Supporting Information for

Two scaffolds from two flips: (α,β)/(β,γ) CH_2/NH "Met-Im" analogues of dTTP

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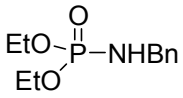
I. General Information

All the reagents and solvents were purchased from VWR, Sigma-Aldrich, or Alfa Aesar and used without further purification, unless noted otherwise. ^{1}H, ^{13}C and ^{31}P NMR spectra were obtained on Varian 400-MR or VNMRS-500 spectrometers. ^{13}C and ^{31}P NMR spectra were proton-decoupled unless stated otherwise. All chemical shifts (δ) are reported in parts per million (ppm) relative to residual CH_3OH in CD_3OD (δ 3.34, ^{1}H NMR), $CHCl_3$ in $CDCl_3$ (δ 7.26, ^{1}H NMR), HDO in D_2O (δ 4.80, ^{1}H NMR), external 85% H_3PO_4 (δ 0.00, ^{31}P NMR) or internal $CDCl_3$ (δ 77.00, ^{13}C NMR). The concentration of the NMR samples was 2 – 15 mg/ml. 1D and 2D NMR spectra processing was performed with MestReNova 9.0.0. Multiplicities are reported using the following abbreviations: s = singlet, d = doublet, t = triplet, m = multiplet, br = broad resonance. Preparative HPLC was performed using a Varian ProStar equipped with a Shimadzu SPD-10A UV detector (0.5 mm path length) with detection at 260 nm. Strong Anion Exchange (SAX) HPLC was performed using a Macherey Nagel 21.4 mm × 250 mm SP15/25 Nucleogel column, C-18 HPLC was performed using a Phenomenex Luna 5u C18 (21.20 mm × 250 mm). Mass spectrometry was performed on a Finnigan LCQ Deca XP Max mass spectrometer equipped with an ESI source in the negative ion mode. Microwave-assisted synthesis was performed using a Milestone Ethos Synth Microwave Labstation. Compound IUPAC names were assigned with the assistance of MarvinSketch 6.1.5. The molar yields of the final products were determined by UV absorbance using the extinction coefficient of dTTP in phosphate buffer at pH 7.0 at 260 nm, ε = 9600.[1]

II. Experimental Procedures and Compound Characterization

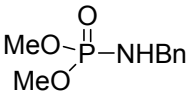


2a

Diethyl benzylphosphoramidate (2a)

Compound **2a** was previously made by the reaction of diethyl phosphite and benzylamine in the presence of hydrogen peroxide and a catalytic amount of I_2,[2] however, we found that it could be conveniently obtained by adapting the procedure of Glidewell.[3]

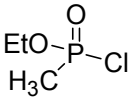
Benzylamine (2.14 g, 0.02 mol) was added to a solution of diethylphosphite (1.38 g, 0.01 mol) in CCl_4 (20 mL). A white precipitate appeared immediately, and the reaction mixture was stirred overnight at r. t. The white precipitate was filtered, washed with CH_2Cl_2, and volatiles were removed from the combined filtrate and washings by rotary evaporation (water pump). The residue was dried under vacuum at r.t. (<1 mm), giving 2.4 g of the product **2a** as a colorless oil (99%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.36-7.27 (m, 5H, C_6H_5), 4.13-3.99 (m, 6H, $CH_2C_6H_5$ and CH_2CH_3), 2.89 (br, 1H, NH), 1.31 (t, J = 7.1 Hz, 6H, 2 × OCH_2CH_3). ^{31}P NMR (202 MHz, $CDCl_3$): δ 8.36. Lit. data:[2] ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.33-7.25 (m, 5H, C_6H_5), 4.11-3.98 (m, 6H, $CH_2C_6H_5$ and CH_2CH_3), 3.22 (br, 1H, NH), 1.29 (t, J = 7.1 Hz, 6H, 2 × OCH_2CH_3). ^{31}P NMR (202 MHz, $CDCl_3$): δ 8.50.



2b

Dimethyl benzylphosphoramidate (2b)[3]

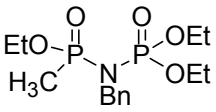
Benzylamine (3.3 g, 0.03 mol) was added to a solution of dimethyl phosphite (1.65 g, 0.015 mol) in CCl_4 (20 mL). A white precipitate appeared immediately, and the reaction mixture was stirred overnight at r. t. The white precipitate was filtered, washed with CH_2Cl_2, and volatiles were removed from the combined filtrate by rotary evaporation (water pump). The residue was dried under vacuum at r. t. (<1 mm), giving 2.98 g of the product **2b** as a colorless oil that crystallized in the refrigerator (~ -20°C) but remelted on rewarming to r.t. (92%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.36-7.28 (m, 5H, C_6H_5), 4.10 (dd, J = 9.7 Hz, J = 7.0 Hz, 2H, $CH_2C_6H_5$), 3.72 (d, J = 10.7 Hz, 6H, 2 × OCH_3), 2.88 (br, 1H, N*H*). ^{31}P NMR (202 MHz, $CDCl_3$): δ 10.93. Lit. data:[3] ^{1}H NMR (60 MHz, CCl_4) δ 7.30 (s, 5H), 5.38 (dt, J = 11 Hz, J = 6 Hz, lH), 3.98 (dd, J = 11 Hz, J = 6 Hz, 2H), 3.58 (d, J = 11 Hz, 6H). ^{31}P NMR (32 MHz, CCl_4): δ 11.7.



Ethyl methylphosphonochloridate[4]

Oxalyl chloride (1.27 g, 0.01 mol) was added in one portion to a solution of diethyl methylphosphonate (1.52 g, 0.01 mol) in 15 mL of dry CH_2Cl_2. The reaction mixture was stirred for 16 h at r.t. The solvent was removed by evaporation, and the residue was distilled in vacuo, yielding 1.03 g of the product as a colorless liquid (72%): bp 75-76 °C/14 mmHg (lit. bp 62-64 °C/8 mmHg).[4] ^{1}H NMR (500 MHz, $CDCl_3$): δ 4.32 (m, 1H, CH_2CH_3), 4.23 (m, 1H, CH_2CH_3),

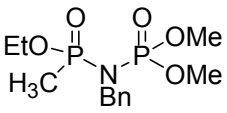
1.97 (d, J = 17.5 Hz, 3H, P-CH_3), 1.40 (t, J = 7.1 Hz, 3H, CH_2CH_3). ^{31}P NMR (202 MHz, $CDCl_3$): δ 40.06. Lit. data:[4] ^{1}H NMR (300/500 MHz, $CDCl_3$): δ 4.41-4.17 (m, 2H, OCH_2), 1.98 (d, J = 18 Hz, 3H, P-CH_3), 1.41 (t, J = 7 Hz, 3H, CH_3).



3a

Benzyl(diethoxyphosphoryl)[ethoxy(methyl)phosphoryl]amine (3a)

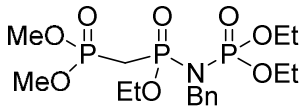
To a suspension of *t*-BuOK (800 mg, 7.16 mmol) in 15 mL of dry toluene was slowly added a solution of compound **2a** (1.16 g, 4.77 mmol) in 5 mL of dry toluene. The reaction mixture was stirred for 5 min, and a solution of ethyl methylphosphonochloridate (1.02 g, 7.16 mmol) in 2 mL of dry toluene was added. The reaction mixture was stirred overnight, and then quenched by addition of a saturated aqueous solution of NH_4Cl. The product was extracted into EtOAc (3 × 15 mL), and the combined organic layers were washed with brine, then dried over Na_2SO_4. After evaporation of the solvent, the residual product was purified by column chromatography on silica gel (EtOAc:Hexanes (3:1) → EtOAc), giving 1.176 g of **3a** as a colorless oil (71%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.49-7.21 (m, 5H, $CH_2C_6H_5$), 4.67-4.42 (m, 2H, C$H_2C_6H_5$), 4.11-3.67 (m, 6H, 3 × OCH_2CH_3), 1.71 (d, J_{HP} = 17.5 Hz, 3H, PCH_3), 1.27 (t, J = 7.1 Hz, 3H, OCH_2CH_3), 1.22 (t, J = 7.1 Hz, 3H, OCH_2CH_3), 1.16 (t, J = 7.1 Hz, 3H, OCH_2CH_3). ^{31}P NMR (202 MHz, $CDCl_3$): δ 32.99 (d, J = 18.1 Hz, 1P, $CH_3(EtO)P(O)$), 3.55 (d, J = 18.1 Hz, 1P, $P(O)(OEt)_2$). ^{13}C NMR (126 MHz, $CDCl_3$): δ 138.6, 128.9, 128.1, 127.4, 63.02 (d, J_{CP} = 5.9 Hz), 62.98 (d, J_{CP} = 5.1 Hz), 60.9 (d, J_{CP} = 6.7 Hz), 49.0, 16.1 (d, J_{CP} = 7.1 Hz), 15.9 (d, J_{CP} = 7.2 Hz), 15.8 (d, J_{CP} = 7.5 Hz), 15.2 (d, J_{CP} = 134 Hz). MS (*m/z*): calcd for $C_{14}H_{25}NNaO_5P_2^+$ $[M+Na]^+$: 372.1, found: 372.4 $[M+Na]^+$.



3b

Benzyl(dimethoxyphosphoryl)[ethoxy(methyl)phosphoryl]amine (3b)

To a suspension of *t*-BuOK (400 mg, 3.6 mmol) in 7 mL of dry toluene was slowly added a solution of compound **2b** (645 mg, 3 mmol) in 5 mL of dry toluene. The reaction mixture was stirred for 5 min, and a solution of ethyl methylphosphonochloridate (442 mg, 3.1 mmol) in 3 mL of dry toluene was added. The mixture was stirred overnight, and then quenched by addition of a saturated aqueous solution of NH_4Cl. The product was extracted into EtOAc (3 × 15 mL), and the combined organic layers were washed with brine, then dried over Na_2SO_4. After evaporation of the solvent, the residual product was purified by column chromatography on silica gel (EtOAc → 3% MeOH in EtOAc), giving 0.88 g of **3b** as a colorless oil (91%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.52-7.25 (m, 5H, $CH_2C_6H_5$), 4.69-4.43 (m, 2H, $CH_2C_6H_5$), 4.15-4.07 (m, 1H, OCH_2CH_3), 3.97-3.89 (m, 1H, OCH_2CH_3), 3.64 (d, J = 11.4 Hz, 3H, OCH_3), 3.53 (d, J = 11.4 Hz, 3H, OCH_3), 1.71 (d, J = 17.4 Hz, 3H, $CH_3P(O)$), 1.30 (t, J = 7.1 Hz, 3H, OCH_2CH_3). ^{31}P NMR (202 MHz, $CDCl_3$): δ 32.89 (d, J = 18.2 Hz, 1P, $H_3C(EtO)P(O)$), 6.54 (d, J = 18.2 Hz, 1P, $(MeO)_2P(O)$). ^{13}C NMR (101 MHz, $CDCl_3$): δ 138.4, 128.9, 128.2, 127.6, 61.1 (d, J_{CP} = 6.5 Hz), 53.5 (d, J_{CP} = 5.4 Hz), 53.4 (d, J_{CP} = 5.4 Hz), 49.1, 16.1 (d, J_{CP} = 7.6 Hz), 15.1 (d, J_{CP} = 134 Hz). MS (*m/z*): calcd for $C_{12}H_{22}NO_5P_2^+$: 322.1 $[M+H]^+$, found: 322.4 $[M+H]^+$.



4a

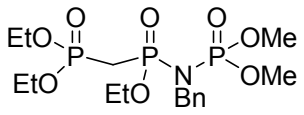
Dimethyl ({[benzyl(diethoxyphosphoryl)amino](ethoxy)phosphoryl}methyl)phosphonate (4a)

A solution of BuLi in hexane (2.8 mL, 4.4 mmol) was added to a dry flask under N_2 and cooled to -20 °C. Then freshly distilled diisopropylamine (b.p. = 82-84°C) (655 μL, 4.68 mmol) in 3 mL of dry THF was added. After stirring for 5 min, the reaction mixture was cooled to -78 °C, and a solution of **3b** (680 mg, 2.12 mmol) in 3 mL of dry THF was added.

The reaction mixture was stirred for 1.5 min at -78 °C, and a solution of diethyl chlorophosphate (439 mg, 2.54 mmol) was slowly added, after which the reaction mixture was allowed to warm up gradually to r.t. and left to stand overnight.

After cooling to 0 °C, the reaction mixture was quenched with saturated NH_4Cl, and the product extracted into EtOAc (3 × 15 mL). The combined organic fractions were washed with brine and dried with Na_2SO_4. The product **4a** was purified by column chromatography on silica gel (2% → 5% MeOH in EtOAc) and isolated as a colorless oil (800 mg, 83%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.52-7.23 (m, 5H, $CH_2C_6H_5$), 4.76-4.39 (m, 2H, $CH_2C_6H_5$), 4.18-4.09 (m, 2H, OCH_2CH_3), 4.02-3.95 (m, 3H, OCH_2CH_3), 3.88-3.82 (m, 1H, OCH_2CH_3), 3.84 (d, J = 11.3 Hz, 3H, OCH_3), 3.79 (d, J = 11.3 Hz, 3H, OCH_3), 3.06-2.77 (m, 2H, PCH_2P), 1.28 (t, J = 7.0 Hz, 6H, OCH_2CH_3), 1.15 (t, J = 7.1 Hz, 3H, OCH_2CH_3). ^{31}P NMR (202 MHz, $CDCl_3$): δ 23.22 (dd, J = 19.8, 4.3 Hz, 1P, PCH_2PNP), 22.70 (d, J = 4.3 Hz, 1P, PCH_2PNP), 2.79 (d, J = 19.8 Hz, 1P, PCH_2PNP). ^{13}C NMR (126 MHz, $CDCl_3$): δ 138.3, 129.3, 128.1, 127.6, 63.5 (d, J_{CP} = 5.2 Hz), 63.1 (d, J_{CP} = 5.7 Hz), 62.0 (d, J_{CP} = 6.8 Hz), 53.2 (d, J_{CP} = 6.1 Hz), 52.9 (d, J_{CP} = 6.1 Hz), 49.9,

27.2 (dd, J_{CP} = 135.1 Hz, J_{CP} = 127.3 Hz), 16.1 (d, J_{CP} = 7.3 Hz), 16.0 (d, J_{CP} =7.6 Hz), 15.8 (J_{CP} = 7.6 Hz).



4b

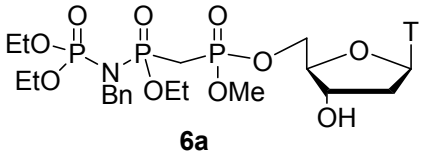
Diethyl ({[benzyl(dimethoxyphosphoryl)amino](ethoxy)phosphoryl}methyl)phosphonate (4b)

A solution of butyllithium in hexane (3.75 mL, 6 mmol) was added to a dry flask under N_2 and cooled to -20 °C. Then freshly distilled diisopropylamine (924 μL, 6.6 mmol) in 3 mL of dry THF was added. After stirring for 5 min, the reaction mixture was cooled to -78 °C, and a solution of **3a** (1.047 g, 3 mmol) in 6 mL of dry THF was added.

The resulting solution was stirred for 1.5 min at -78 °C, and a solution of dimethyl chlorophosphate (520 mg, 3.6 mmol) in dry THF (2 mL) was slowly added. The reaction mixture was allowed to warm up gradually to r.t. and left to stand overnight.

After cooling to 0 °C, the reaction mixture was quenched with a saturated aqueous solution of NH_4Cl and extracted into EtOAc (3 × 15 mL). The combined organic fractions were washed with brine and dried with Na_2SO_4. The product **4b** was purified by column chromatography on silica gel (EtOAc → 2% MeOH in EtOAc) and isolated as a colorless oil (805 mg, 59%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.52-7.25 (m, 5H, $CH_2C_6H_5$), 4.76-4.37 (m, 2H, $CH_2C_6H_5$), 4.26-3.93 (m, 6H, 3 × OCH_2CH_3), 3.70 (d, J = 11.4 Hz, 3H, OCH_3), 3.54 (d, J = 11.5 Hz, 3H, OCH_3), 3.02-2.72 (m, 2H, PCH_2P), 1.35 (t, J = 7.2 Hz, 3H, OCH_2CH_3), 1.34 (t, J = 7.1 Hz, 3H, OCH_2CH_3), 1.27 (t, J = 7.1 Hz, 3H, OCH_2CH_3). ^{31}P NMR (202 MHz, $CDCl_3$): δ 23.38 (dd, J = 19.8, 4.0 Hz,

1P, PCH_2PNP), 19.65 (d, J = 4.0 Hz, 1P, PCH_2PNP), 5.76 (d, J = 19.8 Hz, 1P, PCH_2PNP). ^{13}C NMR (126 MHz, $CDCl_3$): δ 138.2, 129.3, 128.2, 127.6, 62.7 (d, J = 6.3 Hz), 62.3 (d, J = 6.2 Hz), 62.0 (d, J = 6.7 Hz), 53.8 (d, J = 5.3 Hz), 53.4 (d, J = 5.7 Hz), 49.9, 28.0 (dd, J_{CP} = 134.7 Hz, J_{CP} = 127.0 Hz), 16.4 (d, J_{CP} = 6.4 Hz), 16.3 (d, J_{CP} =6.4 Hz), 16.1 (J_{CP} = 7.1 Hz). MS (*m/z*): calcd for $C_{16}H_{31}NO_8P_3^+$ 458.1 $[M+H]^+$, found: 458.5 $[M+H]^+$.



6a

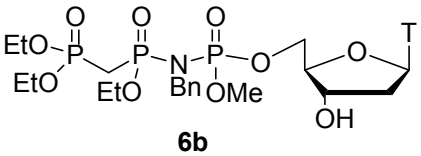
[(2*R*,3*S*,5*R*)-3-Hydroxy-5-(5-methyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-1-yl)oxolan-2-yl]methyl methyl ({[benzyl(diethoxyphosphoryl)amino](ethoxy)phosphoryl}methyl)phosphonate (6a)

A solution of **4a** (320 mg, 0.7 mmol) and 3 mL of triethylamine in 3 mL of acetonitrile was refluxed for 12 h. The reaction progress was monitored by ^{31}P NMR. After completion, solvents were removed under reduced pressure and the residue dried in vacuo. The resulting oil was passed through a column of DOWEX H^+, the acidic fractions were collected and the solvent removed under reduced pressure, yielding 279 mg (90%) of the corresponding acid **5a** as a colorless oil, which was used without further purification.

Acid **5a** (279 mg, 0.63 mmol), thymidine (153 mg, 0.63 mmol), and PPh_3 (247 mg, 0.945 mmol) were co-evaporated with 5 mL of dry DMF 3 times, and then dissolved in 2 mL of dry DMF. DIAD (186 μL, 0.945 mmol) was added dropwise and the resulting mixture allowed to stand at r.t.

After 3 d, the solvent was removed under reduced pressure and the residue dissolved in EtOAc and washed with 10 mL of 10% $NaHCO_3$. The aqueous layer was extracted with EtOAc (3 × 15

mL), and the combined organic layers were washed with brine and dried over Na_2SO_4. Product **6a** was isolated by column chromatography (EtOAc → 10% MeOH in EtOAc) as a mixture of 4 isomers (247 mg, 59%). The ^1H and ^{31}P NMR spectra are presented in **Figs. S20** and **S21**, respectively. ^1H NMR (500 MHz, $CDCl_3$): δ 8.73-8.71 (1H, N*H*), 7.52-7.24 (6H, $CH_2C_6H_5$, C*H*-T), 6.43-6.16 (1H, H-1'), 4.72-3.78 (15H, $CH_2C_6H_5$, 3 × OCH_2CH_3, H-5', H-4', H-3', OCH_3), 3.43-2.68 (2H, PCH_2P), 2.49-2.19 (2H, H-2'), 1.94-1.90 (3H, CH_3-T), 1.41-1.15 (9H, 3 × OCH_2CH_3). ^{31}P NMR (202 MHz, $CDCl_3$): δ 23.80-19.84 (2P, P_β and P_α), 2.64-2.26 (1P, P_γ).



6b

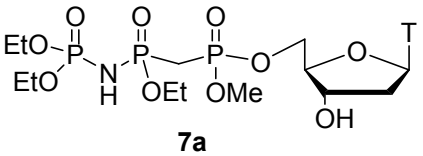
Diethyl ({[benzyl({[(2*R*,3*S*,5*R*)-3-hydroxy-5-(5-methyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-1-yl)oxolan-2-yl]methoxy}(methoxy)phosphoryl)amino](ethoxy)phosphoryl}methyl)phosphonate (6b)

A solution of **4b** (220 mg, 0.48 mmol) in 2 mL of MeCN and 2 mL of TEA was refluxed for 3 h. The reaction progress was monitored by ^{31}P NMR. After completion, solvents were removed and the residue was dissolved in water and extracted with EtOAc (10 ml). The aqueous layer was evaporated at reduced pressure and the residue dried in vacuo. It was then dissolved in MeOH (5mL) and stirred with DOWEX H^+ (300 mg) overnight. After removal of the solvent at reduced pressure, 155 mg (73%) of the acid **5b** was obtained as a colorless oil, which was used without further purification.

Thymidine (90 mg, 0.35 mmol), acid **5b** (155 mg, 0.35 mmol), and PPh_3 (137 mg, 0.525 mmol) were co-evaporated with 3 mL of dry DMF 3 times, and then dissolved in 2 mL of dry DMF. To

this solution, DIAD (103 μL, 0.525 mmol) was slowly added and the reaction mixture was allowed to stand at r.t.

After 4 days, the solvent was evaporated and the residue was dissolved in EtOAc and washed with 10 mL of 10% $NaHCO_3$. The aqueous layer was extracted with EtOAc (3×15 mL), and the combined organic layers were washed with brine, then dried over Na_2SO_4. Product **6b** was isolated by column chromatography (EtOAc → 6% MeOH in EtOAc) as a mixture of 4 isomers (72 mg, 31%). The ^1H and ^{31}P NMR spectra are presented in **Fig. S22** and **S23**, respectively. ^1H NMR (400 MHz, $CDCl_3$): δ 9.32-9.28 (1H, N*H*), 7.50-7.24 (6H, $CH_2C_6H_5$, C*H*-T), 6.29-6.15 (1H, H-1'), 4.82-3.71 (12H, $CH_2C_6H_5$, 3 × OCH_2CH_3, H-5', H-4', H-3'), 3.54-3.47 (3H, OCH_3), 3.26-2.54 (2H, PCH_2P), 2.40-2.18 (2H, H-2'), 1.92-1.87 (3H, CH_3-T), 1.41-1.23 (9H, 3 × OCH_2CH_3). ^{31}P NMR (162 MHz, $CDCl_3$): δ 23.28-22.83 (1P, P_β), 19.53-19.27 (1P, P_γ), 5.65-4.79 (1P, P_α).

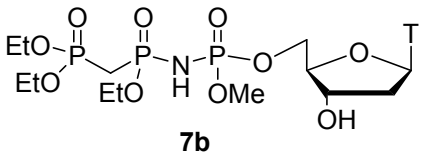


7a

[(2*R*,3*S*,5*R*)-3-Hydroxy-5-(5-methyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-1-yl)oxolan-2-yl]methyl methyl ({[(diethoxyphosphoryl)amino](ethoxy)phosphoryl}methyl)phosphonate (7a)

Compound **6a** (247 mg, 0.37 mmol) was dissolved in methanol (7 mL), and 15 mg (6 wt. %) of $Pd(OH)_2/C$ was added. The reaction mixture was stirred under 1 atm H_2 for 20 h, then filtered through a celite pad, and the volatiles evaporated to yield 206 mg of **7a** as a mixture of 4 isomers as a colorless oil (97%). ^1H NMR (500 MHz, CD_3OD): δ 7.59-7.56 (1H, C*H*-T), 6.29-6.26 (1H, H-1'), 4.47-4.04 (10H, 3 × OCH_2CH_3, H-5', H-4', H-3'), 3.83-3.79 (3H, OCH_3), 3.04-2.84 (2H,

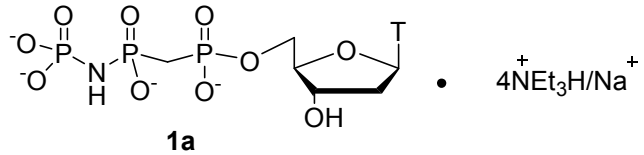
PC*H*$_2$P), 2.29-2.25 (2H, H-2'), 1.91-1.90 (3H, C*H*$_3$-T), 1.37-1.33 (9H, 3 × OCH$_2$C*H*$_3$). ^{31}P NMR (202 MHz, CD$_3$OD): δ 22.89-21.95 (1P, P$_\alpha$), 18.76-18.34 (1P, P$_\beta$), -0.22-(-0.31) (1P, P$_\gamma$). MS (*m/z*): calcd for $C_{18}H_{33}N_3O_{12}P_3^-$ 576.1 $[M-H]^-$, found: 576.1 $[M-H]^-$.



7b

Diethyl ({ethoxy[({[(2*R*,3*S*,5*R*)-3-hydroxy-5-(5-methyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-1-yl)oxolan-2-yl]methoxy}(methoxy)phosphoryl)amino]phosphoryl}methyl)phosphonate (7b)

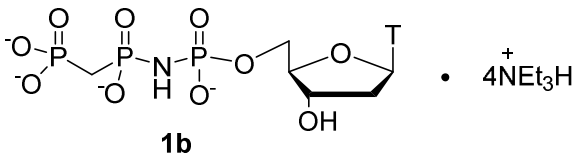
Compound **6b** (72 mg, 0.108 mmol) was dissolved in methanol (3 mL), and 7 mg (10 wt. %) of Pd(OH)$_2$/C was added. The reaction mixture was stirred under 1 atm H_2 for 4 h, then filtered through a celite pad. Volatiles were evaporated from the filtrate, yielding 60 mg of **7b** as a mixture of 4 isomers as a colorless oil (96%). ^{1}H NMR (400 MHz, CD$_3$OD): δ 7.58-7.56 (1H, C*H*-T), 6.30-6.26 (1H, H-1'), 4.48-4.03 (10H, 3 × OC*H*$_2$CH$_3$, H-5', H-4', H-3'), 3.86-3.81 (3H, OC*H*$_3$), 2.95-2.74 (2H, PC*H*$_2$P), 2.27-2.24 (2H, H-2'), 1.91-1.90 (3H, C*H*$_3$-T), 1.38-1.32 (9H, 3 × OCH$_2$C*H*$_3$). ^{31}P NMR (162 MHz, CD$_3$OD): δ 19.75-19.66 (1P, P$_\beta$), 18.70-18.55 (1P, P$_\gamma$), 1.91-1.59 (1P, P$_\alpha$).



1a

{[({[(2*R*,3*S*,5*R*)-3-Hydroxy-5-(5-methyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-1-yl)oxolan-2-yl]methyl phosphonato}methyl)phosphinato]amino}phosphonate (TEA/Na salt) [(α,β)-CH_2-(β,γ)-NH-dTTP (1a)

A mixture of **7a** (10 mg, 17 μmol), dry DMF (20 μL) and BTMS (200 μL, 1.52 mmol) was microwaved at 40 °C for 30 min. The reaction mixture was allowed to cool to r. t., volatiles were removed in vacuo, and the residue was treated with 1 mL of Et_3N-MeOH (1:5). The product was purified by two-stage preparative HPLC: first, on a SAX column (0-0.5 M TEAB gradient in 20 min); then, on a C-18 column (0.1 M TEAB:5% MeCN, pH = 6.9). The fractions containing **1a** (identified by MS) were collected and combined, and evaporated yielding the TEA salt of compound **1a**. The solution of **1a** in water was treated with DOWEX Na^+ (20 mg) and lyophilized again to yield Na-TEA salt of compound **1a** (2.2 mg, 27%). ^{1}H NMR (500 MHz, D_2O): δ 7.62 (s, 1H, C*H*-T), 6.39 (t, *J* = 7.0 Hz, 1H, H-1'), 4.64-4.62 (m, 1H, H-3'), 4.17-4.07 (m, 3H, H-4', H-5'), 2.40 (t, *J* = 19.5 Hz, 2H, PCH_2P), 2.39-2.30 (m, 2H, H-2'), 1.92 (s, 3H, CH_3-T). ^{31}P NMR (202 MHz, D_2O): δ 19.89 (d, *J* = 9.8 Hz, 1P, P_α), 12.28 (d, *J* = 9.8 Hz, 1P, P_β), -0.31 (s, 1P, P_γ). MS (*m/z*): calcd for $C_{11}H_{19}N_3O_{12}P_3^-$ 478.0 $[M-H]^-$, found: 478.1 $[M-H]^-$.



1b

({[({[(2*R*,3*S*,5*R*)-3-Hydroxy-5-(5-methyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-1-yl)oxolan-2-yl]methoxy}phosphinato)amino]phosphinato}methyl)phosphonate (TEA salt) [(α,β)-NH-(β,γ)-CH_2-dTTP] (1b)

A mixture of **7b** (5.0 mg, 8.7 μmol), dry DMF (100 μL) and BTMS (100 μL, 760 μmol) was microwaved at 40 °C for 8 min. The reaction mixture was cooled to r.t., all volatiles removed in

vacuo, and 1 mL of Et_3N-MeOH (1:5) added. The crude product was purified by two-stage preparative HPLC: first, on a SAX column (0-0.5 M TEAB gradient over 20 min); second, on a C-18 (0.1 M TEAB:5% MeCN, pH = 7.0). The fractions containing **1b** (identified by MS) were collected and combined, and then lyophilized to dryness to provide a TEA salt of compound **1b** (1.9 mg, 46%). ^{1}H NMR (400 MHz, D_2O): δ 7.71 (s, 1H, C*H*-T), 6.38 (t, *J* = 6.9 Hz, 1H, H-1'), 4.68-4.65 (m, 1H, H-3'), 4.19-4.08 (m, 3H, H-4', H-5'), 2.44-2.32 (m, 2H, H-2'), 2.08 (t, *J* = 19.2 Hz, 2H, PC*H*$_2$P), 1.93 (d, *J* = 1.1 Hz, 3H, C*H*$_3$-T). ^{31}P NMR (162 MHz, D_2O): δ 15.32 (dd, *J* = 8.3, *J* = 3.9 Hz, 1P, P_β), 12.30 (d, *J* = 8.3 Hz, 1P, P_γ), 0.68 (d, *J* = 3.9, 1P, P_α). MS (*m/z*): calcd for $C_{11}H_{19}N_3O_{12}P_3^-$ 478.0 $[M-H]^-$, found: 478.1 $[M-H]^-$.

DNA synthesis, purification, radiolabeling, and annealing

Primer (5′-TAT TAC CGC GCT GAT GCG C), template, (5′-GCG TTG TTC CGA CAG CGC ATC AGC GCG GTA ATA), and 5′-phosphorylated downstream (5′-GTC GGA ACA ACG C) oligomers were synthesized on a solid phase DNA synthesizer and purified by 16% denaturing polyacrylamide gel electrophoresis, followed by desalting using oligonucleotide purification cartridges. 1 mol equiv primer was 5′-end labeled with 0.4 U/μL T4 polynucleotide kinase and 0.7 mol equiv [γ-^{32}P]ATP with the supplied buffer at 37 °C for 30 minutes, followed by heat inactivation at 95 °C for 10 minutes. The primer was annealed by mixing with 1.2 mol equiv template and 1.5 mol equiv downstream oligomers. The mixture was heated to 95 °C and cooled slowly to room temperature. The reaction buffer consisted of 50 mM Tris-Cl, 20 mM KCl, 20 mM NaCl, 10 mM MgCl2, 1 mM DTT, and 6% glycerol at pH 8.0, 37 °C.

DNA synthesis kinetics

Radiolabeled 1 nt gapped DNA (100 nM) was incubated with pol β (600 nM) in reaction buffer (2x mixture) for 3 minutes at 37 °C. Equal volumes of the DNA/pol β mixture and a 2x solution

of the **1a**/**1b** at different concentrations and dTTP at 0.2 μM in reaction buffer were combined and incubated at 37 °C. After the appropriate reaction time, an aliquot of the reaction mixture was quenched with 0.5 M EDTA at pH 8.0. Reaction products were separated by 20% denaturing polyacrylamide gel electrophoresis (39 cm x 33 cm x 0.4 mm). Dehydrated gels were exposed to a phosphor screen and detected by phosphorescence emission.

For each set of reactions, the percentage of primer extended is plotted versus time, and the data for each concentration of analog is fit to the first order exponential $y = a(1-e^{-kt})$, where a is the maximum percent of primer extension and k is the observed rate constant. The observed rate constant (k_{obs}) is then plotted versus the corresponding inhibitor concentration and the data fit to the hyperbolic decay equation $k_{obs} = k_{pol} \times K_i/(K_i + [\text{inhibitor}])$ to give the K_i parameter.

Crystallization of the Pol β Substrate Complex

Binary complex crystals of human pol β with a dideoxy-terminated primer in a 1-nucleotide gapped DNA were grown as described previously.[5] Briefly, the sequence of the template strand (16-mer) was 5′-CCGACAGCGCATCAGC-3′. The primer strand (9-mer) sequence was 5′-GCTGATGCG-3′. The downstream oligonucleotide (5-mer) was 5′-phosphorylated, and the sequence was 5′-GTCGG-3′. These oligonuleotides were annealed in a ratio of 1:1:1 by heating at 90 °C for 10 minutes and cooling to 4 °C (1 °C/min) using a PCR thermocycler, resulting in a 2-nucleotide gapped duplex DNA. These annealed oligonucleotides were further incubated with pol β in a solution containing dideoxyCTP to create a dideoxyCMP-terminated primer. This pol β-DNA binary complex was crystallized by sitting-drop vapor diffusion at 18 °C by mixing 2 μl of the complex with 2 μl of crystallization buffer. The crystallization buffer consisted of 16% PEG-3350, 350 mM sodium acetate, and 50 mM imidazole, pH 7.5. Crystals grew in approximately 2-4 days after seeding. The ternary complexes were obtained by soaking crystals of the binary (one nucleotide gapped DNA) complex in artificial mother liquor containing 50 mM $MgCl_2$, 20% PEG-3350, 12% ethylene glycol, and 4 mM (α,β)-CH_2-(β,γ)-NH-dTTP or (α,β)-NH-(β,γ)-CH_2-dTTP

for 1-2 hours. The crystals were flash-frozen in a liquid nitrogen stream. Diffraction quality data were then collected for the ternary complex crystals as described below.

Data collection and structure determination

Data were collected at 100 °K on a CCD detector system mounted on a MiraMax®-007HF (Rigaku Corporation) rotating anode generator. Data were integrated and reduced with HKL2000 software.[6] All crystals belong to the space group $P2_1$. The ternary complex structures were solved by molecular replacement using 2FMP[5] as a reference model. The structure was refined using PHENIX and manual model building using Coot. The crystallographic statistics are reported in Table S1.

Structure factors and the coordinates for the pol β complexes with (α,β)-CH_2-(β,γ)-NH-dTTP and (α,β)-NH-(β,γ)- CH_2-dTTP have been deposited with Protein Data Bank with accession codes 4RT2 and 4RT3, respectively.

Table S1. Crystallographic Statistics

	(α,β)-CH_2-(β,γ)-NH-dTTP	**(α,β)-NH-(β,γ)-CH_2-dTTP**
PDB Code	4RT2	4RT3
Data Collection		
Space Group	$P2_1$	$P2_1$
a (Å)	50.7	50.6
b (Å)	79.9	79.4
c (Å)	55.3	55.3
β (°)	107.5	107.3
d_{min} (Å)	1.92	1.92
R_{merge} (%)[a, b]	0.084 (0.493)	0.079 (0.479)
Completeness (%)	94.0 (67.4)	89.8 (48)
Unique Reflections	30067 (2142)	28973 (1547)
Total Reflections	106757	96986
I/s	13.8 (2.0)	16.6 (1.9)
Refinement		
r.m.s. deviations		
Bond lengths (Å)	0.007	0.008
Bond angles (°)	1.020	1.040
R_{work} (%)[c]	17.6	18.0
R_{free} (%)	21.1	22.6
Average B Factors (Å)		
Protein	27.4	33.6
DNA	34.4	40.5
Analog	18.7	21.8
Ramachandaran Analysis		
Favored	98.5	98.5
Allowed	100	100

[a] $R_{merge}=100 \times \Sigma_h\Sigma_i |I_{h,i}-I_h| \Sigma_h \Sigma_i I_{h,j}$, where I_h is the mean intensity of symmetry related reflections $I_{h,j}$.

[b] Numbers in the parentheses refer to the highest resolution shell of data (10%).

[c] $R_{work} = 100 \times \Sigma |F_{obs}|-|F_{calc}|/\Sigma|F_{obs}|$.

III. NMR Spectra of Compounds 1-7

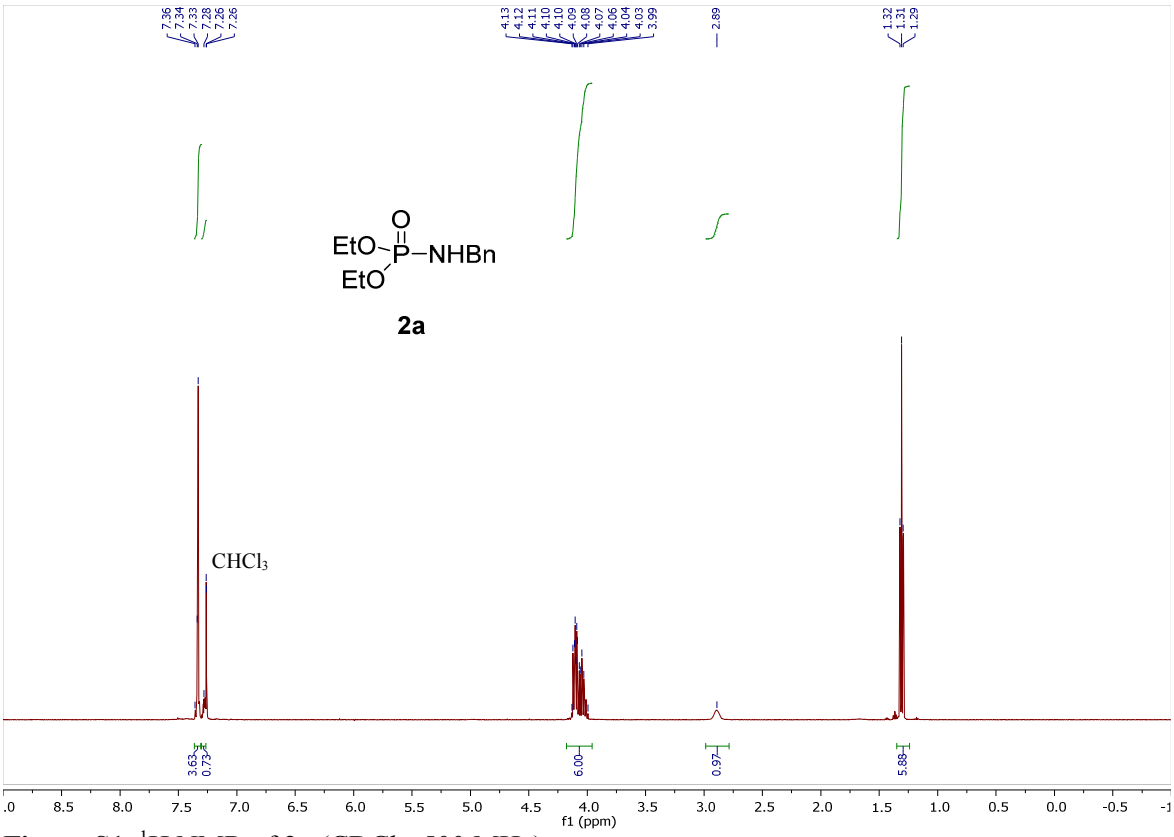


Figure S1. ^{1}H NMR of **2a** ($CDCl_3$, 500 MHz)

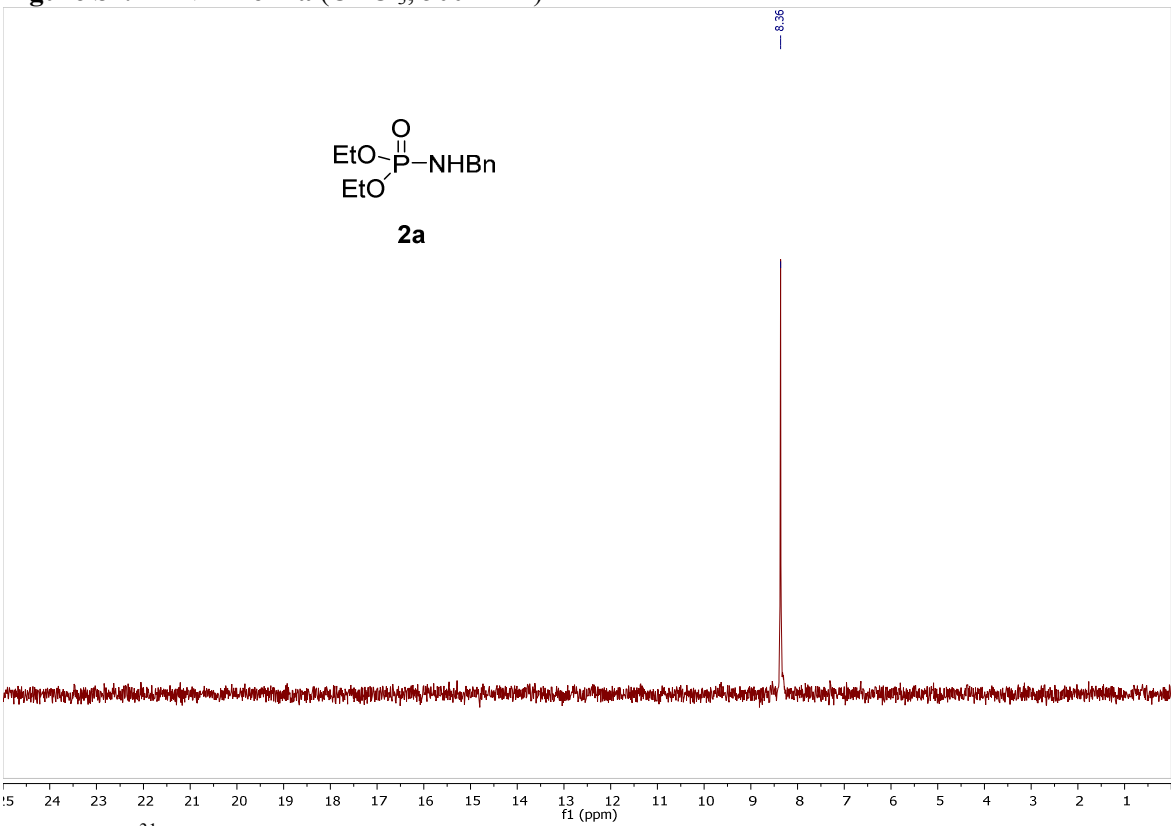


Figure S2. ^{31}P NMR of **2a** ($CDCl_3$, 202 MHz)

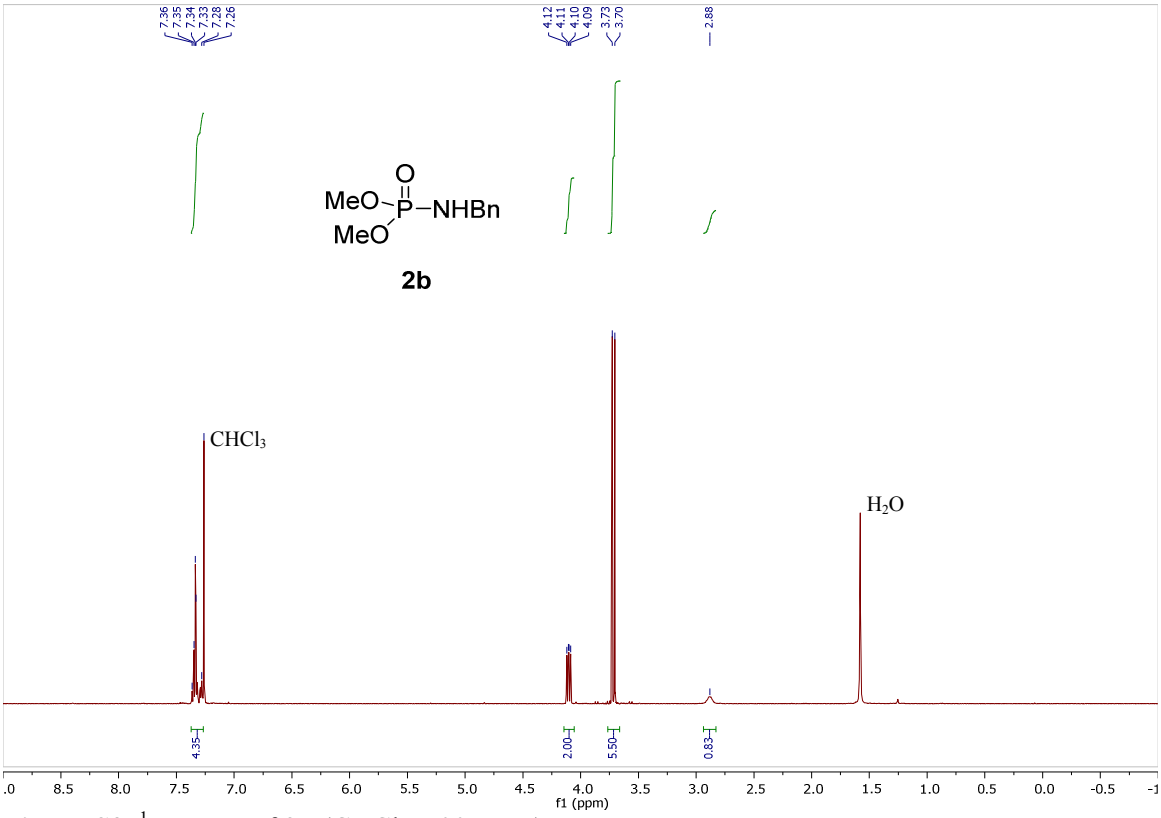


Figure S3. ^{1}H NMR of **2b** ($CDCl_3$, 500 MHz)

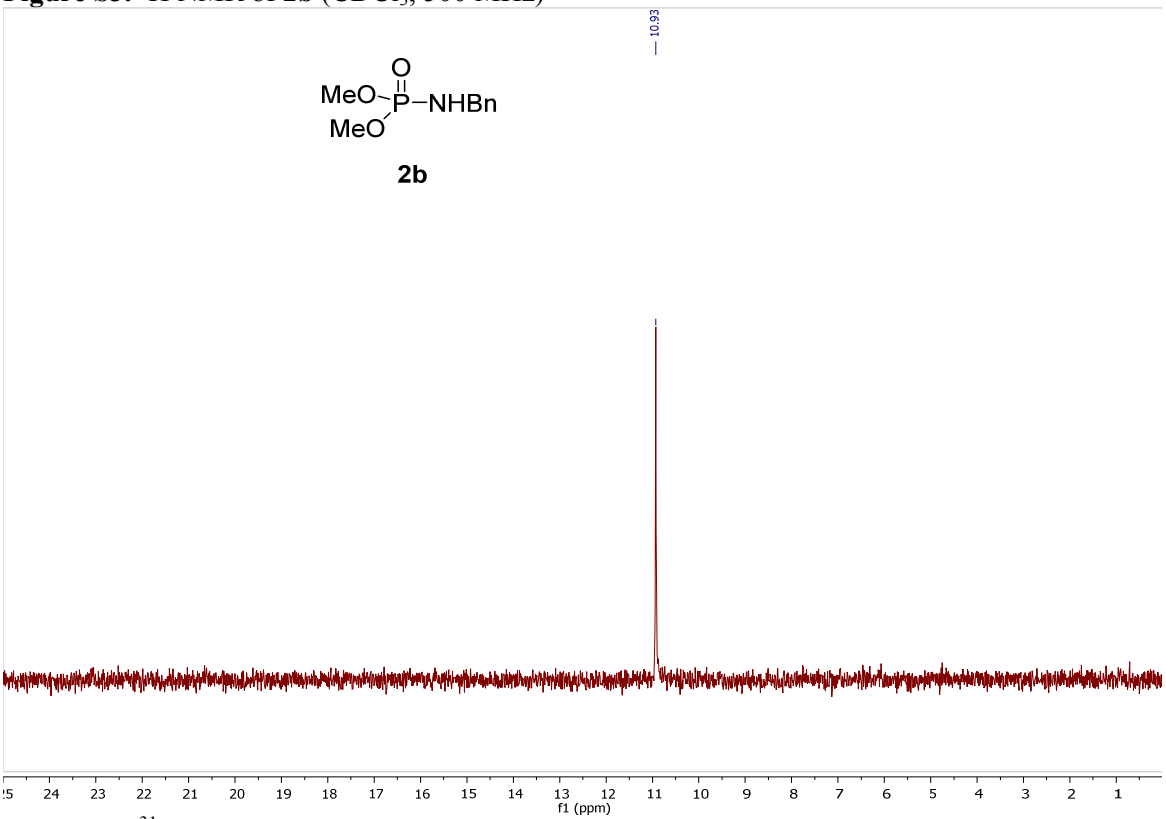


Figure S4. ^{31}P NMR of **2b** ($CDCl_3$, 202 MHz)

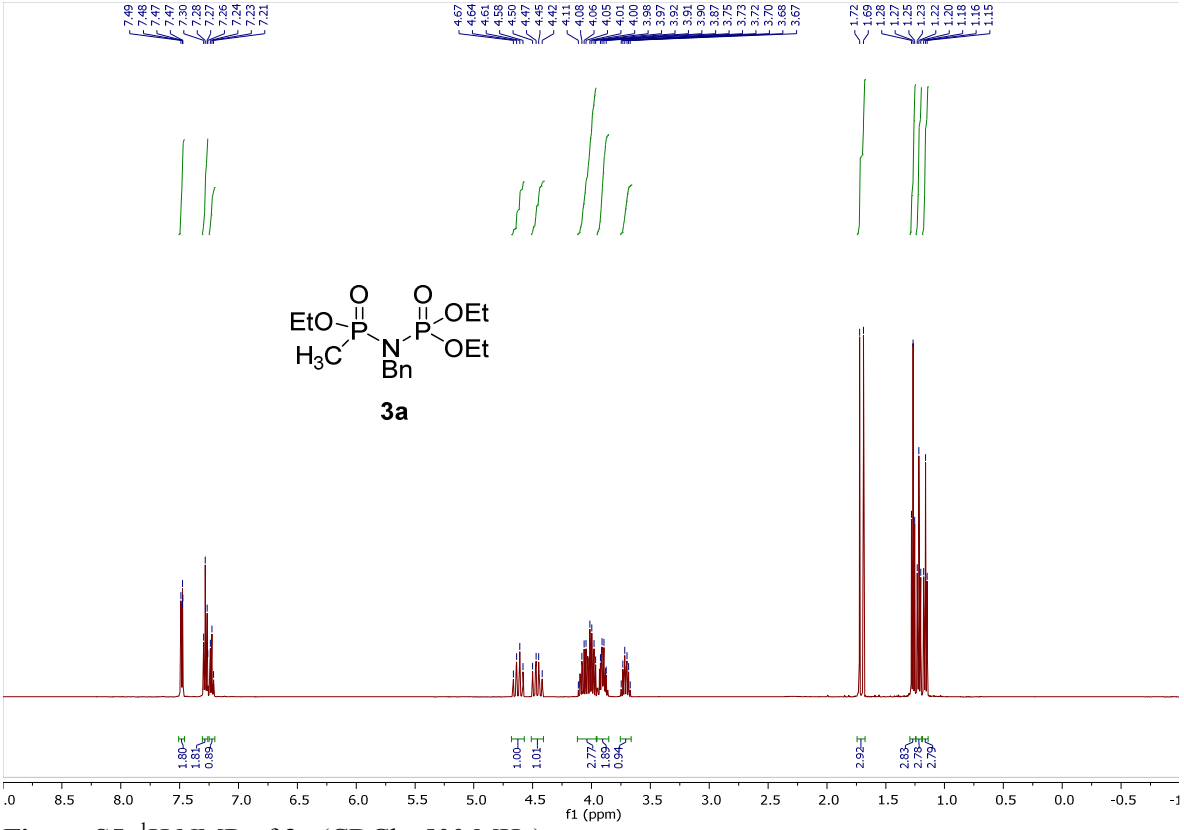


Figure S5. ^{1}H NMR of **3a** ($CDCl_3$, 500 MHz)

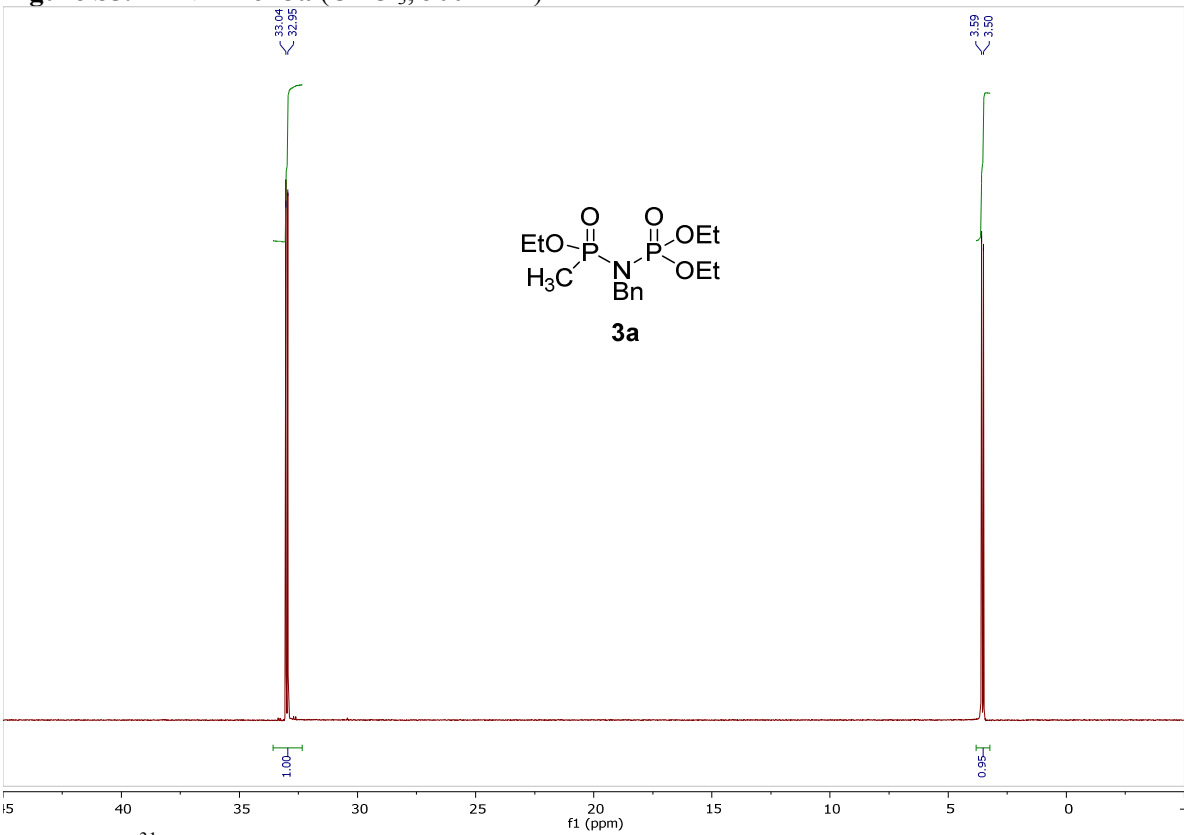


Figure S6. ^{31}P NMR of **3a** ($CDCl_3$, 202 MHz)

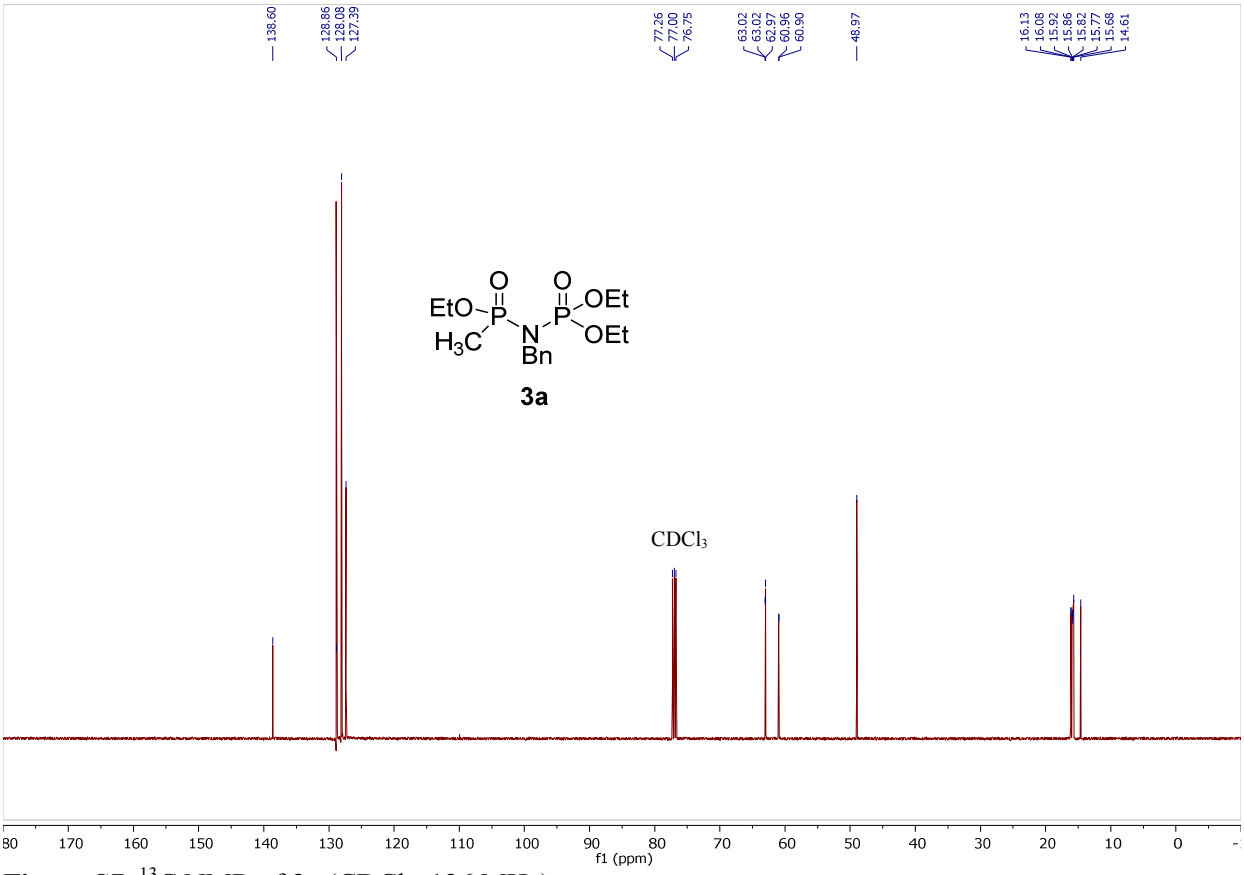


Figure S7. ^{13}C NMR of **3a** ($CDCl_3$, 126 MHz)

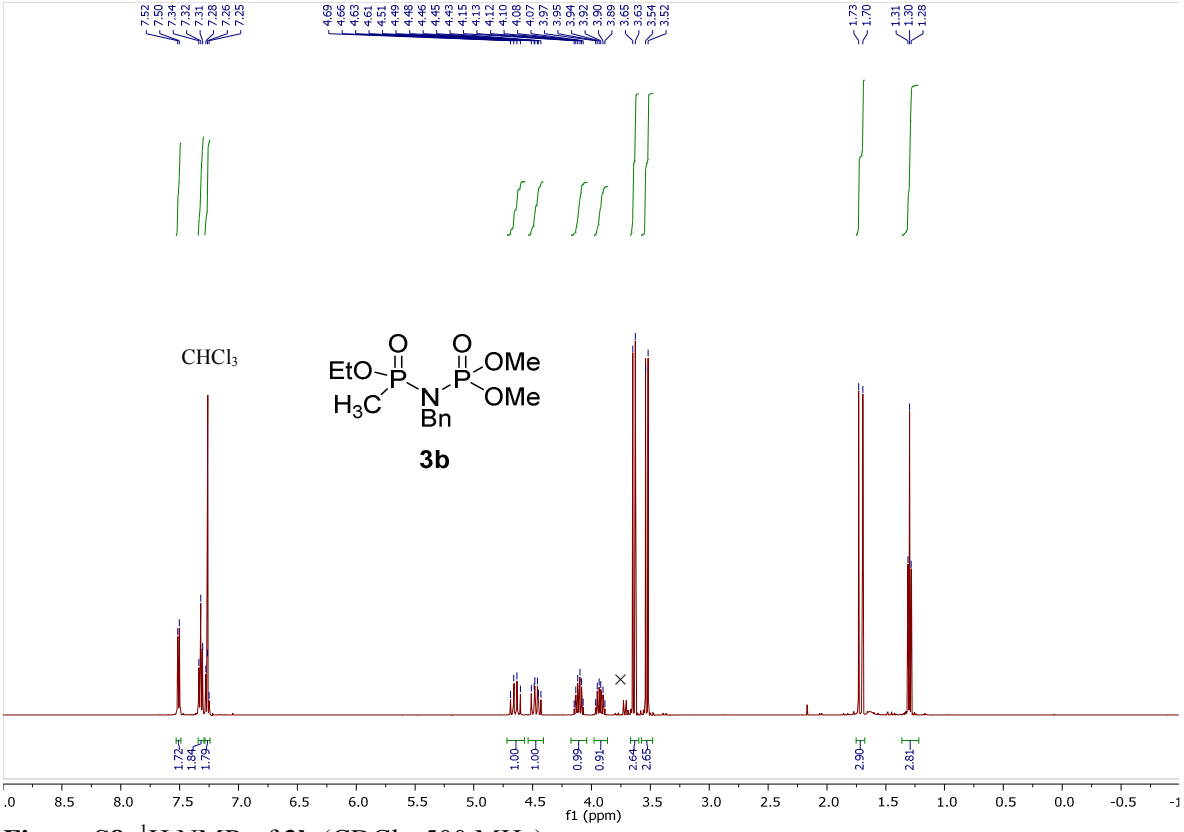


Figure S8. ^{1}H NMR of **3b** ($CDCl_3$, 500 MHz)

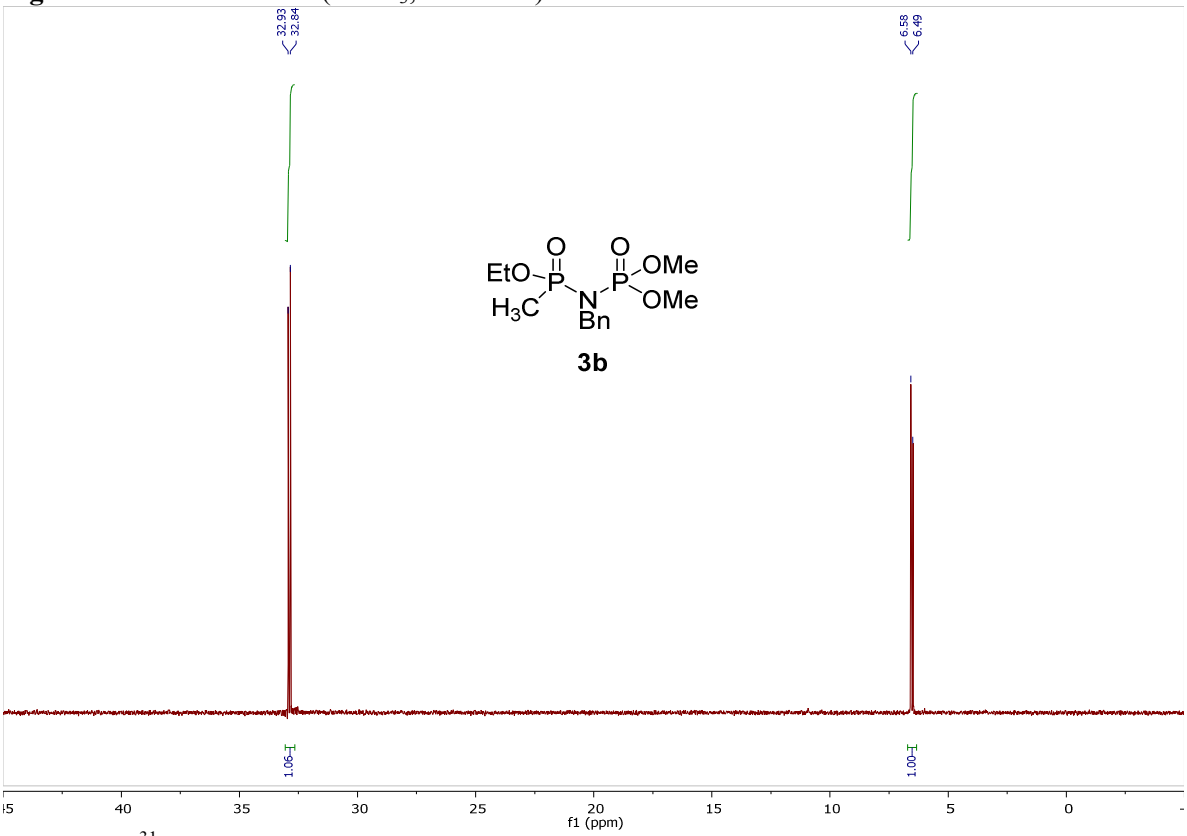


Figure S9. ^{31}P NMR of **3b** ($CDCl_3$, 202 MHz)

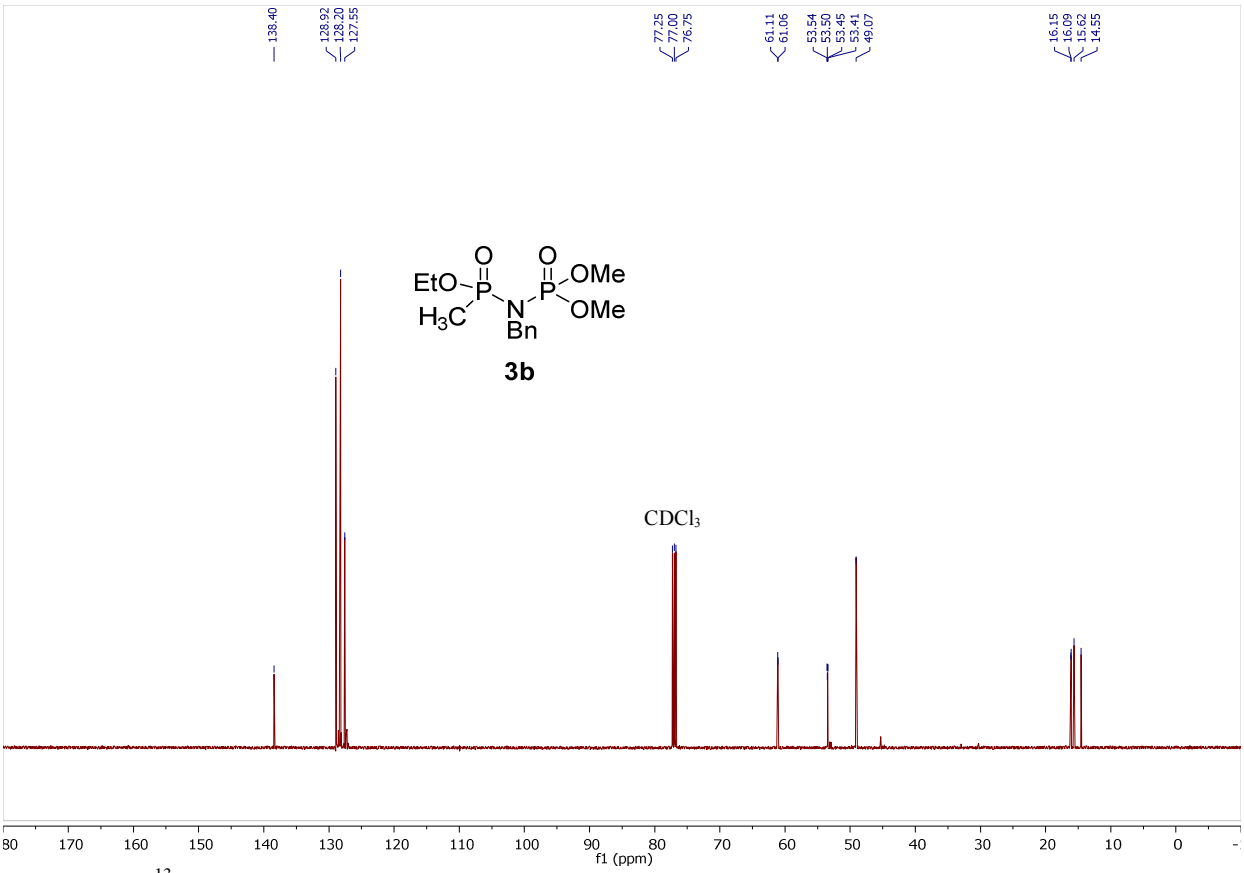


Figure S10. ^{13}C NMR of **3b** ($CDCl_3$, 126 MHz)

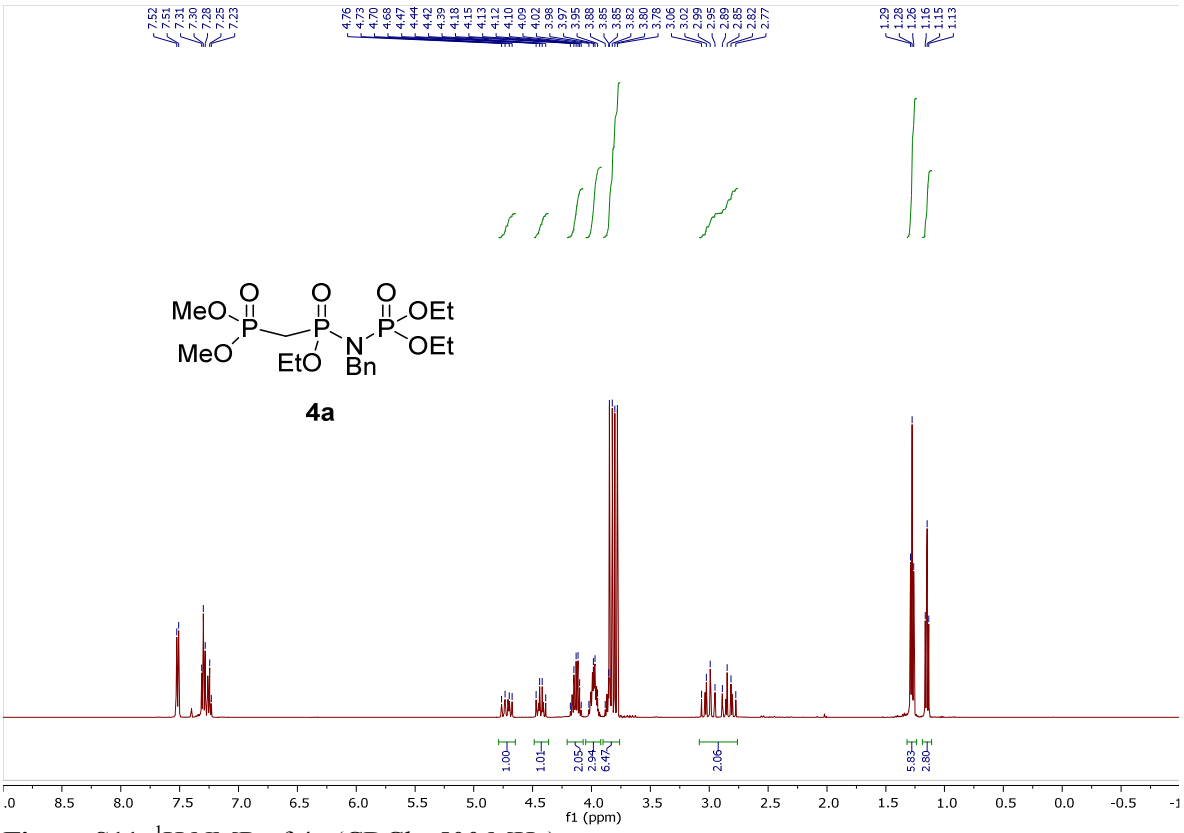


Figure S11. ^{1}H NMR of **4a** ($CDCl_3$, 500 MHz)

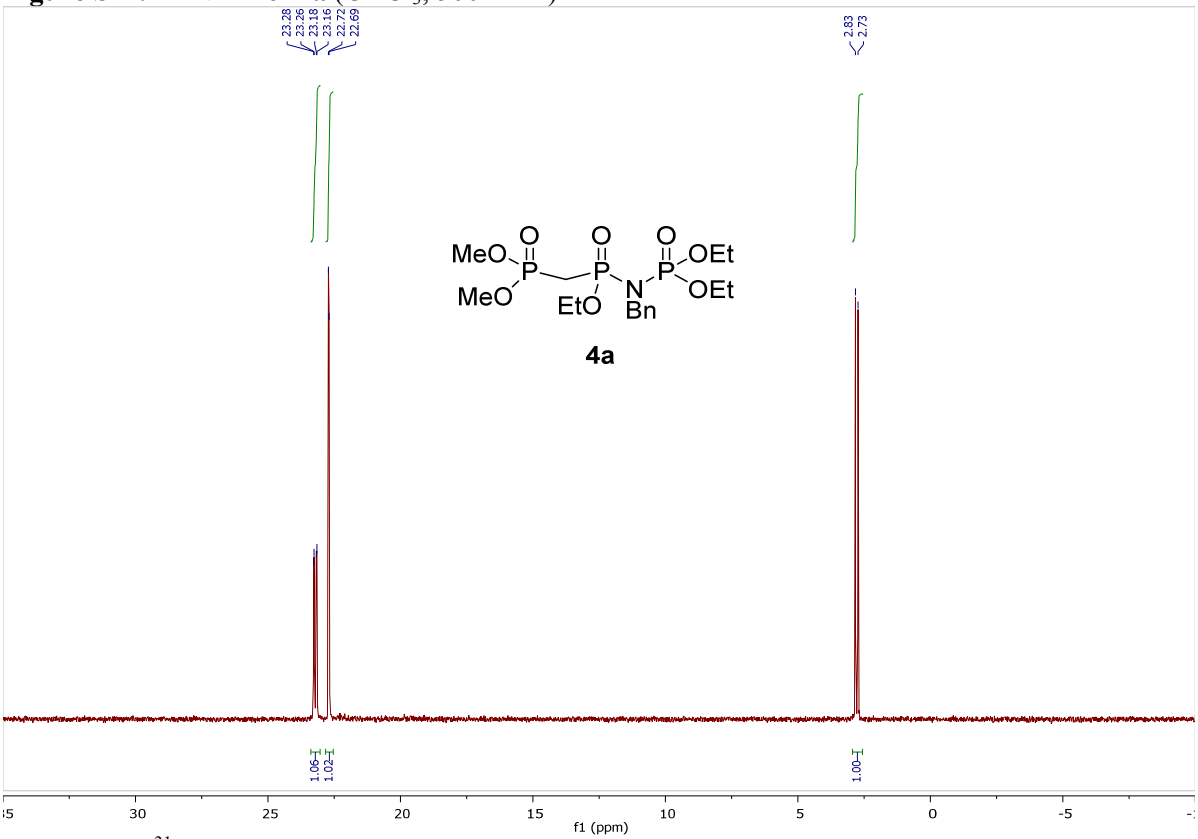


Figure S12. ^{31}P NMR of **4a** ($CDCl_3$, 202 MHz)

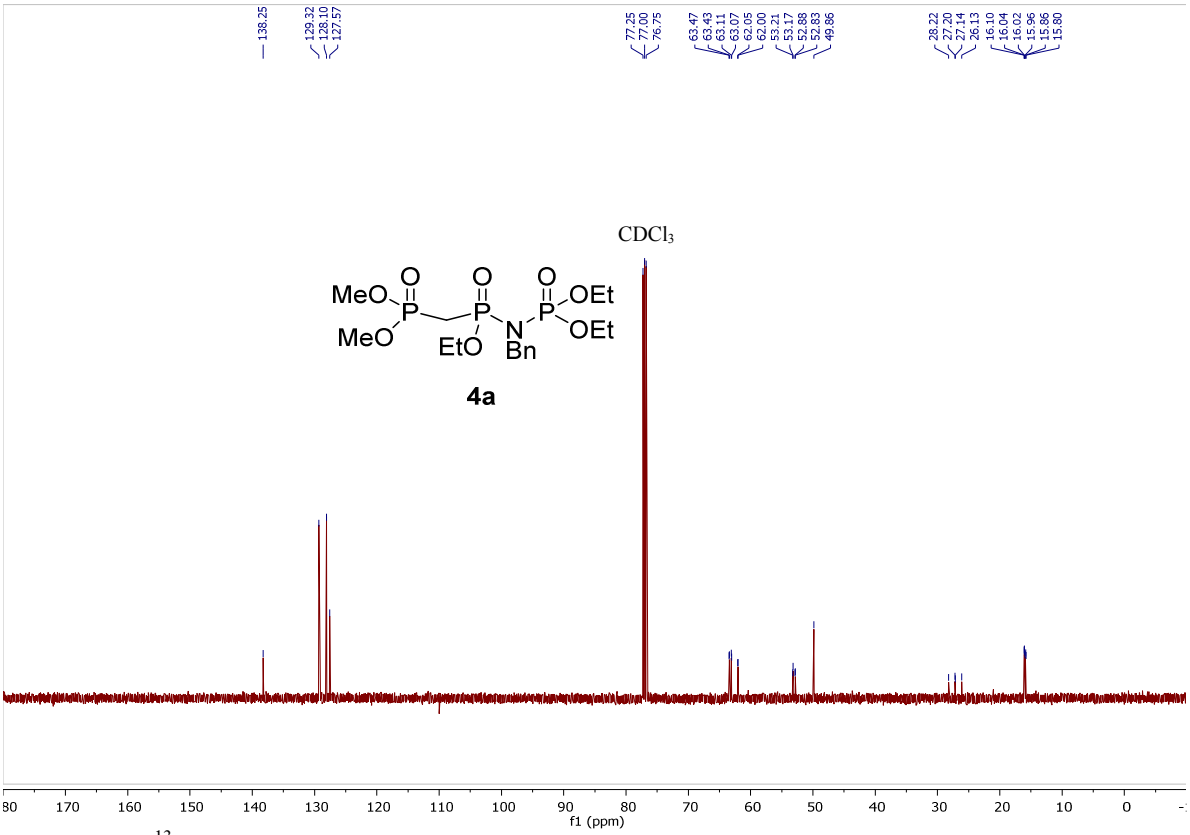


Figure S13. ^{13}C NMR of **4a** ($CDCl_3$, 126 MHz)

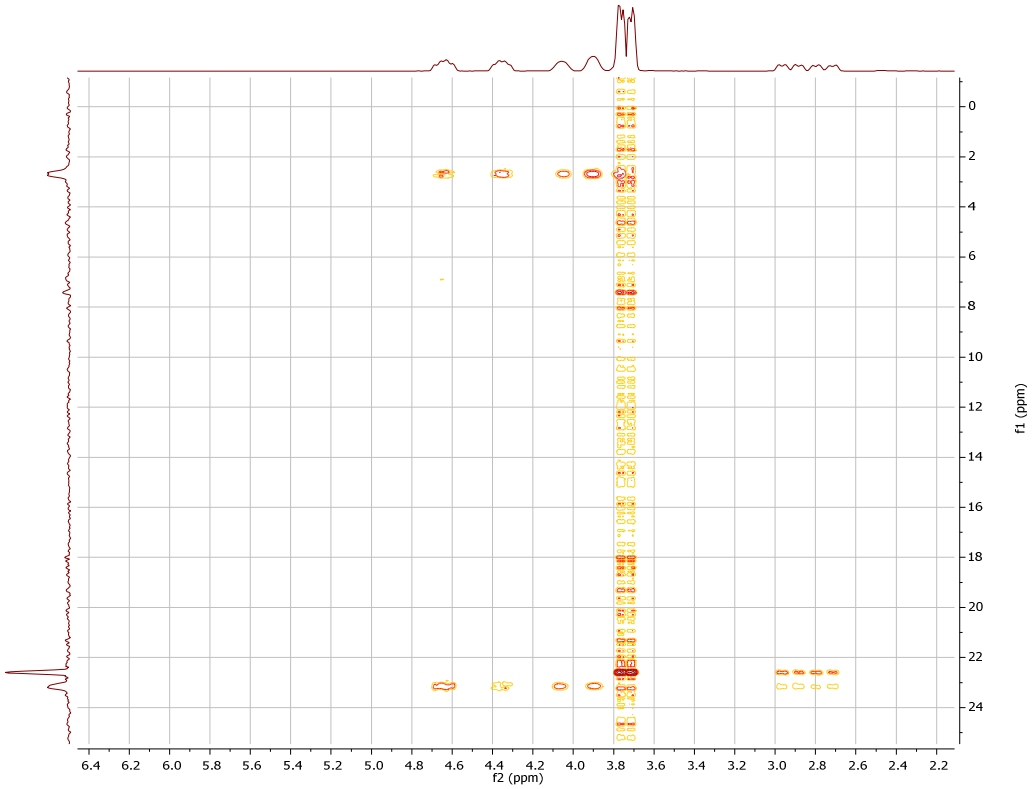


Figure S14. ^{1}H - ^{31}P gHMBC NMR of **4a** ($CDCl_3$, 500 MHz)

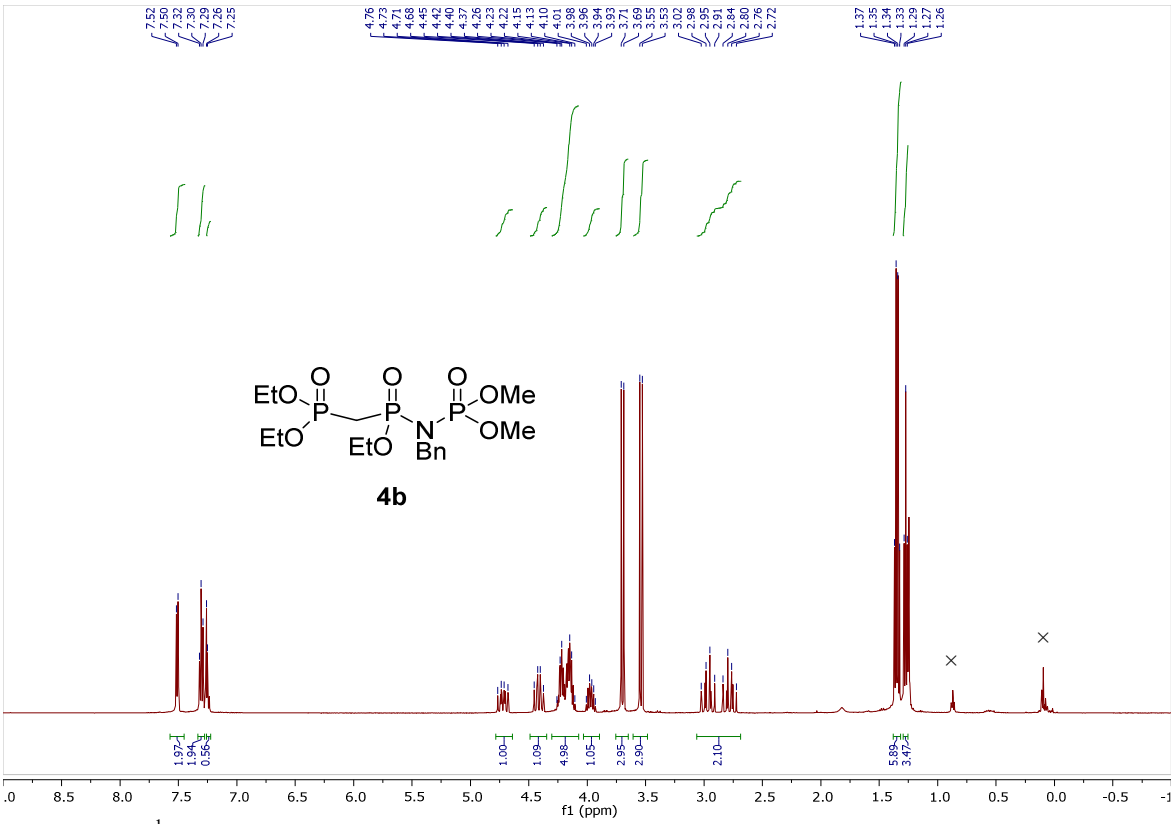


Figure S15. ^{1}H NMR of **4b** ($CDCl_3$, 500 MHz)

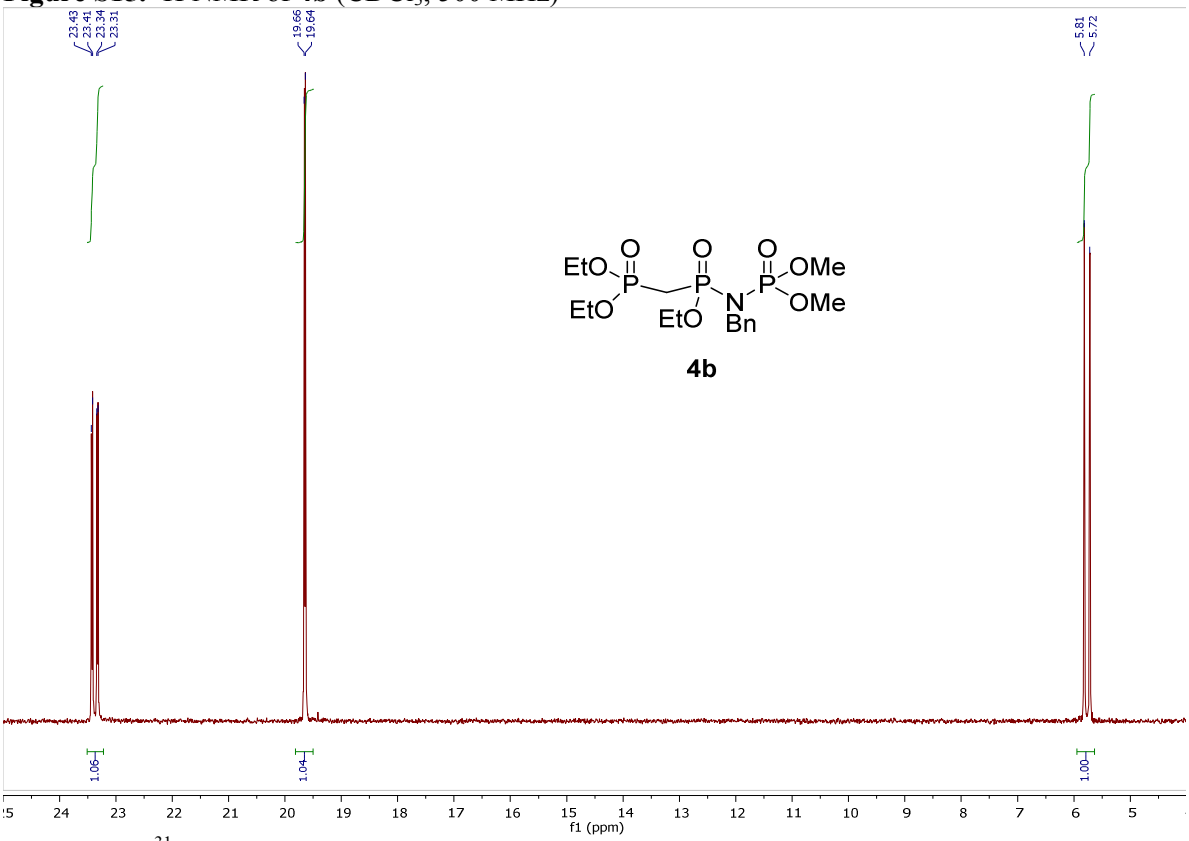


Figure S16. ^{31}P NMR of **4b** ($CDCl_3$, 202 MHz)

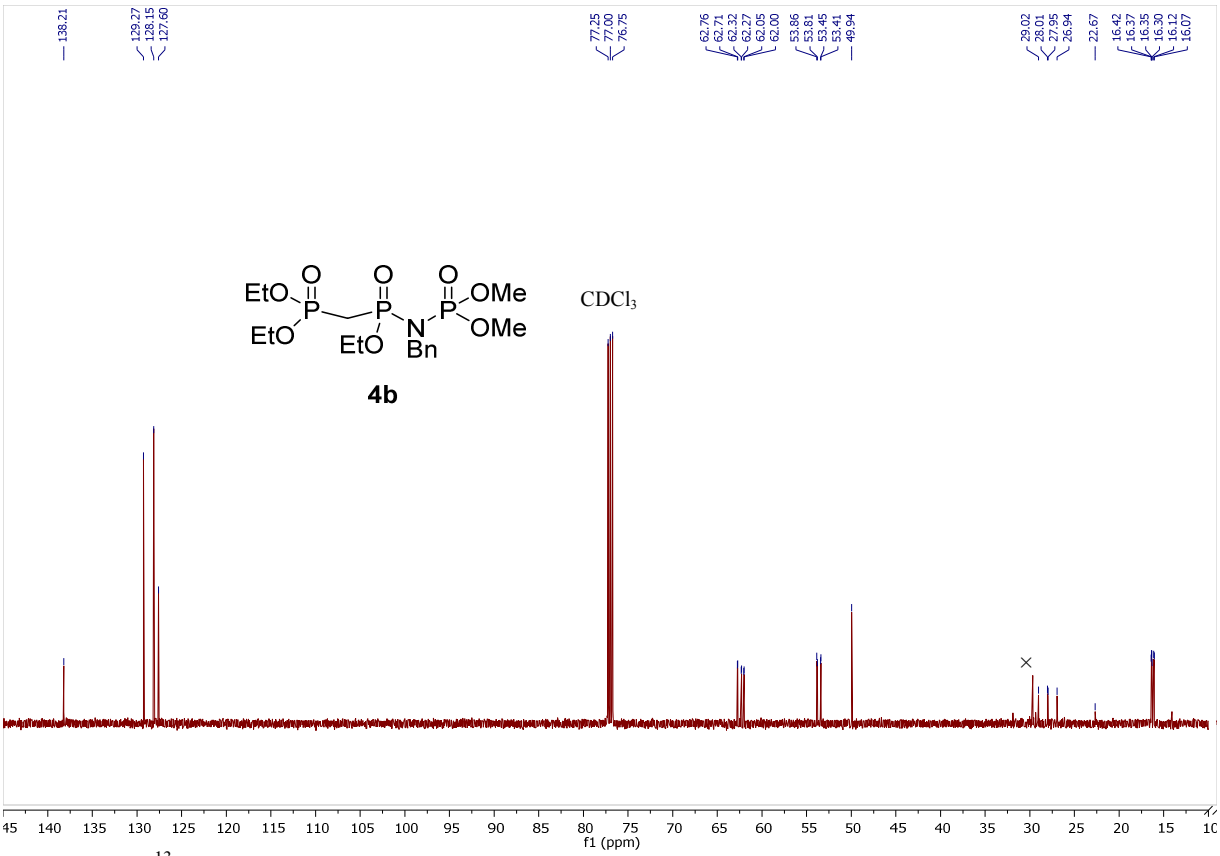


Figure S17. ^{13}C NMR of **4b** ($CDCl_3$, 126 MHz)

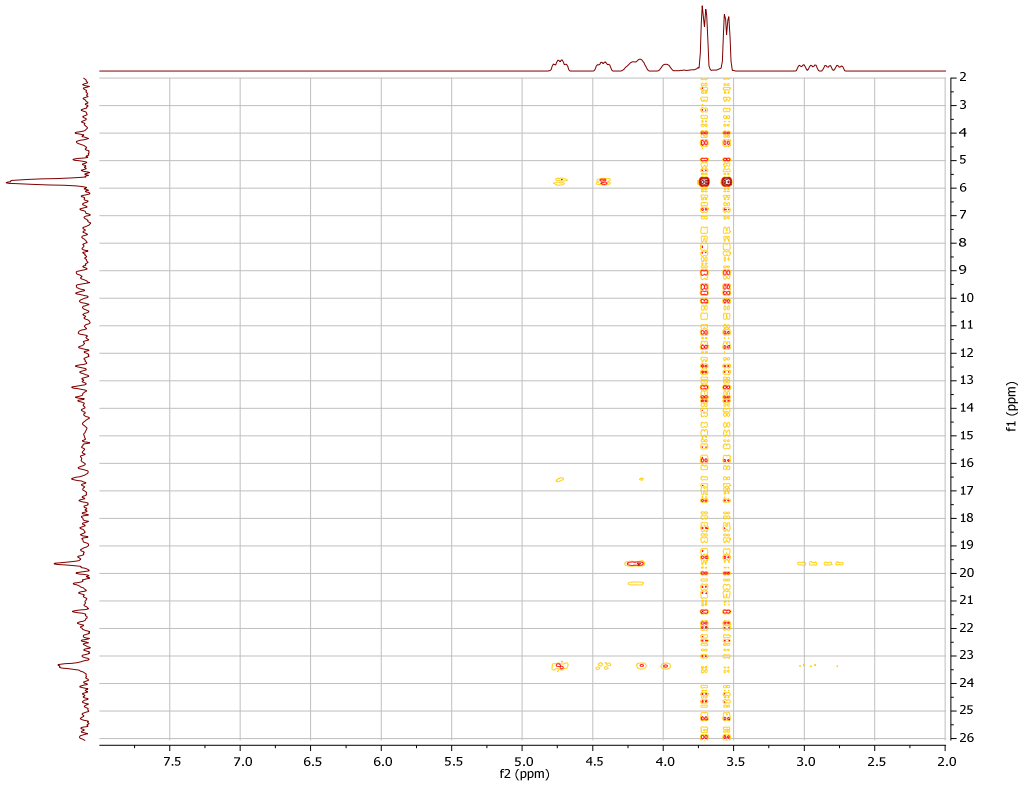


Figure S18. ^{1}H – ^{31}P gHMBC NMR of **4b** ($CDCl_3$, 500 MHz)

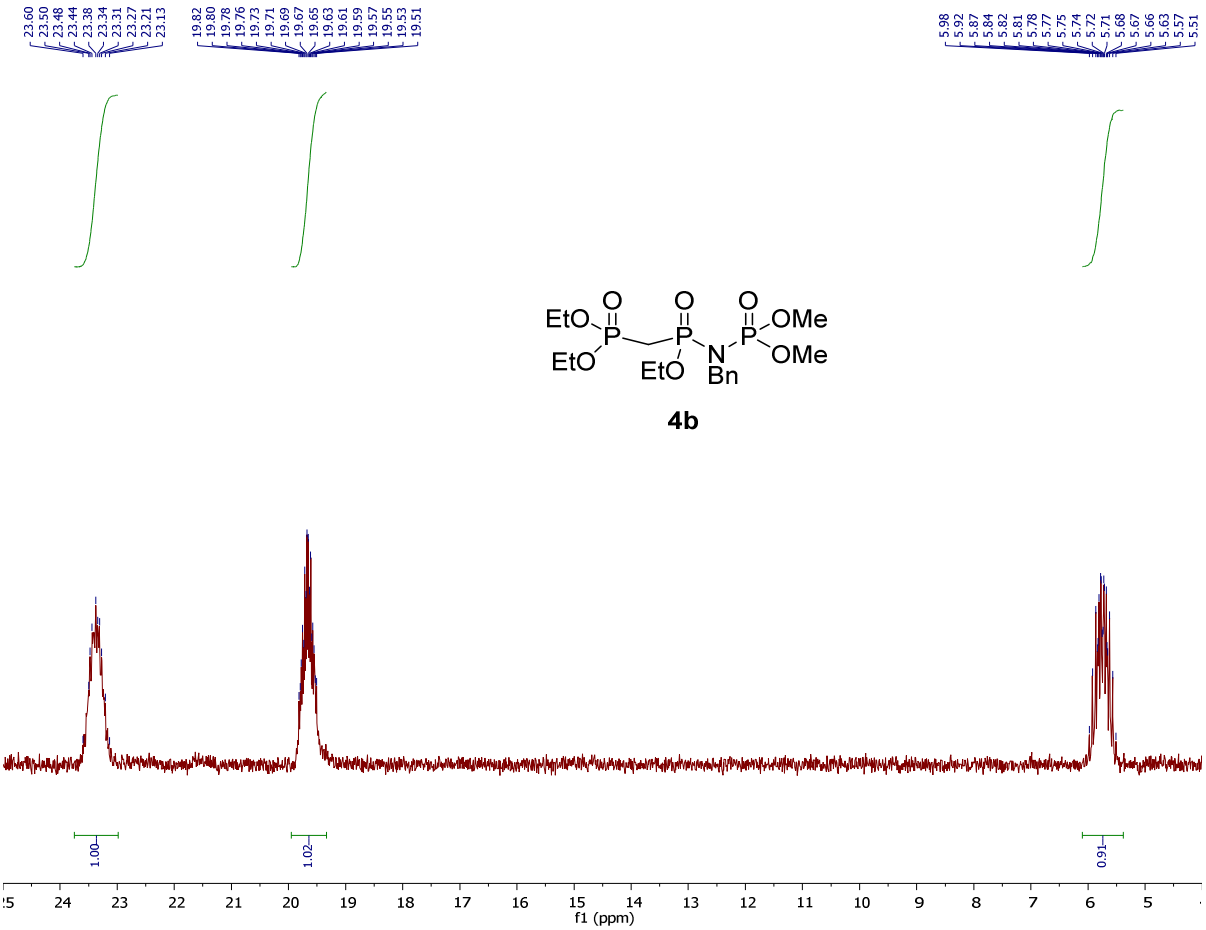


Figure S19. $^{31}P - {}^{1}H$ NMR of **4b** ($CDCl_3$, 202 MHz)

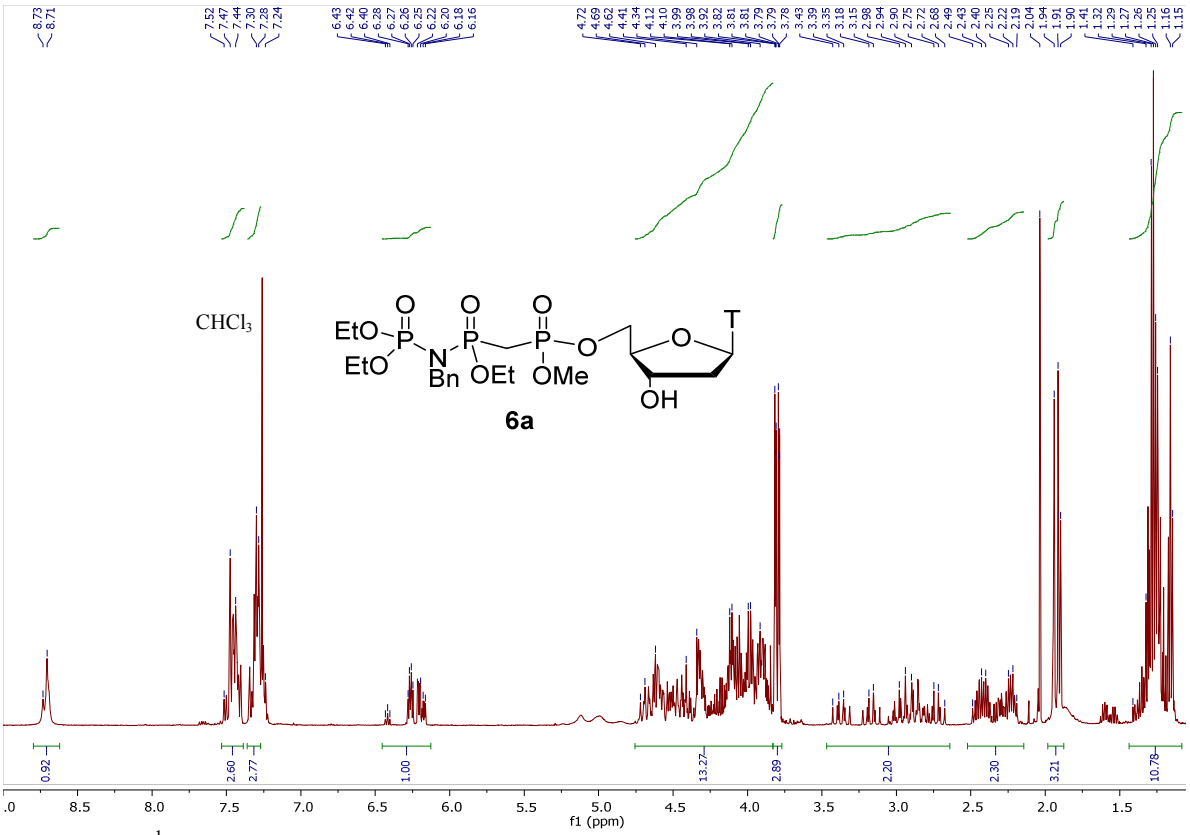


Figure S20. ^{1}H NMR of **6a** (mixture of 4 isomers) ($CDCl_3$, 500 MHz)

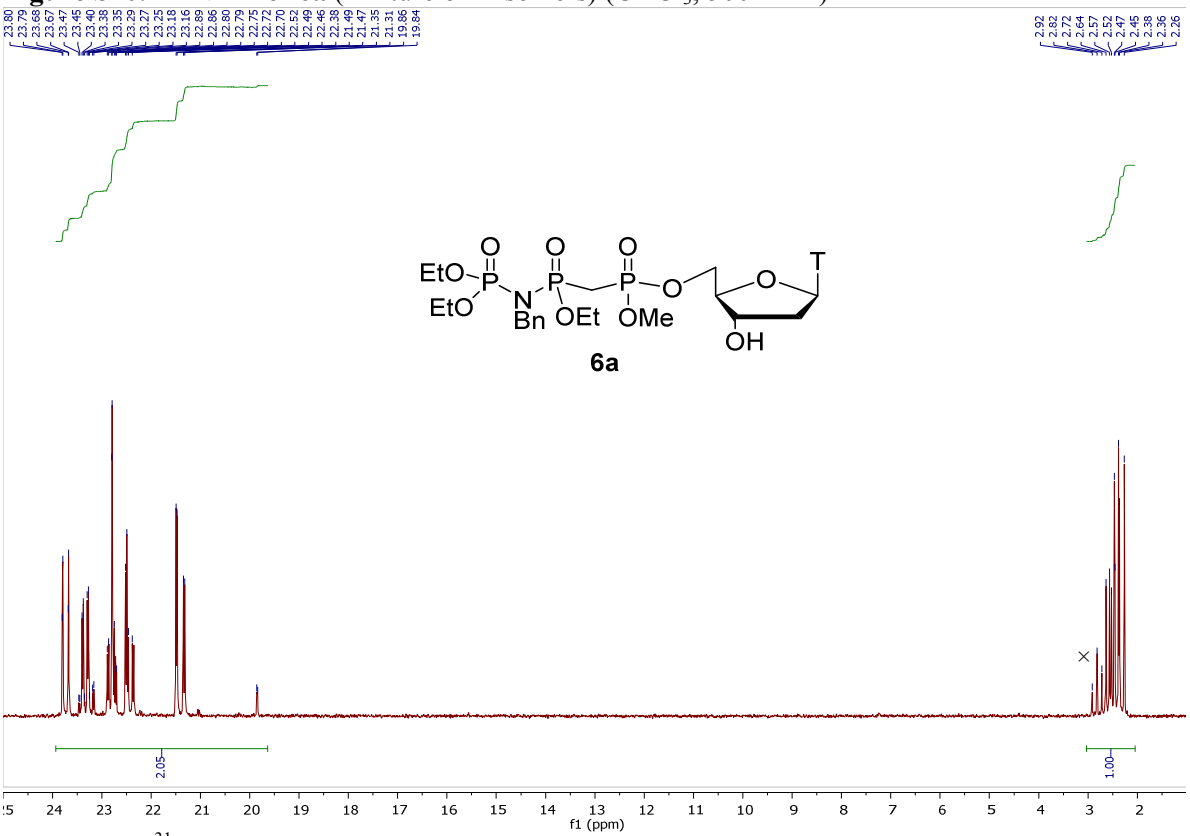


Figure S21. ^{31}P NMR of **6a** (mixture of 4 isomers) ($CDCl_3$, 202 MHz)

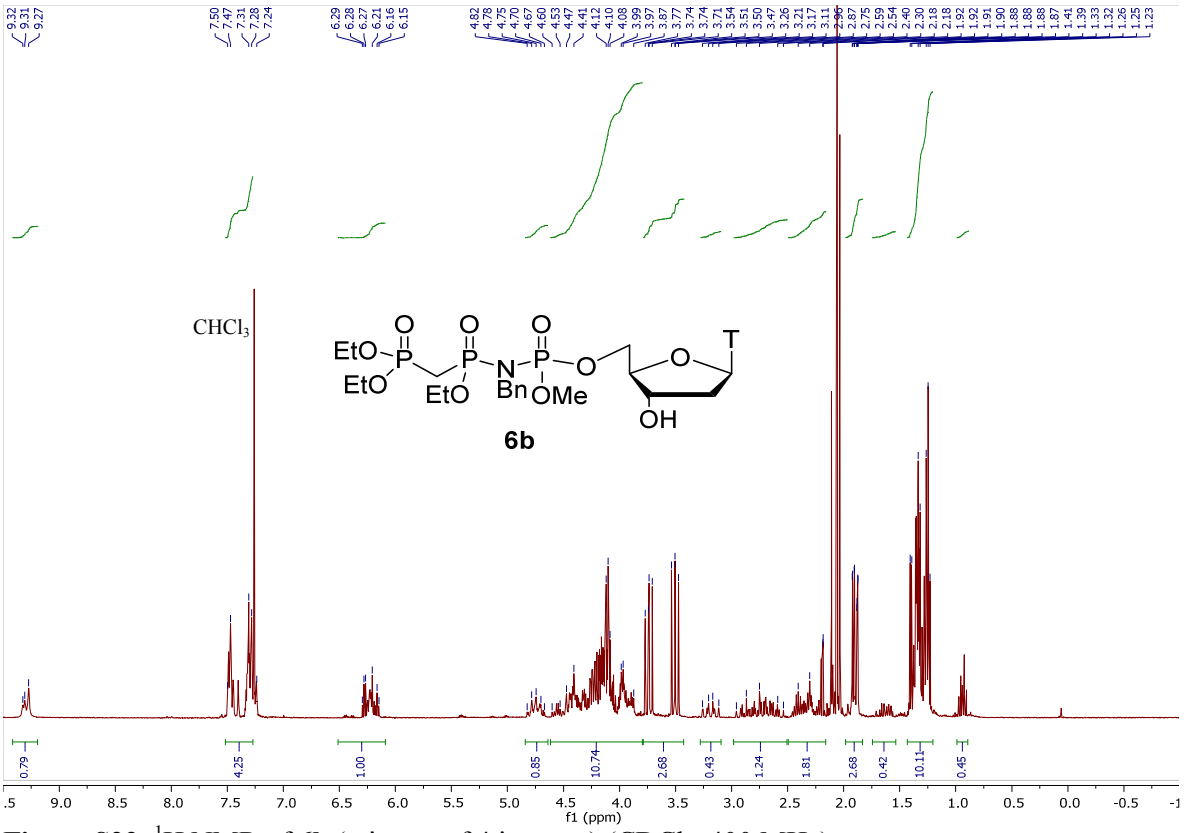


Figure S22. ^{1}H NMR of **6b** (mixture of 4 isomers) ($CDCl_3$, 400 MHz)

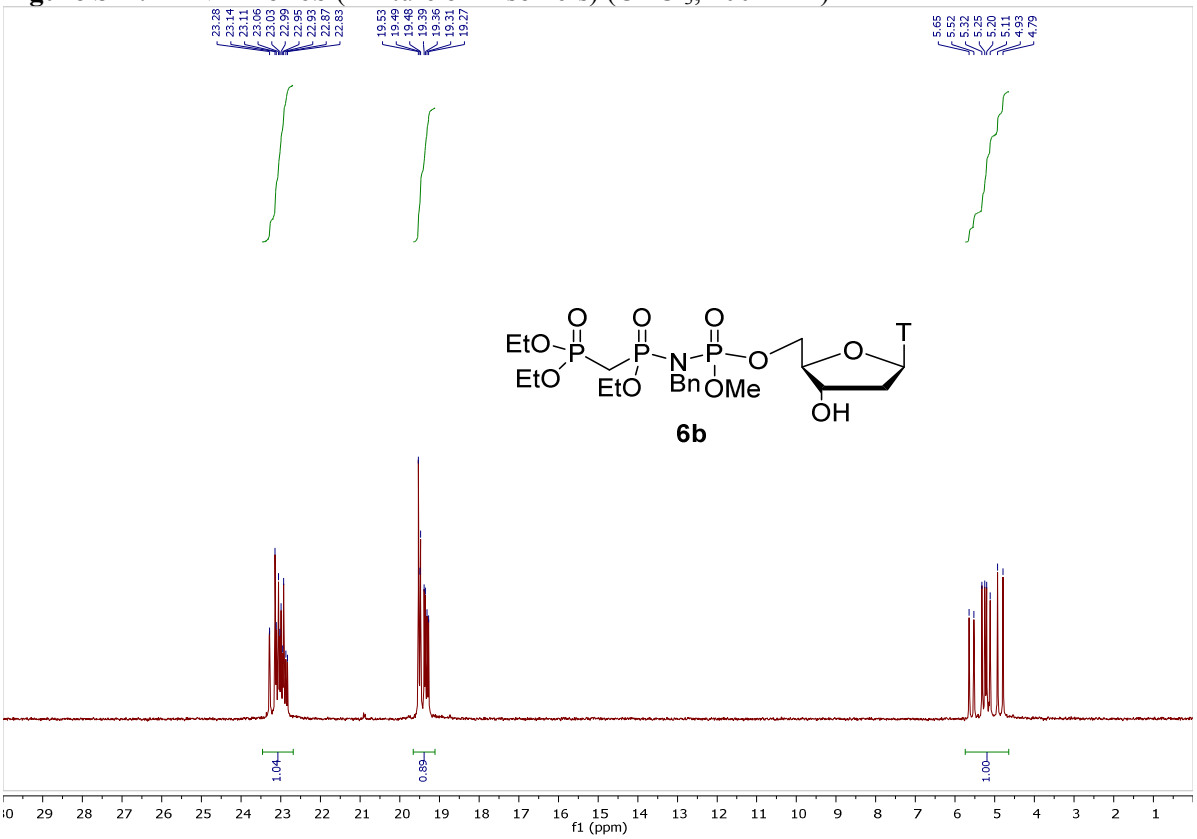


Figure S23. ^{31}P NMR of **6b** (mixture of 4 isomers) ($CDCl_3$, 162 MHz)

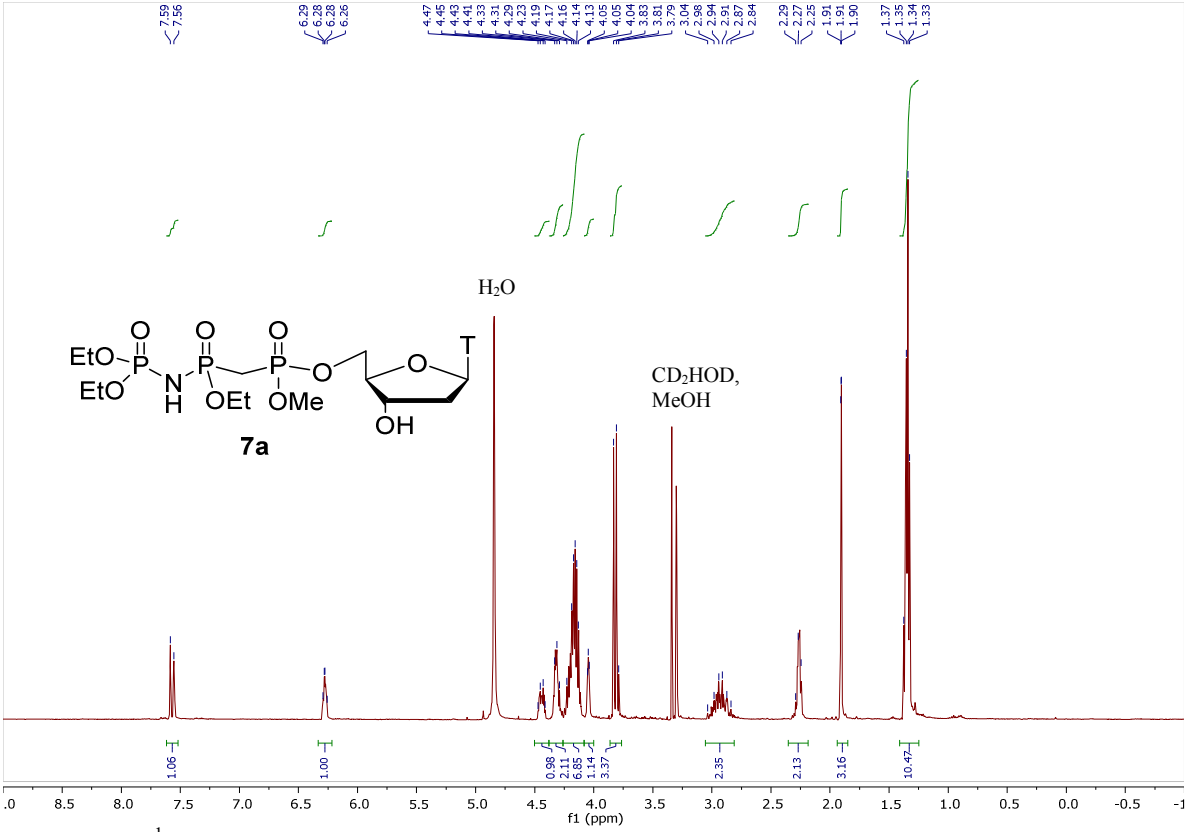


Figure S24. ^{1}H NMR of **7a** (mixture of 4 isomers) (CD_3OD, 500 MHz)

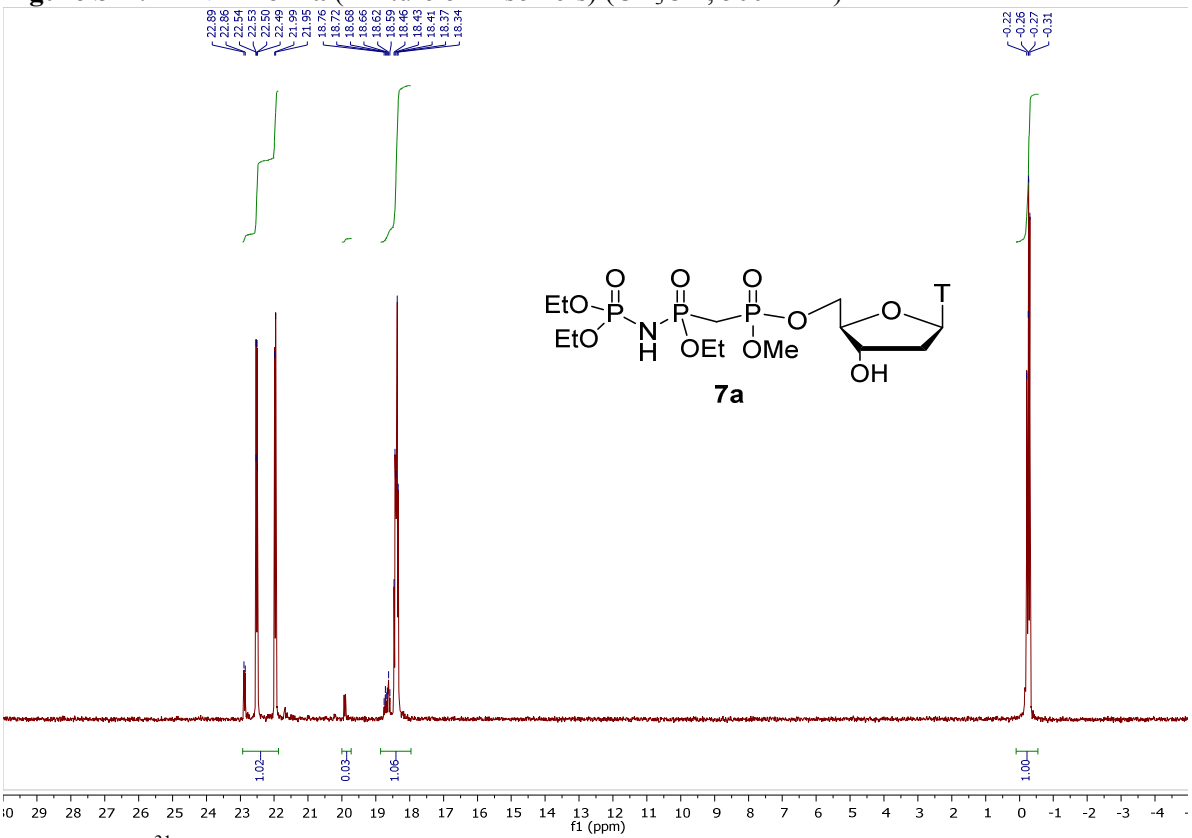


Figure S25. ^{31}P NMR of **7a** (mixture of 4 isomers) (CD_3OD, 202 MHz)

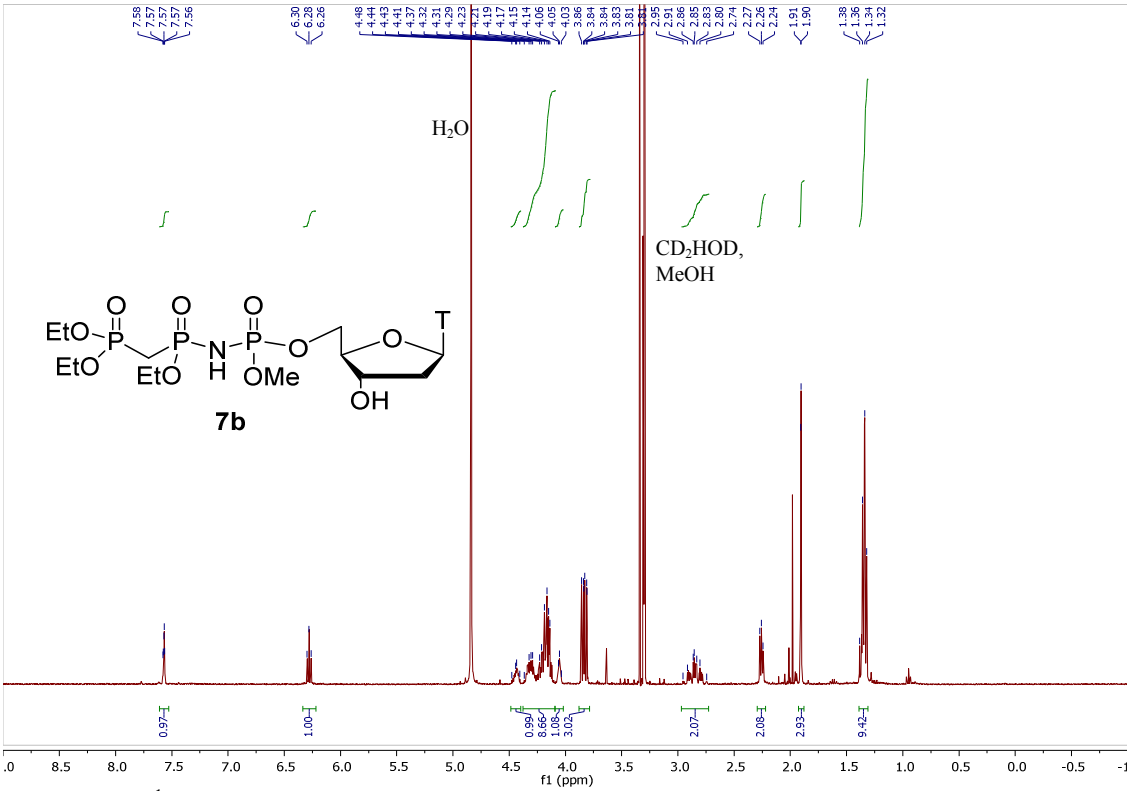


Figure S26. ^{1}H NMR of **7b** (mixture of 4 isomers) (CD_3OD, 400 MHz)

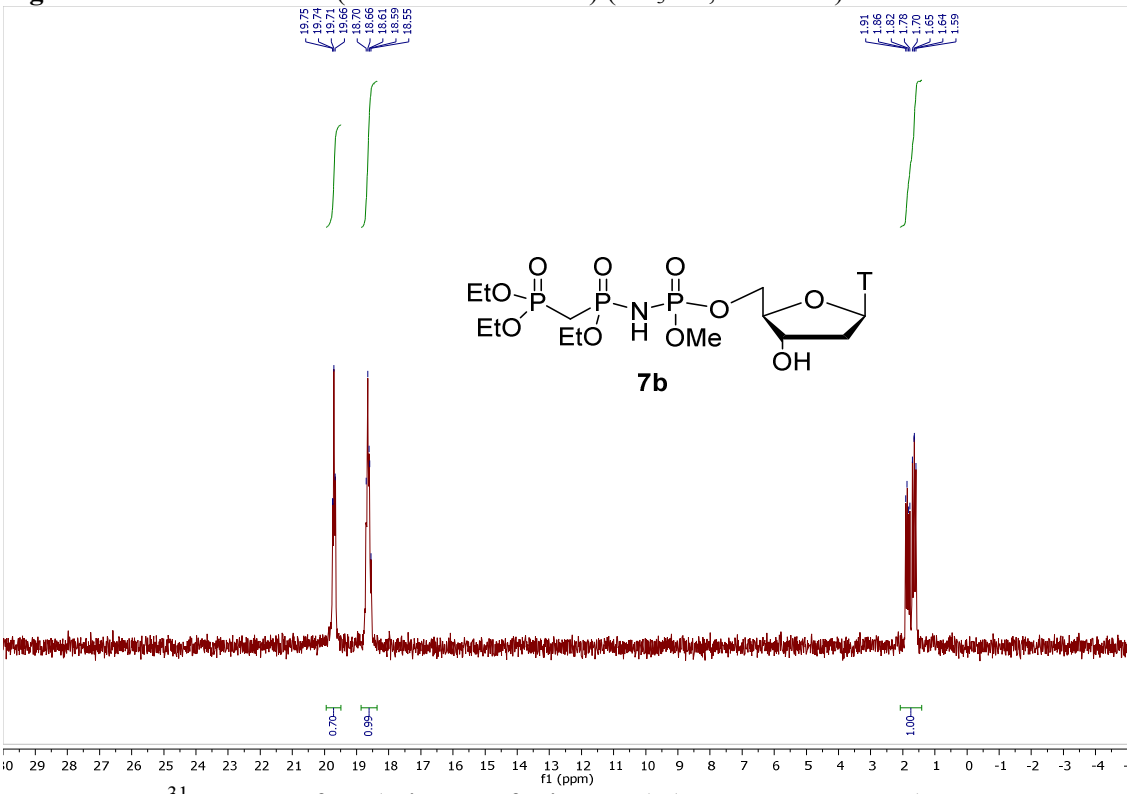


Figure S27. ^{31}P NMR of **7b** (mixture of 4 isomers) (CD_3OD, 162 MHz)

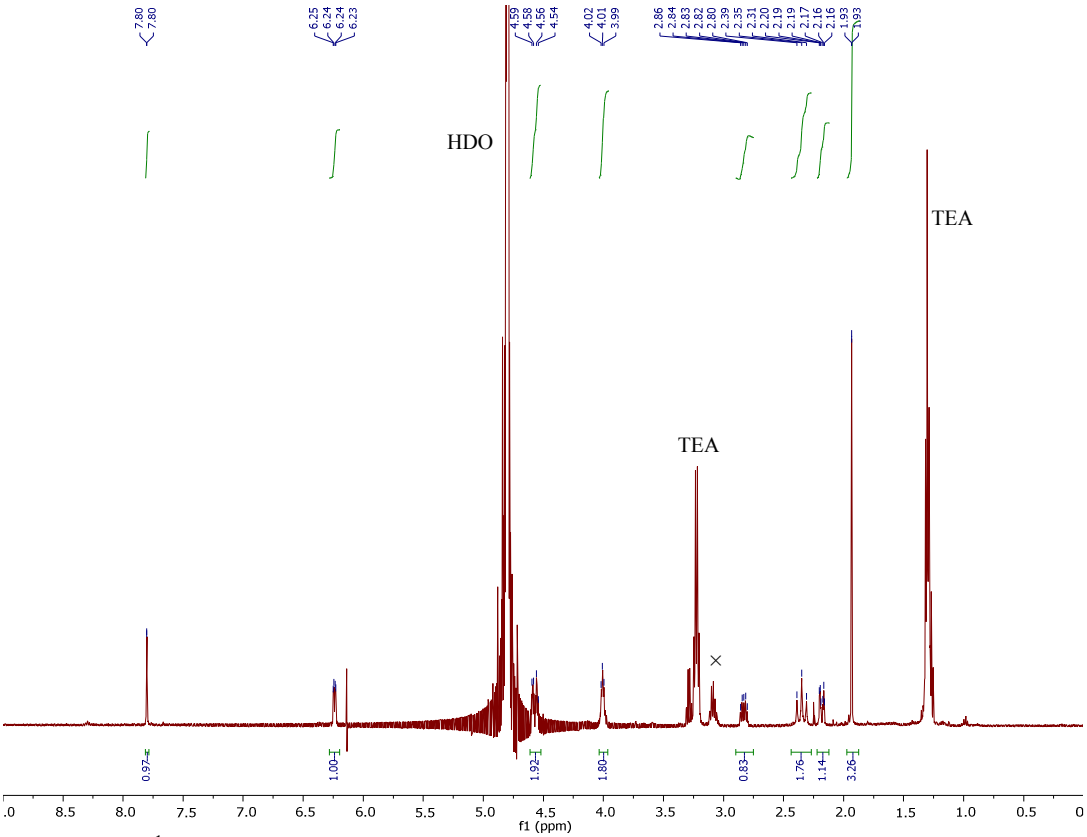


Figure S28. ^{1}H NMR of α-**1a** (D_2O, 500 MHz)

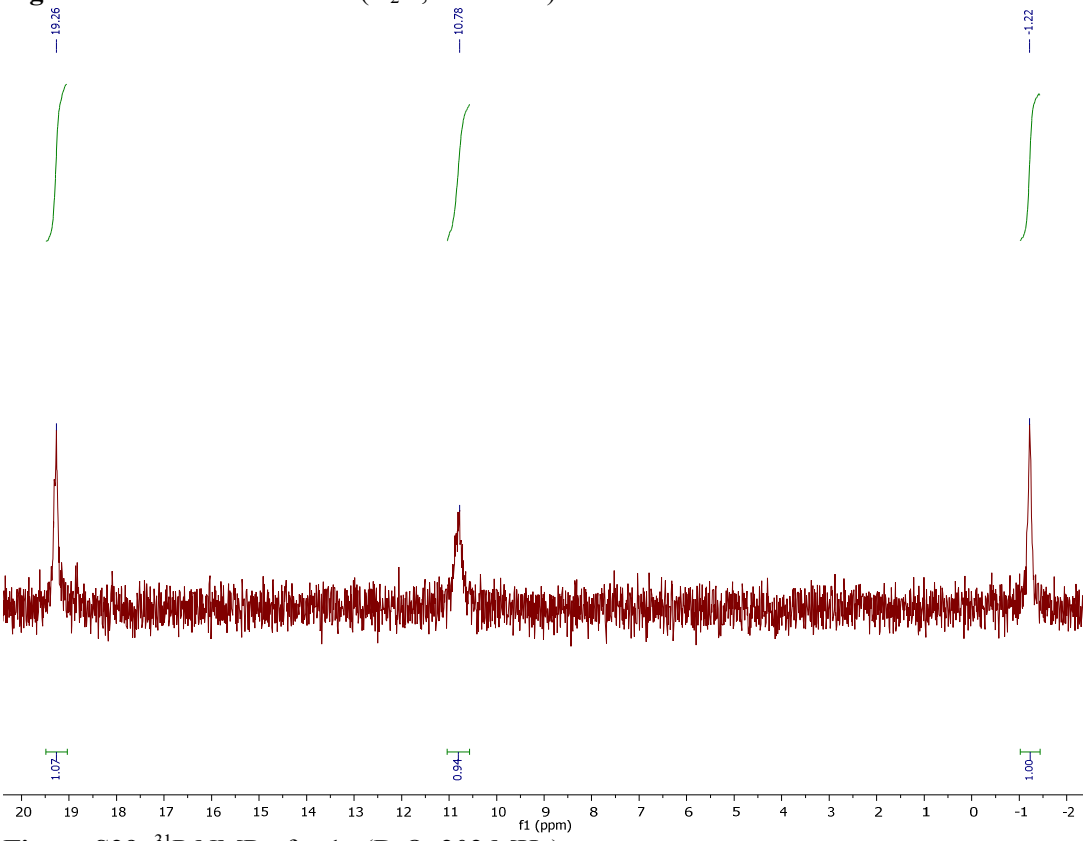


Figure S29. ^{31}P NMR of α-**1a** (D_2O, 202 MHz)

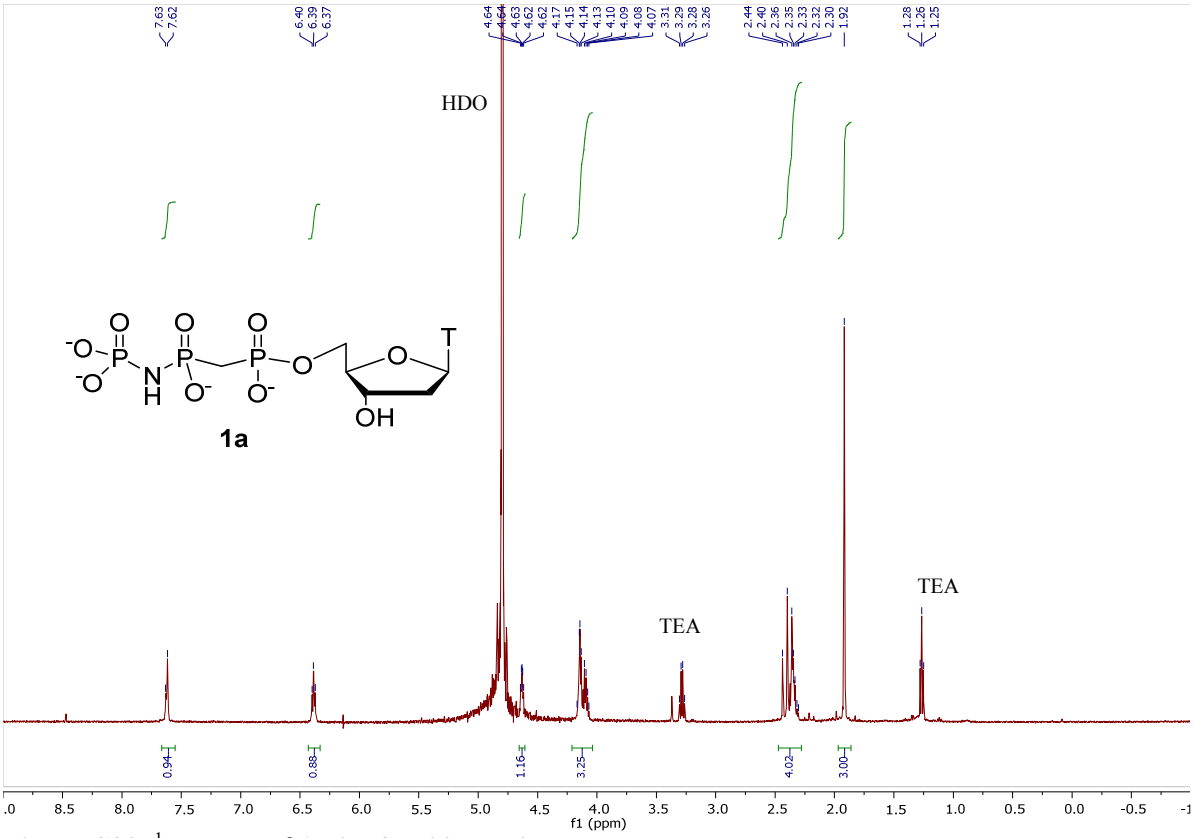


Figure S30. ^{1}H NMR of **1a** (D_2O, 500 MHz)

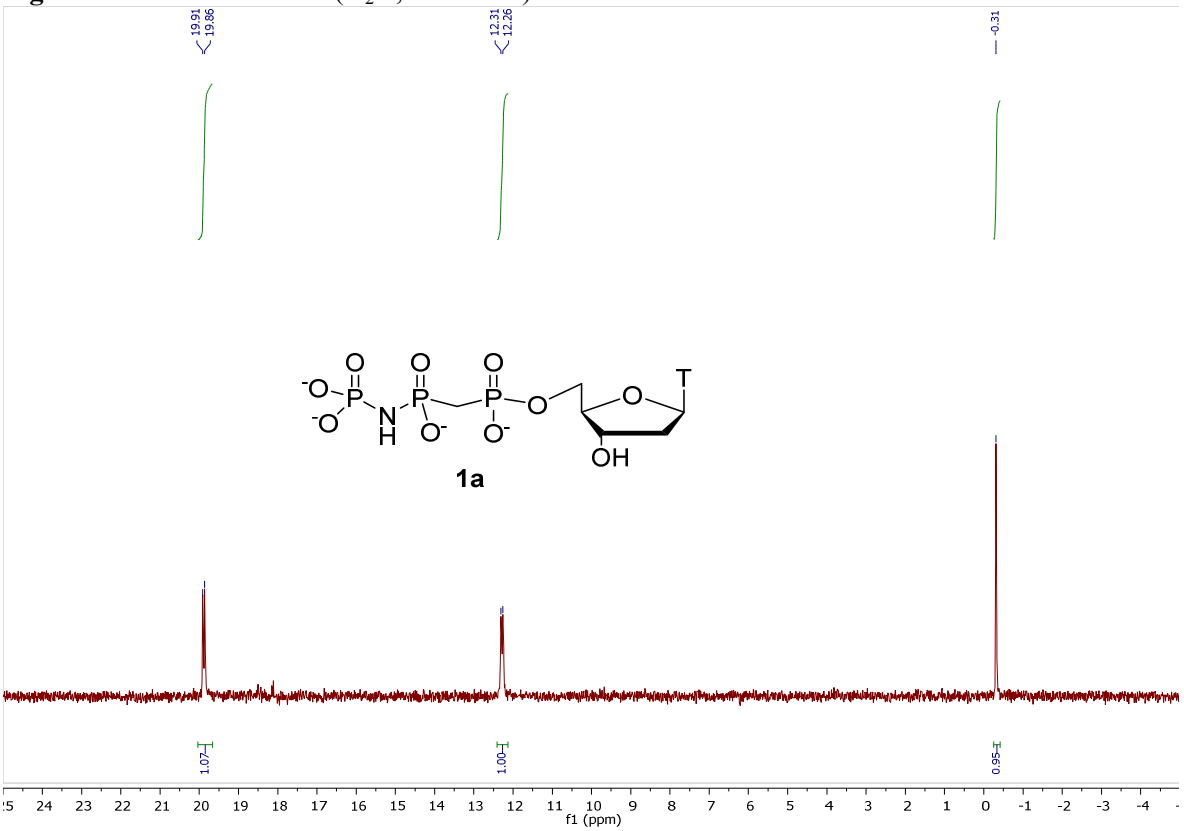


Figure S31. ^{31}P NMR of **1a** (D_2O, 202 MHz)

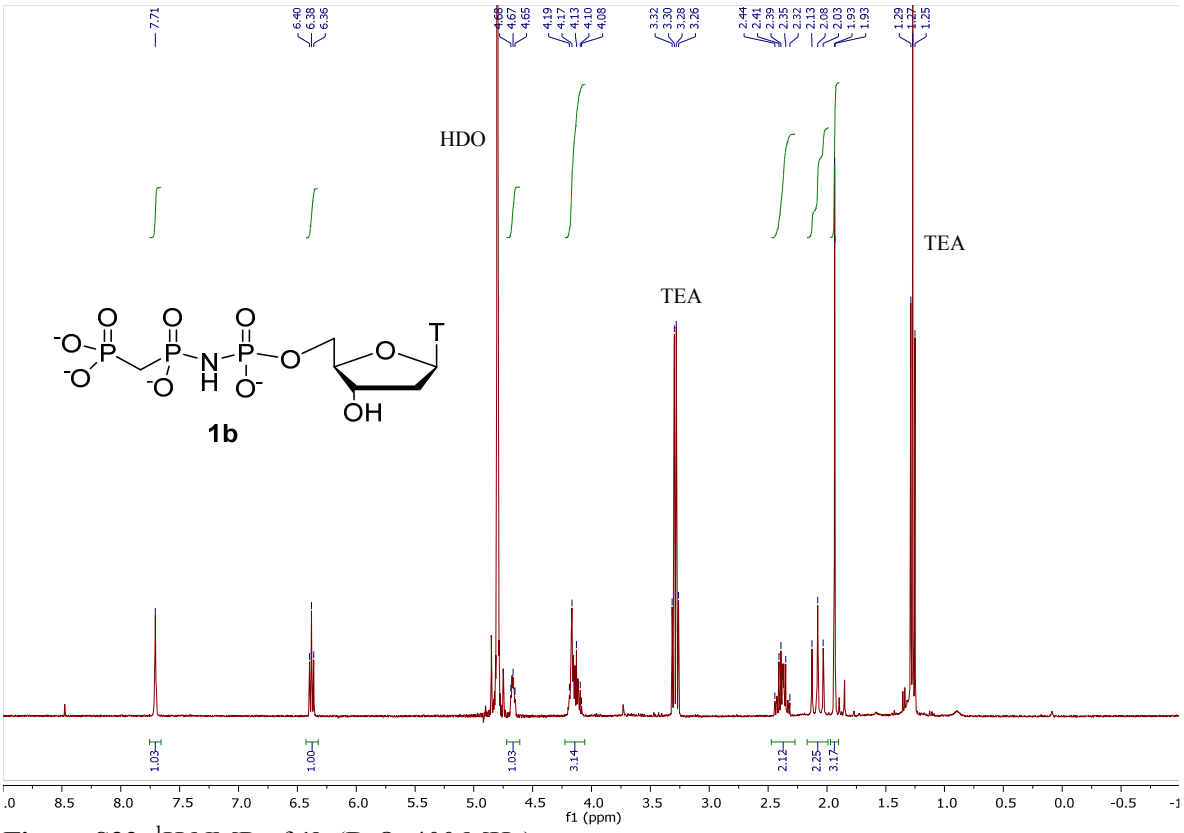


Figure S32. ^{1}H NMR of **1b** (D_2O, 400 MHz)

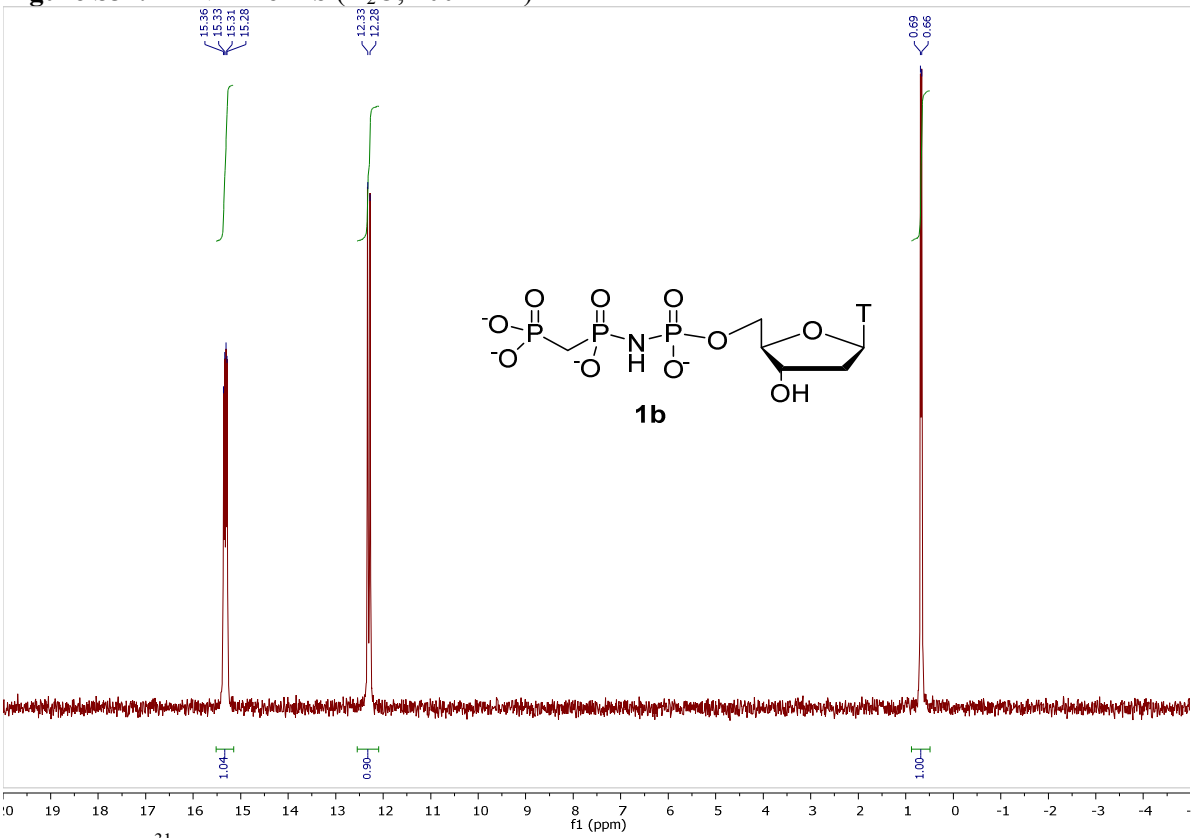


Figure S33. ^{31}P NMR of **1b** (D_2O, 162 MHz)

IV. References

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