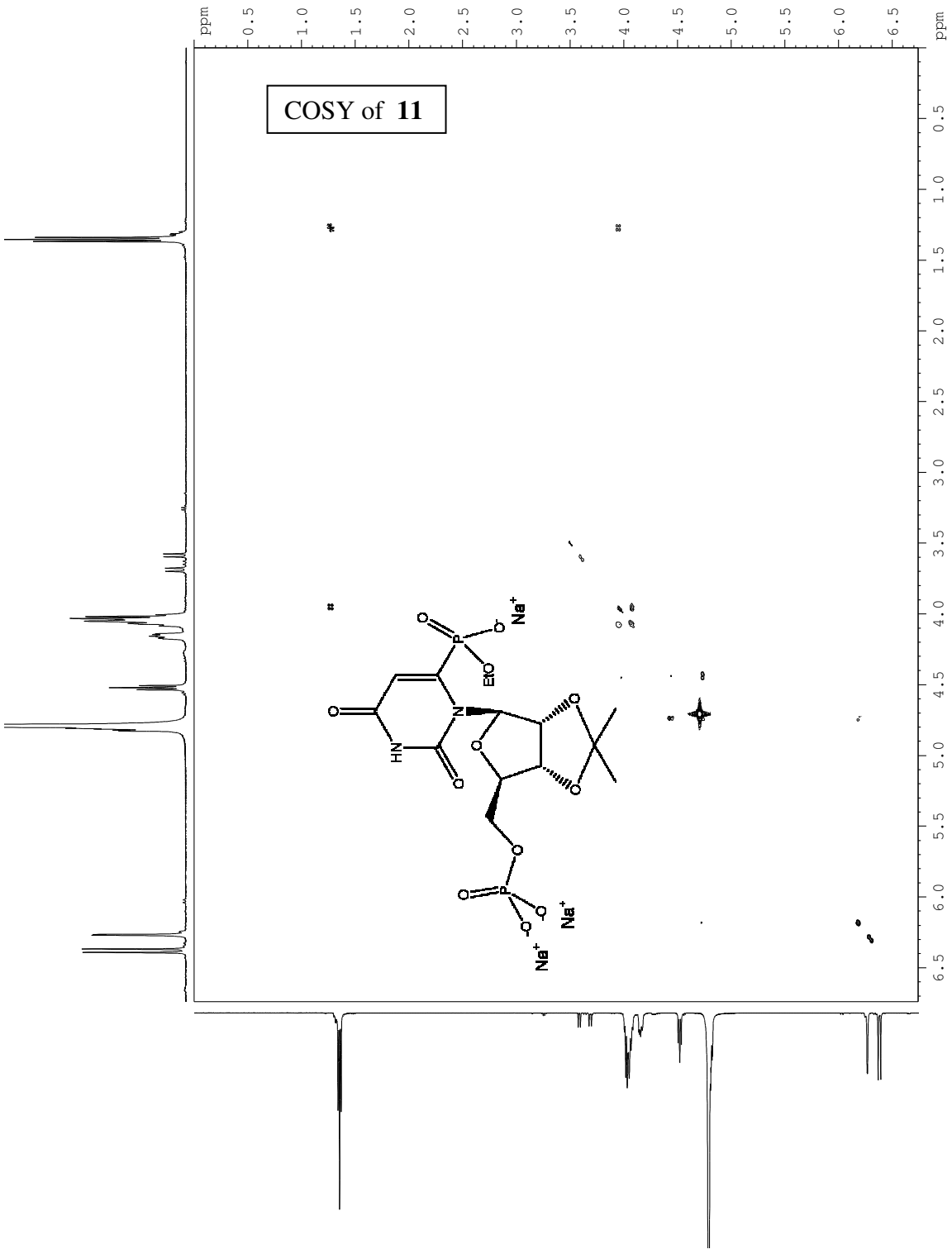

0.928
-0.866
31P-NMR of 10 – decoupled spectrum
HN
O
N
P
Na+
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
ppm


^{1}H-NMR of **11**
ppm
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
1.340
1.354
1.368
3.576
3.597
4.006
4.019
4.032
4.041
4.052
4.068
4.073
4.080
4.086
4.140
4.146
4.157
4.161
4.168
4.173
4.177
4.507
4.522
4.535
4.790
4.808
4.814
4.822
4.827
6.264
6.269
6.366
6.391
3.203
0.173
0.220
4.348
1.105
1.043
0.998
1.000


13C-NMR of 11
15.696
15.747
62.885
62.931
64.405
64.446
69.387
71.895
81.856
81.921
95.056
95.078
109.347
109.426
151.571
151.653
151.904
165.147
165.279
EtO
Na+
Na+
Na+
HN
N
O
P
ppm
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170


1.840
0.715
0.655
31P-NMR of 11 – coupled spectrum
HN
O
N
P
EtO
Na+
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
ppm


1.829
0.679
31P-NMR of 11 – decoupled spectrum
HN
O
N
P
EtO
Na+
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
ppm


COSY of 11
ppm
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
Na+
EtO
HN

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