β,γ-CHF- and β,γ-CHCl-dGTP diastereomers: synthesis, discrete ^{31}P NMR signatures and absolute configurations of new stereochemical probes for DNA polymerases

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Supporting Information

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Materials and Methods

2'-Deoxyguanosine 5'-monophosphate monosodium salt monohydrate (**8**) and (*R*)-(-)-methyl mandelate (ee: 97%) were purchased from Aldrich. Monohalomethylenebisphosphonic acids (**1a,b**)[1] and 2'-deoxyguanosine 5'-monophosphate morpholidate (dGMP-morpholidate) (**9**)[2] were prepared according to literature procedures. Compounds **2a,b-7a,b** and **10a,b-12a,b** were synthesized as described below. All other reagents were purchased from commercial sources and used as obtained, unless specified otherwise. ^{1}H, ^{19}F and ^{31}P NMR spectra were obtained on Varian 400-MR, VNMRS-500 and Bruker AMX-500 2-Channel and VNMRS-600 3-Channel NMR spectrometers. Multiplicities are quoted as singlet (s), doublet (d), triplet (t), unresolved multiplet (m), doublet of doublets (dd), doublet of doublet of doublets (ddd), doublet of triplets (dt) or broad signal (br). All chemical shifts (δ) are 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 4.79, ^{1}H NMR),[3] internal PPh_3O (δ 28, ^{31}P NMR),[4] external 85% H_3PO_4 (δ 0.00, ^{31}P NMR) or external $CFCl_3$ (δ 0.00, ^{19}F NMR). ^{31}P NMR spectra were proton-decoupled, and ^{1}H, ^{19}F, and ^{31}P coupling constants (*J* values) are given in Hz. The concentration of the NMR samples was in the range of 2-5 mg/mL. Preparative HPLC was performed using a Varian ProStar equipped with a Shimadzu SPD-10A UV detector (0.5 mm path length) with detection at the wavelength specified in **Table S1**. Mass spectrometry was performed on a Finnigan LCQ Deca XP Max mass spectrometer equipped with an ESI source in the negative ion mode. Compound IUPAC names were assigned using ACD/Labs, Release 12.00, Product Version 12.01. LC-MS was performed on Finnigan LCQ Deca XP Max mass spectrometer in negative mode with a Finnigan Survey or PDA 158 Plus detector (1 cm path length) and MS Pump Plus, all controlled using Xcalibur software, version 2.0.7.

Synthesis of tetramethyl (chloromethanediyl)bis(phosphonate), 2a.

(Chloromethanediyl)bis(phosphonic acid) **1a**, 1.00 g (4.75 mmol) was dissolved in 10 mL of trimethyl orthoformate (91 mmol, 9.7 g). The reaction mixture was set to reflux for 1 h. Warm water (65˚C) was circulated in the condenser to allow evaporation of the byproducts, MeOH and trimethylformate, in order

to drive the reaction to completion. Excess trimethyl orthoformate was removed under vacuum to yield 1.048 g (83%) of compound **2a**, which was obtained as a colorless oil. The compound was used for the next step without further purification. ^{1}H NMR (500 MHz; $CDCl_3$): δ 4.08 (t, J_{HP} = 17.8 Hz, 1H), 3.91 (m, 12H). ^{31}P NMR (202 MHz; $CDCl_3$): δ 16.24 (s). Lit[5]: ^{1}H ($CDCl_3$): 4.0 (1H, t, J = 18 Hz), 3.7 (12 H, m); ^{31}P ($CDCl_3$): 15.5.

Synthesis of tetramethyl (fluoromethanediyl)bis(phosphonate), 2b.

Following the procedure for synthesis of **2a**, 0.418 g (2.16 mmol) of (fluoromethanediyl)bis(phosphonic acid) **1b** was methylated to yield 0.535 g (>99%) of compound **2b**. After removal of solvents a colorless oil was obtained. ^{1}H NMR (500 MHz; CD_3OD): δ 5.67 (dt, J_{HF} = 44.8 Hz, J_{HP} = 14.3 Hz, 1H), 3.89 (br, 12H,). ^{31}P NMR (202 MHz; CD_3OD): δ 13.11 (d, J_{PF} = 62.3 Hz). ^{19}F NMR (470 MHz; CD_3OD): δ -231.97 (dt, J_{FH} = 44.1 Hz, J_{FP} = 63.1 Hz).

Synthesis of sodium methyl [chloro(dimethoxyphosphoryl)methyl]phosphonate, 3a.

Compound **2a**, 2.36 g (8.85 mmol) was dissolved in 5 mL of acetone, followed by addition of NaI (8.85 mmol, 1.33 g). After the NaI was completely dissolved, the reaction mixture was allowed to stand at rt overnight. White crystals, the di-demethylated compound, slowly precipitated out of the solution and were removed by filtration. The organic phase was concentrated under vacuum. The residue was redissolved in 10 mL of H_2O and unreacted tetramethyl ester was extracted with $CHCl_3$ (20 mL × 3). The aqueous phase was dried under vacuum to yield 1.176 g (49%) of compound **3a**, which was obtained as a colorless, viscous oil. ^{1}H NMR (500 MHz; D_2O; pH 7.0): δ 4.40 (t, J_{HP} = 16.6 Hz, 1H), 3.91-3.88 (m, 6H), 3.69 (d, J_{HP} = 10.8 Hz, 3H). ^{31}P NMR (202 MHz; D_2O; pH 7.0): δ 22.59 (d, J_{PP} = 3.9 Hz, 1P), 9.62 (d, J_{PP} = 3.9 Hz, 1P).

Synthesis of sodium methyl [(dimethoxyphosphoryl)(fluoro)methyl]phosphonate, 3b.

Following the procedure for synthesis of **3a**, 0.53 g (2.11 mmol) of compound **2b** was monodemethylated to yield 0.277 g (51%) of compound **3b**. After removal of solvent a colorless film was obtained. ^{31}P NMR (202 MHz; CD_3OD): δ 19.37 (dd, J_{PP} = 15.2 Hz, J_{PF} = 66.1, 1P), 6.93 (dd, J_{PP} = 15.2 Hz, J_{PF} = 54.7, 1P).

Synthesis of methyl (7*S*)-7-benzyl-4-chloro-3,5-dimethoxy-2,6-dioxa-3,5-diphosphaoctan-8-oate 3,5-dioxide, 4a.

Monosodium salt **3a**, 365 mg (1.33 mmol) was dissolved in 1 mL of MeOH, loaded onto a column of strong cation exchange DOWEX resin in acidic form (5 mL) and then eluted from the column using MeOH. The eluate was concentrated under vacuum and dried by repeated co-evaporation with anhydrous dioxane until the total weight of the flask remained constant. The product was then redissolved in 2 mL of anhydrous dioxane, followed by sequential addition of PPh_3 (1.99 mmol, 523 mg) and (*R*)-(-)-methyl mandelate (1.99 mmol, 332 mg). Distilled anhydrous diisopropylazodicarboxylate (DIAD) (1.99 mmol, 404 mg, 395 μL) was dissolved in 1 mL of anhydrous dioxane and added to the reaction mixture dropwise under N_2. The reaction mixture was stirred under N_2 at rt overnight. After reaction was complete (monitored by ^{31}P NMR), volatiles were removed under vacuum. The crude product was purified by column chromatography on silica gel (10% MeOH/ether) to yield 346 mg (65%) of **4a** as a mixture of four diastereomers (^{31}P NMR). After removal of solvent a colorless oil was obtained. Alternatively, impurities were removed by crystallization (15% hexane/diethyl ether) and the organic phase was concentrated under vacuum to afford **4a**. Mixture **4a** purified by the second method contained some PPh_3O impurity, but was used for the next step without further purification. ^{31}P NMR (202 MHz; $CDCl_3$): δ 16-15 (m).

Synthesis of methyl (7*S*)-7-benzyl-4-fluoro-3,5-dimethoxy-2,6-dioxa-3,5-diphosphaoctan-8-oate 3,5-dioxide, 4b.

Following the procedure for synthesis of **4a**, 270 mg (1.05 mmol) of monosodium salt **3b** was alkylated by (*R*)-(-)-methyl mandelate. Preparative TLC (50% hexane/ethyl acetate) purification gave 298 mg (74%) of the product **4b** as a mixture of four diastereomers (^{31}P NMR). After removal of solvent a colorless oil was obtained. ^{31}P NMR (202 MHz; CD_3OD): δ 14-12 (m).

Synthesis of (2*S*)-({[chloro(phosphono)methyl](hydroxy)phosphoryl}oxy)(phenyl)ethanoic acid, 5a-1/5a-2.

Compound **4a** (32 mg, 82 μmol) was dissolved in 5 mL of anhydrous acetonitrile, followed by addition of 6 eq of freshly redistilled bromotrimethylsilane (BTMS) (494 μmol, 75 mg). The reaction mixture was stirred at rt for 1 h. Completion of the reaction was confirmed by mass spectrometry by monitoring the peak at 357 m/z. Excess BTMS was removed under vacuum. The residue was dissolved in 10 mL of MeOH and stirred at rt for 15 min. Volatiles were removed under vacuum producing a crude mixture of compounds **5a-1/5a-2**, which was obtained as a colorless film. The compound was not further characterized and used in the next step without purification.

Synthesis of (2*S*)-({[fluoro(phosphono)methyl](hydroxy)phosphoryl}oxy)(phenyl)ethanoic acid, 5b-1/5b-2.

Following the procedure for synthesis of **5a-1/5a-2** and monitored by ^{31}P NMR, silyldemethylation of 100 mg (0.26 mmol) of **4b** gave the mixture **5b-1/5b-2**. After removal of solvent a colorless film was obtained. The compound was not further characterized and used in the next step without purification.

Synthesis of (2*S*)-({[(*S*)-chloro(phosphono)methyl](hydroxy)phosphoryl}oxy)(phenyl)ethanoic acid, 6a-1.

The crude mixture of **5a-1/a-2** was dissolved in 10 mL of water and the pH was adjusted to 8 using Na_2CO_3. The solution was washed with $CHCl_3$ (30 mL × 3) to remove contaminating PPh_3O. The aqueous layer was stirred overnight, and then concentrated under vacuum to provide sodium salts **6a-1/6a-2** in 80% yield (by ^{31}P NMR overall from **4a**). Purification and separation of diastereomers **6a-1/6a-2** was performed on HPLC using a Varian Microsorb C_{18} HPLC column (5 μm, 250 mm × 21.4 mm) with 3.5% CH_3CN in 0.1 N triethylammonium bicarbonate (TEAB) buffer pH 7.2 at a flow rate of 15.0 mL/min. The UV detector was operated at 256 nm. Diastereomer **6a-1** eluted at 10.5 min and was obtained as a triethylammonium salt (**Table S1**). After removal of solvent a colorless film was obtained. ^{1}H NMR (500 MHz; D_2O; pH 10.3): δ 7.58-7.35 (m, 5H), 5.54 (d, J_{HP} = 8.8 Hz, 1H), 3.68 (t, J_{HP} = 15.8 Hz, 1H). ^{31}P NMR (202 MHz; D_2O; pH 10.3): δ 15.54 (d, J_{PP} = 4.6 Hz, 1P), 9.76 (d, J_{PP} = 4.9 Hz, 1P).

Synthesis of (2*S*)-({[(*R*)-chloro(phosphono)methyl](hydroxy)phosphoryl}oxy)(phenyl)ethanoic acid, 6a-2.

Following the procedure for synthesis and isolation of **6a-1**, HPLC separation provided individual diastereomer **6a-2**, which eluted at 11.5 min and was obtained as a triethyammonium salt (**Table S1**). After removal of solvent a colorless film was obtained. ^{1}H NMR (500 MHz; D_2O; pH 9.8): δ 7.55-7.36 (m, 5H), 5.56 (d, J_{HP} = 9.0 Hz, 1H), 3.66 (t, J_{HP} = 15.8 Hz, 1H). ^{31}P NMR (202 MHz; D_2O; pH 9.8): δ 14.03 (d, J_{PP} = 5.2 Hz, 1P), 9.71 (d, J_{PP} = 5.6 Hz, 1P).

Synthesis of (2*S*)-({[fluoro(phosphono)methyl](hydroxy)phosphoryl}oxy)(phenyl)ethanoic acid, 6b-1/6b-2.

Following the procedure for synthesis of **6a-1**, the methylmandelate moiety of the crude mixture **5b-1/5b-2** was hydrolyzed to give diastereomers **6b-1/6b-2** in 83% yield (by ^{31}P NMR, overall from **4b**). After removal of solvent a colorless film was obtained. HPLC separation of **6b-1/6b-2** was not successful using similar or modified conditions of separation for **6a-1/6a-2**. ^{31}P NMR (202 MHz; CD_3OD): δ 13.48-12.73 (m, 1P), 7.87-7.43 (m, 1P).

Synthesis of [(*R*)-chloro(hydroxy{[(2*S*)-1-(morpholin-4-yl)-1-oxo-3-phenylpropan-2-yl]oxy}phosphoryl)methyl]morpholin-4-ylphosphinic acid, 7a-1.

The mixture of diastereomers **6a-1/6a-2** (30.4 mg, 88.3 μmol) was dissolved in 10 mL of *t*-BuOH:H_2O (1:1), followed by addition of morpholine (706.4 μmol, 61.5 mg, 61 μL). The reaction mixture was first stirred at rt for 15 min and then set to reflux. Dicyclohexylcarbodiimide (DCC) (1.79 mmol, 379 mg) was dissolved in 3 mL of *t*-BuOH and divided into 12 aliquots. Every 15 min, one aliquot was added dropwise to the reaction mixture under reflux. After 3 h, DCC addition was complete, and reflux was continued for another 2 h. The reaction completion was confirmed by mass spectrometry by monitoring the peak at 481 m/z. After reaction was complete, the mixture was cooled to rt and solvent was removed under vacuum. The residue was resuspended in 2 mL of water. Solids were removed by filtration and the aqueous layer was concentrated under vacuum to yield 33.5 mg (78%, by HPLC) of **7a-1/a-2** as a mixture of

diastereomers. Purification and separation of **7a-1/7a-2** was performed on preparative HPLC using a Varian Microsorb C_{18} HPLC column (5 μm, 250 mm × 21.4 mm) with 15% CH_3CN in 0.1 N triethylammonium bicarbonate (TEAB) buffer pH 7.4 at a flow rate of 8.0 mL/min (**Table S1**). The UV detector was operated at 256 nm. Diastereomer **7a-1** eluted at 14.2 min and was obtained as a triethylammonium salt. After removal of solvent a colorless film was obtained. ^{1}H NMR (500 MHz; D_2O; pH 9.8): δ 7.55-7.46 (m, 5H), 6.20 (d, J_{HP} = 8.7 Hz, 1H), 3.82-3.76 (m, 5H), 3.63 (m, 8H), 3.16-3.14 (m, 4H). ^{31}P NMR (202 MHz; D_2O; pH 9.8): δ 11.79-11.63 (J_{PP} = 5.3 Hz, 2P). MS [M-H]$^-$: calcd for $C_{17}H_{24}ClN_2O_8P_2^-$, 481.1, found 481.1.

Synthesis of [(*S*)-chloro(hydroxy{[(2*S*)-1-(morpholin-4-yl)-1-oxo-3-phenylpropan-2-yl]oxy}phosphoryl)methyl]morpholin-4-ylphosphinic acid, 7a-2.

Following the procedure for synthesis and separation of **7a-1**, HPLC separation provided diastereomer **7a-2**, which eluted at 15.2 min and was obtained as triethylammonium salt (**Table S1**). After removal of solvent a colorless film was obtained. ^{1}H NMR (500 MHz; D_2O; pH 10.0): δ 7.56-7.45 (m, 5H), 6.16 (d, J_{HP} = 8.6 Hz, 1H), 3.84-3.78 (m, 5H), 3.65-3.63 (m, 8H), 3.16-3.13 (m, 4H). ^{31}P NMR (202 MHz; D_2O; pH 10.0): δ 12.46 (d, J_{PP} = 4.5 Hz, 1P), 11.67 (d, J_{PP} = 4.6 Hz, 1P). MS [M-H]$^-$: calcd for $C_{17}H_{24}ClN_2O_8P_2^-$, 481.1, found 481.1.

Synthesis of [(*R*)-fluoro(hydroxy{[(2*S*)-1-(morpholin-4-yl)-1-oxo-3-phenylpropan-2-yl]oxy}phosphoryl)methyl]morpholin-4-ylphosphinic acid, 7b-1.

Following the procedure for synthesis and separation of **7a-1**, 72.2 mg (0.22 mmol) of the diastereomer mixture **6b-1/6b-2** was dimorpholidated to yield 89.2 mg (87%) of **7b-1/7b-2** as a diastereomer mixture. HPLC separation provided individual diastereomer **7b-1**, which eluted at 14.3 min and was obtained as a triethylammonium salt (**Table S1**). After removal of solvent a colorless film was obtained. ^{1}H NMR (500 MHz; D_2O; pH 10.3): δ 7.47-7.40 (m, 5H), 6.09 (d, J_{HP} = 8.3 Hz, 1H), 4.64 (dt, J_{HP} = 12.2 Hz, J_{HF} =45.4 Hz, 1H), 3.73 (m, 4H), 3.60 (m, 8H), 3.02 (m, 4H). ^{31}P NMR (202 MHz; D_2O; pH 10.0): δ 9.77 (dd, J_{PP} =

12.7 Hz, J_{PF} = 62.0 Hz, 1P), 9.62 (dd, J_{PP} = 12.7 Hz, J_{PF} = 58.8 Hz 1P). ^{19}F (470 MHz; CD_3OD): δ -218.46 (dt, J_{FH} = 45.3 Hz, J_{FP} = 59 Hz). LC-MS [M-H]$^-$: calcd for $C_{17}H_{24}FN_2O_8P_2^-$, 465.10, found 465.05.

Synthesis of [(*S*)-fluoro(hydroxy{[(2*S*)-1-(morpholin-4-yl)-1-oxo-3-phenylpropan-2-yl]oxy}phosphoryl)methyl]morpholin-4-ylphosphinic acid, 7b-2.

Following the procedure for synthesis and separation of **7a-1**, HPLC separation provided individual diastereomer **7b-2**, which eluted at 15.5 min and was obtained as a triethylammonium salt (**Table S1**). After removal of solvent a colorless film was obtained. ^{1}H NMR (500 MHz; D_2O; pH 10.3): δ 7.46-7.39 (m, 5H), 6.08 (d, J_{HP} = 8.8 Hz, 1H), 4.66 (dt, J_{HP} = 12.2 Hz, J_{HF} =42.6 Hz, 1H), 3.73 (m, 4H), 3.58 (m, 8H), 3.05 (m, 4H). ^{31}P NMR (202 MHz; D_2O; pH 10.0): δ 10.22 (dd, J_{PP} = 12.7 Hz, J_{PF} = 63.6 Hz, 1P), 9.70 (dd, J_{PP} = 12.7 Hz, J_{PF} = 60.4 Hz 1P). ^{19}F (470 MHz; CD_3OD): δ -218.26 (dt, J_{FH} = 45.3 Hz, J_{FP} = 61 Hz). LC-MS [M-H]$^-$: calcd for $C_{17}H_{24}FN_2O_8P_2^-$, 465.10, found 465.09.

Synthesis of [(*R*)-chloro{hydroxy[(1*S*)-2-(morpholin-4-yl)-2-oxo-1-phenylethoxy]phosphoryl}methyl]phosphonic acid, 10a-1.

The triethylammonium salt of the individual dimorpholidate diastereomer **7a-1** 24 mg (50 μmol) was dissolved in 15 mL of water, followed by addition of strong cation exchange Dowex resin in acidic form (5 mL), and the mixture was stirred at rt for 30 min. The Dowex resin was removed by filtration. A couple of drops of 1 M HCl were added to the filtrate, and stirring continued for another 30 min to complete the hydrolysis of the phosphoroamidite. Completion of hydrolysis was confirmed by mass spectrometry by monitoring the peak at 412 m/z. The solvent was removed under vacuum to yield 20.6 mg (>99%) of compound **10a-1**. After removal of solvent a colorless film was obtained. The compound was not further characterized and used in next step without purification.

Synthesis of [(*S*)-chloro{hydroxy[(1*S*)-2-(morpholin-4-yl)-2-oxo-1-phenylethoxy]phosphoryl}methyl]phosphonic acid, 10a-2.

Following the procedure for synthesis of **10a-1**, 30.1 mg (62.5 μmol) of **7a-2** was hydrolyzed to yield 25.8 mg (>99%) of compound **10a-2**. After removal of solvent a colorless film was obtained. The compound was not further characterized and used in the next step without purification.

Synthesis of [(*R*)-fluoro{hydroxy[(1*S*)-2-(morpholin-4-yl)-2-oxo-1-phenylethoxy]phosphoryl}methyl]phosphonic acid, 10b-1.

Following the procedure for synthesis of **10a-1**, 30 mg (64.3 μmol) of **7b-1** was hydrolyzed to yield 25.4 mg (>99%) of compound **10b-1**. After removal of solvent a colorless film was obtained. The compound was not further characterized and used in the next step without purification.

Synthesis of [(*S*)-fluoro{hydroxy[(1*S*)-2-(morpholin-4-yl)-2-oxo-1-phenylethoxy]phosphoryl}methyl]phosphonic acid, 10b-2.

Following the procedure for synthesis of **10a-1**, 35 mg (75.1 μmol) of **7b-2** was hydrolyzed to yield 30 mg (>99%) of compound **10b-2**. After removal of solvent a colorless film was obtained. The compound was not further characterized and used in the next step without purification.

Synthesis of 5'-*O*-[({[(*S*)-chloro{hydroxy[(1*S*)-2-(morpholin-4-yl)-2-oxo-1-phenylethoxy]phosphoryl}methyl](hydroxy)phosphoryl}oxy)(hydroxy)phosphoryl]-2'-deoxyguanosine, 11a-1.

The individual monomorpholidate diastereomer **10a-1**, 25 mg (60.5 μmol) was dissolved in 5 mL of EtOH. Tributylamine in EtOH (1:10) was slowly added to the mixture to reach pH 4.5. After mixing for 30 min at rt, the solvent was removed under vacuum and dried by co-evaporation with anhydrous DMF (3 mL × 3). The compound was then mixed with 2 mL solution of 1.5 eq of dGMP-morpholidate (90.8 μmol, 37.7 mg) in anhydrous DMSO. The reaction mixture was stirred under rt for 72 h. Completion of reaction was confirmed by mass spectrometry by monitoring the peak at 741 m/z and by ^{31}P NMR. Purification of **11a-1** was performed on a Macherey-Nagel Nucleogel SAX 1000-10 25 mm × 15 cm preparative column, using a gradient (0-10 min, 55%; 10-16 min, 55%; 16-25 min, 100%) of 0.5 N triethylammonium

bicarbonate (TEAB) buffer pH 7.4 at a flow rate of 9 mL/min (**Table 1**). Compound **11a-1** was eluted at 18.5 min to give 15.77 mg (35%) and obtained as a triethylammonium salt with 2-5% impurity of diguanosine diphosphate (dG*pp*dG). After removal of solvent a colorless film was obtained. ^{1}H NMR (500 MHz; D_2O; pH 9.8): δ 8.03 (s, 1H), 7.53-7.41 (m, 5H), 6.30 (dd, J = 8.1 Hz, 6.3 Hz, 1H), 6.22 (d, J_{HP} = 8.7 Hz, 1H), 4.74 (m, 1H), 4.23-4.04 (m, 4H), 3.73-3.56 (m, 8H), 2.75-2.69 (m, 1H), 2.50-2.45 (m, 1H). ^{31}P NMR (202 MHz; D_2O; pH 9.8): δ 10.96 (d, J_{PP} = 8.0 Hz, 1P), 2.68 (dd, J_{PP} = 26.6 Hz, J_{PP} = 8.4 Hz, 1P), -10.39 (d, J_{PP} = 26.8 Hz, 1P).

Synthesis of 5'-*O*-[({[(*R*)-chloro{hydroxy[(1*S*)-2-(morpholin-4-yl)-2-oxo-1-phenylethoxy]phosphoryl}methyl](hydroxy)phosphoryl}oxy)(hydroxy)phosphoryl]-2'-deoxyguanosine, 11a-2.

Following the procedure of synthesis and purification for **11a-1**, 20 mg (48.4 μmol) of compound **10a-2** was conjugated with dGMP-morpholidate (**9**) to yield 18.5 mg (50%) of compound **11a-2** obtained as a triethylammonium salt after HPLC purification, eluted at 18.8 min (**Table 1**) with 2-5% diguanosine diphosphate (dG*pp*dG). After removal of solvent a colorless film was obtained. ^{1}H NMR (500 MHz; D_2O; pH 10.0): δ 8.05 (s, 1H), 7.54-7.41 (m, 5H), 6.31 (dd, J = 7.7 Hz, 6.8 Hz, 1H), 6.21 (d, J_{HP} = 8.3 Hz 1H), 4.75 (m, 1H), 4.24-4.06 (m, 4H), 3.64-3.54 (m, 8H), 2.79-2.74 (m, 1H), 2.51-2.46 (m, 1H). ^{31}P NMR (202 MHz; D_2O; pH 10.0): δ 11.38 (d, J_{PP} = 8.1 Hz, 1P), 2.76 (dd, J_{PP} = 26.7 Hz, J_{PP} = 8.5 Hz, 1P), -10.43 (d, J_{PP} = 27.2 Hz, 1P).

Synthesis of 2'-deoxy-5'-*O*-[({[(*S*)-fluoro{hydroxy[(1*S*)-2-(morpholin-4-yl)-2-oxo-1-phenylethoxy]-phosphoryl}methyl](hydroxy)phosphoryl}oxy)(hydroxy)phosphoryl]guanosine, 11b-1.

Following the procedure of synthesis for **11a-1**, 18.6 mg (47 μmol) of compound **10b-1** was conjugated with dGMP-morpholidate (**9**) to give compound **11b-1**. HPLC purification was performed using the same system as for **11a-1** except flow rate was at 8 mL/min (**Table 1**). Compound **11b-1** was eluted at 21.2 min to give 21.5 mg (63%) and obtained as a triethylammonium salt with 2-5% diguanosine diphosphate (dG*pp*dG). After removal of solvent a colorless film was obtained. ^{1}H NMR (500 MHz; CD_3OD): δ 8.01 (s, 1H), 7.52 (d, J = 7.4 Hz, 2H), 7.38-7.27 (m, 3H), 6.23 (t, J = 6.8 Hz, 1H), 6.17 (d, J = 9.3 Hz, 1H), 5.03

(dt, J_{HP} = 12.7 Hz, J_{HF} = 46.9 Hz, 1H), 4.71 (m, 1H), 4.25 (m, 1H), 4.15 (m, 1H), 4.12 (m, 1H), 3.56-3.08 (m, 8H), 2.81 (m, 1H), 2.30 (ddd, J = 2.4, 5.4, 12.7 Hz, 1H). ^{31}P NMR (202 MHz; CD_3OD): δ 9.46 (dd, J_{PP} = 17.4 Hz, J_{PF} = 58.8 Hz, 1P), 1.69 (ddd, J_{PP} = 17.5 Hz, J_{PP} = 25.5 Hz, J_{PF} = 60.4 Hz, 1P), -10.00 (d, J_{PP} = 25.5 Hz, 1P). ^{19}F NMR (470 MHz; CD_3OD): δ -220.37 (br, J_{FH} = 47.7 Hz, J_{FP} = 60 Hz).

Synthesis of 2'-deoxy-5'-*O*-[({[(*R*)-fluoro{hydroxy[(1*S*)-2-(morpholin-4-yl)-2-oxo-1-phenylethoxy]-phosphoryl}methyl](hydroxy)phosphoryl}oxy)(hydroxy)phosphoryl]guanosine, 11b-2.

Following the procedure of synthesis for **11a-1**, 20.5 mg (52 μmol) of compound **10b-2** was conjugated with dGMP-morpholidate (**9**) to give compound **11b-2**. HPLC purification was performed using the same system as for **11a-1** except the flow rate was 8 mL/min (**Table 1**) with 2-5% diguanosine diphosphate (dG*pp*dG). After removal of solvent a colorless film was obtained. ^{1}H NMR (500 MHz; CD_3OD): δ 8.05 (s, 1H), 7.53 (d, J = 7.3 Hz, 2H), 7.38-7.31 (m, 3H), 6.25 (t, J = 6.9 Hz, 1H), 6.19 (d, J = 8.8 Hz, 1H), 5.07 (dt, J_{HP} = 12.8 Hz, J_{HF} = 47.0 Hz, 1H), 4.77 (m, 1H), 4.26 (m, 1H), 4.18 (m, 1H), 4.12 (m, 1H), 3.62-3.01 (m, 8H), 2.85 (m, 1H), 2.32 (ddd, J = 3.0, 6.4, 13.7 Hz, 1H). ^{31}P NMR (202 MHz; CD_3OD): δ 9.51 (dd, J_{PP} = 17.5 Hz, J_{PF} = 58.8 Hz, 1P), 1.44 (ddd, J_{PP} = 17.5 Hz, J_{PP} = 25.5 Hz, J_{PF} = 60.4 Hz, 1P), -10.00 (d, J_{PP} = 25.5 Hz, 1P). ^{19}F NMR (470 MHz; CD_3OD): δ -220.00 (br).

Synthesis of 5'-*O*-[({[(*S*)-chloro(phosphono)methyl](hydroxy)phosphoryl}oxy)(hydroxy)phospho-ryl]-2'-deoxyguanosine, 12a-1.

The triethylammonium salt of compound **11a-1**, 5.7 mg (7.7 μmol, determined by UV) was dissolved in 5 ml of 0.1 N TEAB:MeOH (1:1, pH 8), followed by addition of 10 wt. % Pd/C (2.8 mg, 34 mol %) and a stir bar. The pH of the solution was re-adjusted to 8 by bubbling in CO_2. The reaction mixture was first frozen (dry ice/acetone), and then degassed by alternating application of vacuum and flushing N_2 over 30 minutes. The system was then flushed with H_2 gas several times. After the last fill of H_2 gas, the solution was allowed to melt. The mixture was stirred under rt under H_2 for 3 h. Completion of reaction was confirmed by mass spectrometry by monitoring the peak at 538 m/z and by ^{31}P NMR. Purification was performed on a Varian Microsorb C_{18} HPLC column (5 μm, 250 mm × 21.4 mm) eluted isocratically with

3.5% CH_3CN in 0.1 N triethylammonium bicarbonate (TEAB) buffer pH 7.4 at a flow rate of 9.0 mL/min (**Table S1**). Compound **12a-1** was eluted at 14.4 min. After removal of solvent a colorless film was obtained, 2.6 mg (62%) as a triethylammonium salt. ^{1}H NMR (400 MHz; D_2O; pH 10.6): δ 8.05 (s, 1H), 6.32 (dd, J = 7.8, 6.5 Hz, 1H), 4.27-4.12 (m, 3H), 3.90 (dd, J_{HP} = 16.7 Hz, J_{HP} = 15.4 Hz, 1H), 2.85-2.75 (m, 1H), 2.53-2.47 (m, 1H). ^{31}P NMR (202 MHz; D_2O; pH 10.6): δ 9.24 (d, J_{PP} = 5.9 Hz, 1P), 7.32 (dd, J_{PP} = 28.2 Hz, J_{PP} = 6.0 Hz, 1P), -10.05 (d, J_{PP} = 28.2 Hz, 1P).

Synthesis of 5'-*O*-[({[(*R*)-chloro(phosphono)methyl](hydroxy)phosphoryl}oxy)(hydroxy)phosphoryl]-2'-deoxyguanosine, 12a-2.

Following the procedure for synthesis and purification of **12a-1**, 5.4 mg (7.3 μmol, determined by UV) of compound **11a-2** was deprotected by hydrogenolysis. HPLC purification gave compound **12a-2** (eluted at 14.2 min, **Table 1**) as a triethylammonium salt (2.48 mg, 63%). After removal of solvent a colorless film was obtained. ^{1}H NMR (600 MHz; D_2O; pH 10.3): δ 8.09(s, 1H), 6.32 (dd, J = 8.0, 7.0 Hz, 1H), 4.26-4.15 (m, 3H), 3.90 (dd, J_{HP} = 16.7 Hz, J_{HP} = 15.5 Hz, 1H), 2.85-2.80 (m, 1H), 2.52-2.48 (m, 1H). ^{31}P NMR (202 MHz; D_2O; pH 10.3): δ 9.21 (d, J_{PP} = 6.1 Hz, 1P), 7.19 (dd, J_{PP} = 28.1 Hz, J_{PP} = 5.9 Hz, 1P), -10.08 (d, J_{PP} = 28.2 Hz, 1P).

Synthesis of 2'-deoxy-5'-*O*-[({[(*S*)-fluoro(phosphono)methyl](hydroxy)phosphoryl}oxy)(hydroxy)-phosphoryl]guanosine, 12b-1.

Following the procedure for synthesis of **12a-1**, 13 mg (17.9 μmol, determined by UV) of compound **11b-1** was deprotected by hydrogenolysis to give compound **12b-1**. Purification was performed using the same system as for **12a-1** except the flow rate was 8 mL/min (**Table S1**). Compound **12b-1** was eluted at 14.1 min to give 8.2 mg (88%) of the product as a triethylammonium salt. After removal of solvent a colorless film was obtained. ^{1}H NMR (500 MHz; D_2O; pH 10.3): δ 8.06 (s, 1H), 6.32 (dd, J = 6.4, 7.8 Hz, 1H), 4.83 (m, 1H), 4.77 (m, 1H), 4.25 (m, 1H), 4.21-4.12 (m, 2H), 2.80 (m, 1H), 2.49 (ddd, J = 3.5, 6.4, 14.2 Hz, 1H). ^{31}P NMR (202 MHz; D_2O; pH 10.3): δ 6.80 (dd, J_{PP} = 14.3 Hz, J_{PF} = 55.7 Hz, 1P), 4.50 (ddd, J_{PP} = 14.3 Hz, J_{PP} = 28.6 Hz, J_{PF} = 65.2 Hz, 1P), -11.07 (d, J_{PP} = 30.2 Hz, 1P). ^{19}F NMR (470 MHz;

D_2O; pH 10.3): δ -216.24 (ddd, J_{FH} = 45.3 Hz, J_{FP} = 56.0 Hz, J_{FP} = 65.6 Hz). Lit[6]: ^{19}F (D_2O; pH 10) -218.61 (calculated from ^{19}F NMR of ~1:1 synthetic mixture by NMR simulation).

Synthesis of 2'-deoxy-5'-*O*-[({[(*R*)-fluoro(phosphono)methyl](hydroxy)phosphoryl}oxy)(hydroxy)-phosphoryl]guanosine, 12b-2.

Following the procedure for synthesis of **12a-1**, 15.2 mg (20.9 μmol, determined by UV) of compound **11b-1** was deprotected by hydrogenolysis to give compound **12b-2**. Purification was performed using the same system as for **12a-1** except the flow rate was 8 mL/min (**Table S1**). Compound **12b-2** eluted at 14.5 min to give 10 mg (92%) of the product as a triethylammonium salt. After removal of solvent a colorless film was obtained. ^1H NMR (500 MHz; D_2O; pH 10.5): δ 8.09 (s, 1H), 6.31 (dd, J = 6.4, 7.8 Hz, 1H), 4.83 (dt, J_{HP} = 12.7 Hz, J_{HF} = 45.5 Hz, 1H), 4.80 (m, 1H), 4.24 (m, 1H), 4.21-4.13 (m, 2H), 2.83 (m, 1H), 2.49 (ddd, J = 3.4, 6.4, 13.7 Hz, 1H). ^{31}P NMR (202 MHz; D_2O; pH 10.5): δ 6.85 (dd, J_{PP} = 14.3 Hz, J_{PF} = 55.6 Hz, 1P), 4.51 (ddd, J_{PP} = 14.3 Hz, J_{PP} = 30.2 Hz, J_{PF} = 65.2 Hz, 1P), -11.07 (d, J_{PP} = 30.2 Hz, 1P). ^{19}F NMR (470 MHz; D_2O; pH 10.5): δ -216.30 (ddd, J_{FH} = 46.5 Hz, J_{FP} = 56.1 Hz, J_{FP} = 66.8 Hz). HPLC: T_{ret} = 14.5 min. Lit[6]: ^{19}F (D_2O; pH 10) -218.67 (calculated from ^{19}F NMR of ~1:1 synthetic mixture by NMR simulation).

Crystallization of the pol *β* substrate complexes.

Binary complex crystals of Human pol β with dideoxy-terminated primer in a 1-nucleotide gapped DNA were grown as previously described.[7] The sequence of the template strand (16-mer) was 5′- CCG ACC GCG CAT CAG C- 3′. The primer strand (9-mer) sequence was 5′- GCT GAT GCG -3′. The downstream oligonucleotide (5-mer) was phosphorylated, and the sequence was 5′- GTC GG - 3′. The soaking of binary complex crystals with artificial mother liquor (50 mM imidazole, pH 7.5, 20% PEG3350, 90 mM sodium acetate, 100 mM $MgCl_2$ with 2.5 mM of **12a-1**, **12a-2**, **12b-1** or **12b-2**, and 12% ethylene glycol) resulted in ternary complex crystals. Diffraction quality data were then collected for the ternary complex crystal 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.[8] The ternary complex structure was solved by molecular replacement using 2PXI[7] as a reference model. The structure was refined using PHENIX and manual model building using O. The crystallographic statistics are reported in **Table S2**. **Fig. 3** in the main paper was prepared using Chimera.[9]

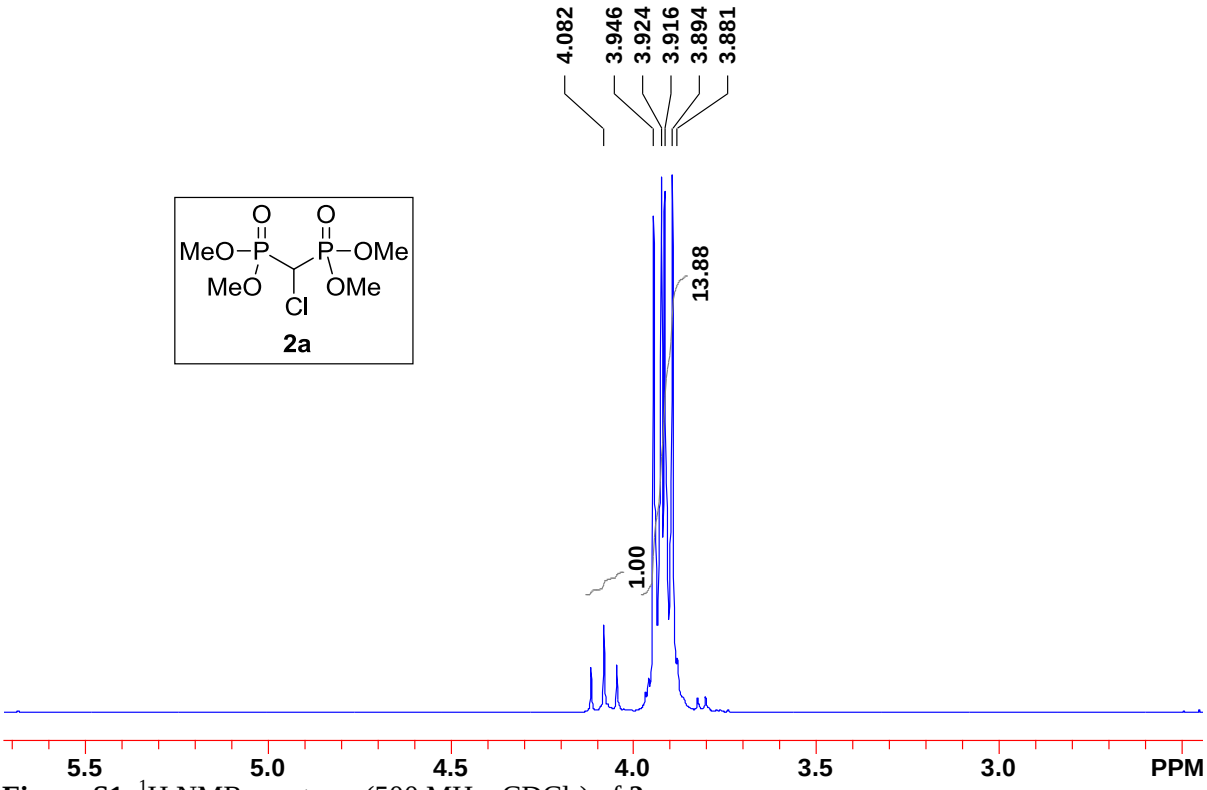

Figure S1. ^{1}H NMR spectrum (500 MHz; $CDCl_3$) of **2a**.

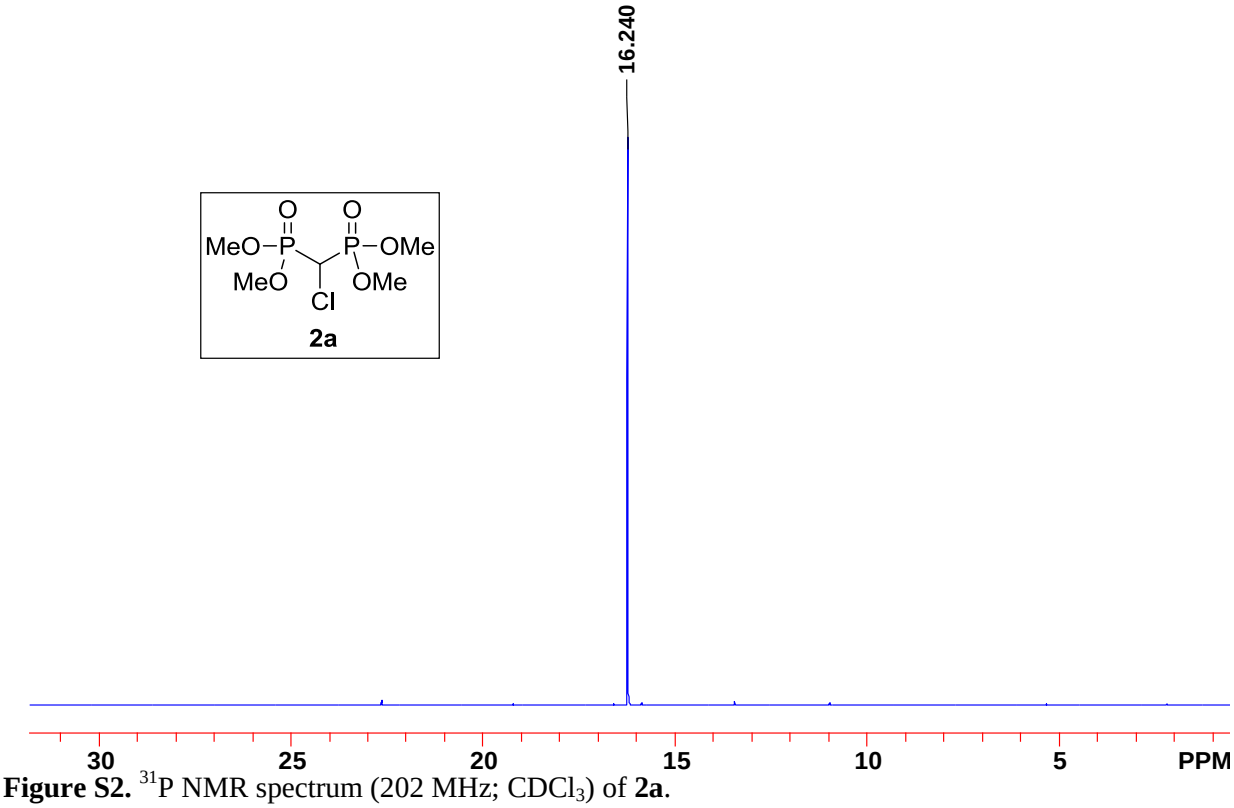

Figure S2. ^{31}P NMR spectrum (202 MHz; $CDCl_3$) of **2a**.

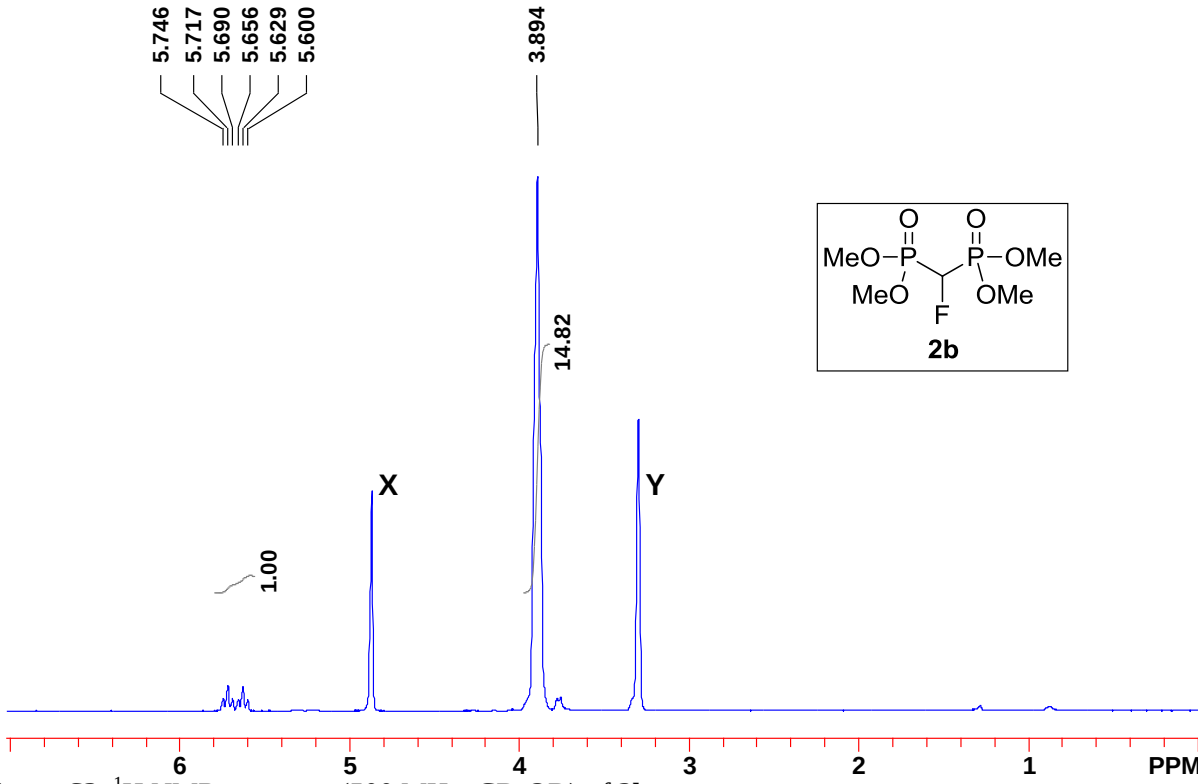

Figure S3. ^{1}H NMR spectrum (500 MHz; CD_3OD) of **2b**.
X = HDO; Y = CHD_2OD

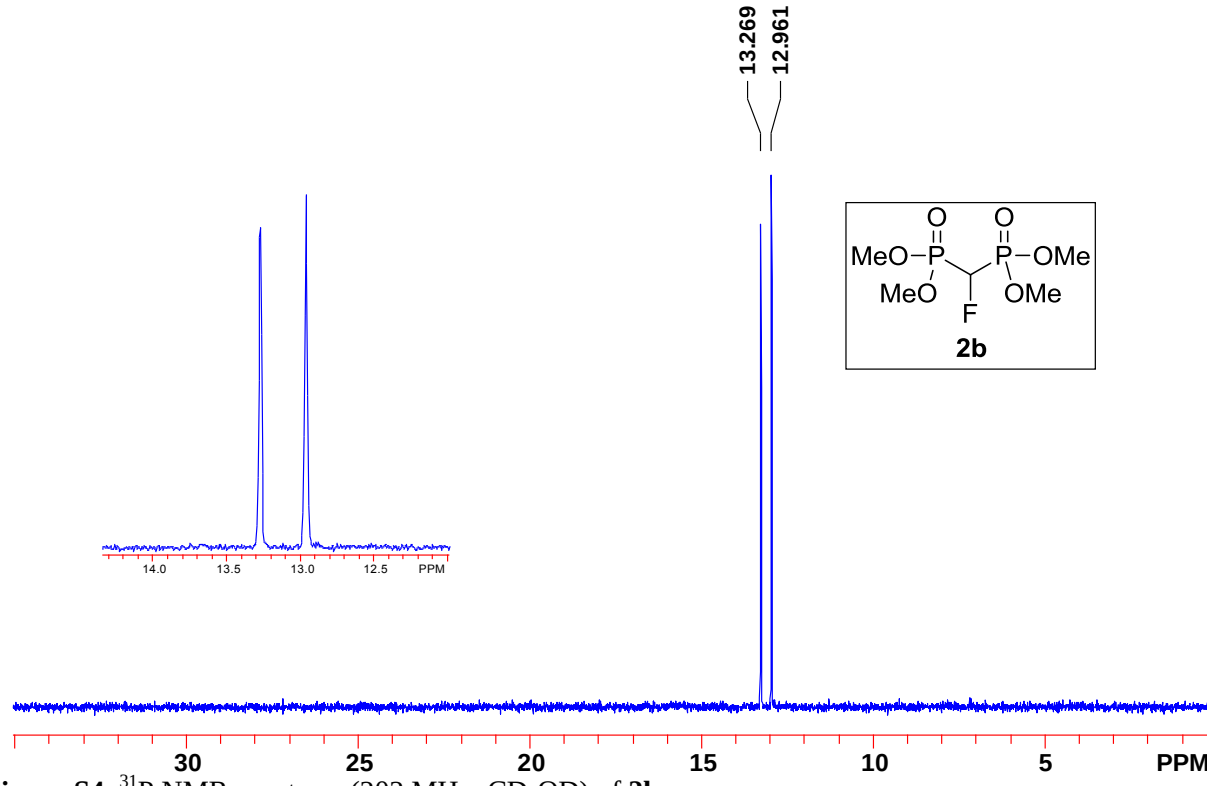

Figure S4. ^{31}P NMR spectrum (202 MHz; CD_3OD) of **2b**.

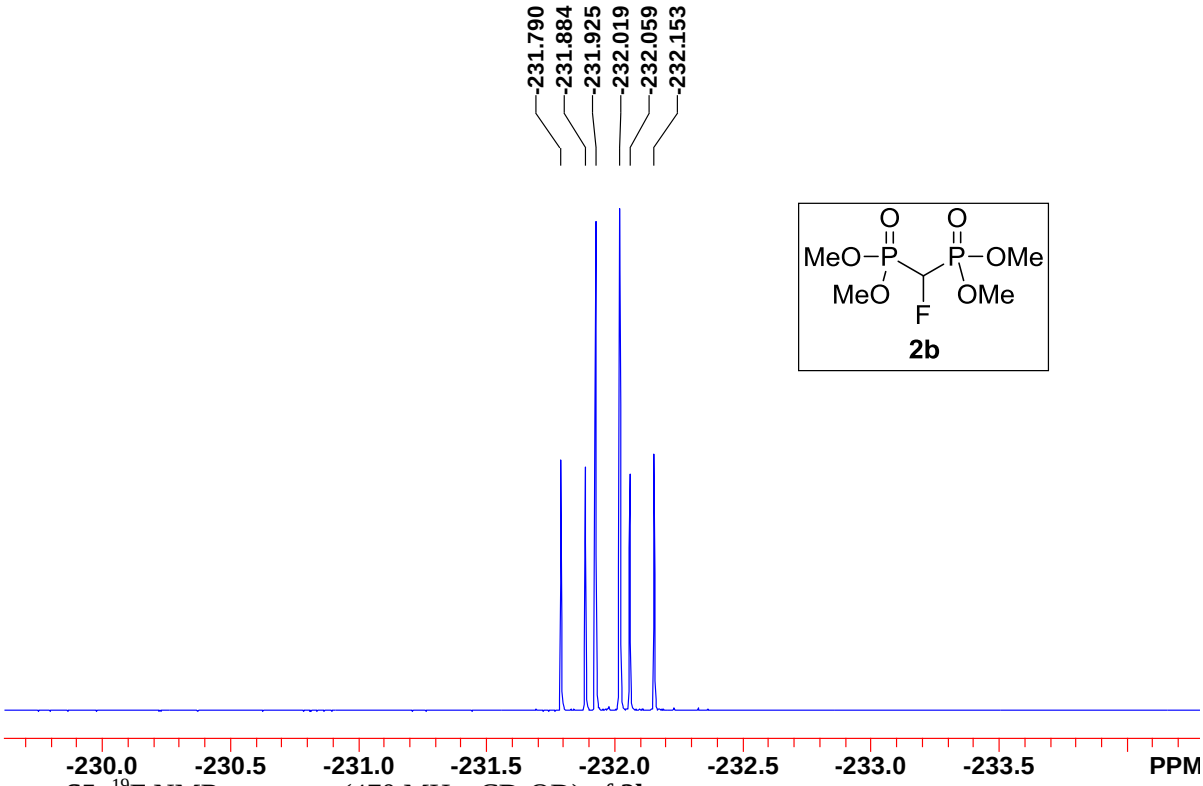

Figure S5. ^{19}F NMR spectrum (470 MHz; CD_3OD) of **2b**.

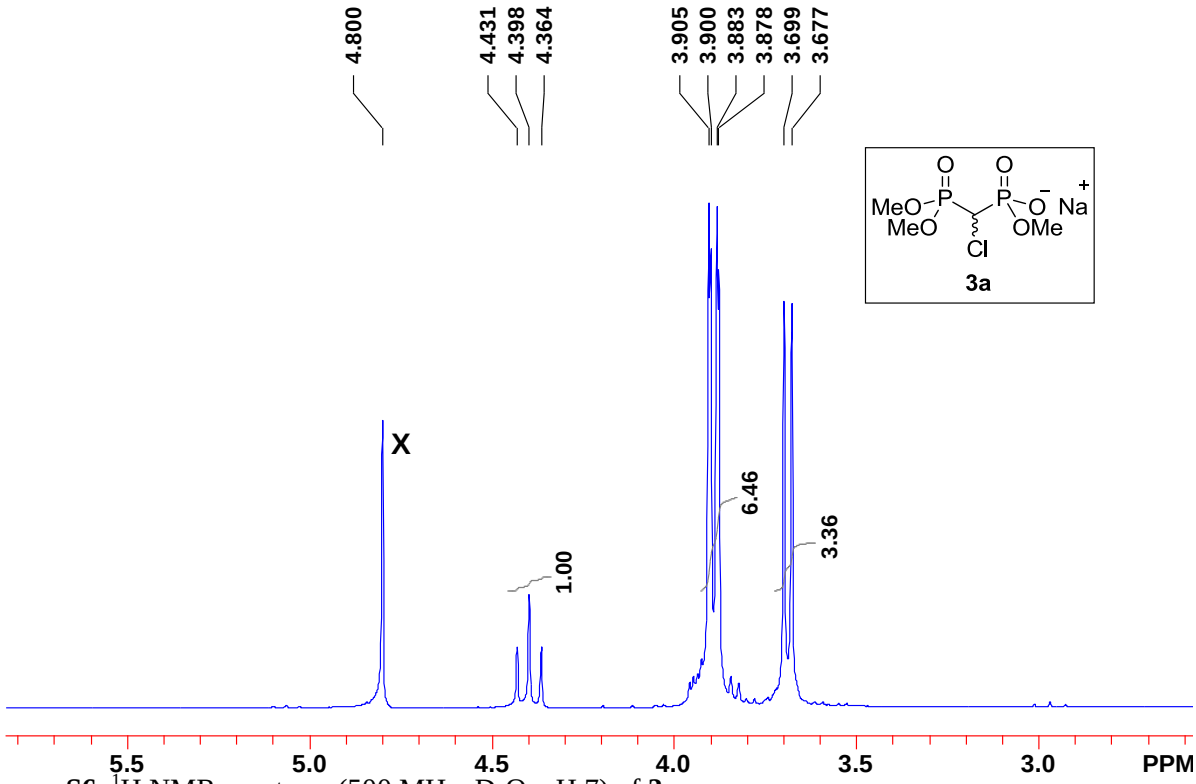

Figure S6. ^{1}H NMR spectrum (500 MHz; D_2O, pH 7) of **3a**.
X = HDO

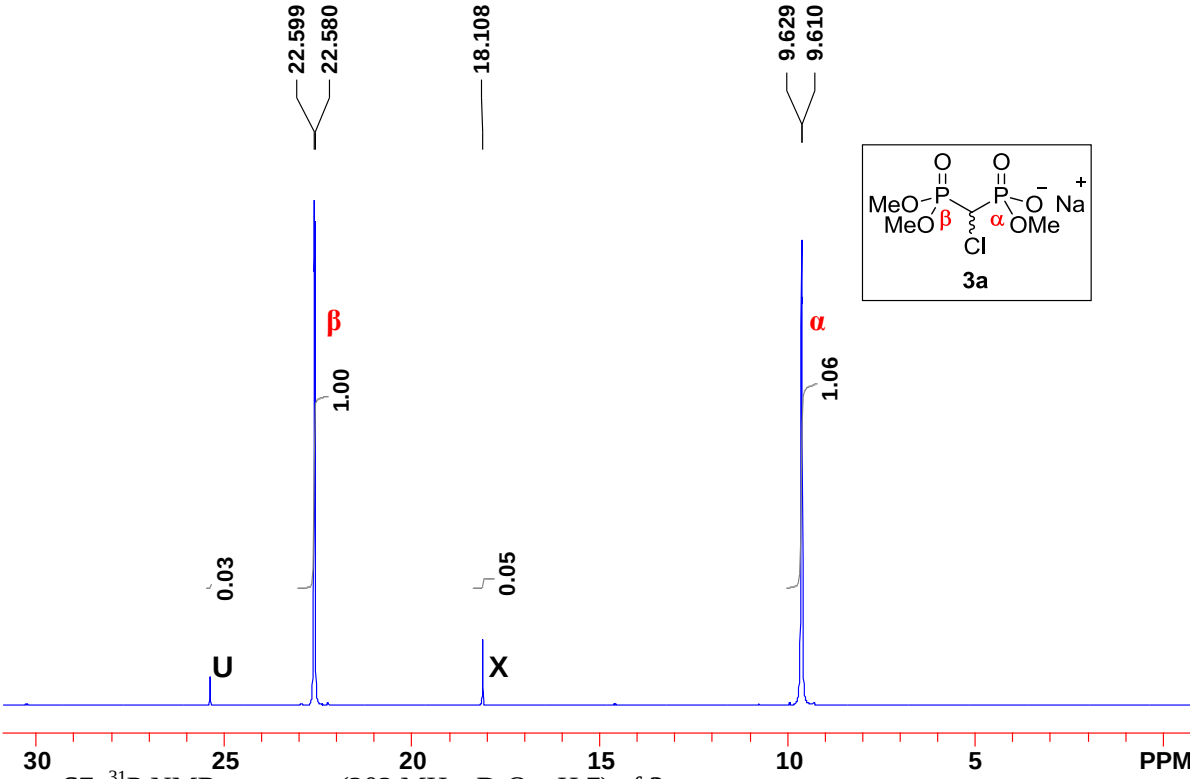

Figure S7. ^{31}P NMR spectrum (202 MHz; D_2O, pH 7) of **3a**.
U = unidentified byproduct; X = **2a**
Assignment of phosphorus signals is based on H-coupled ^{31}P-NMR. (Spectrum not shown)

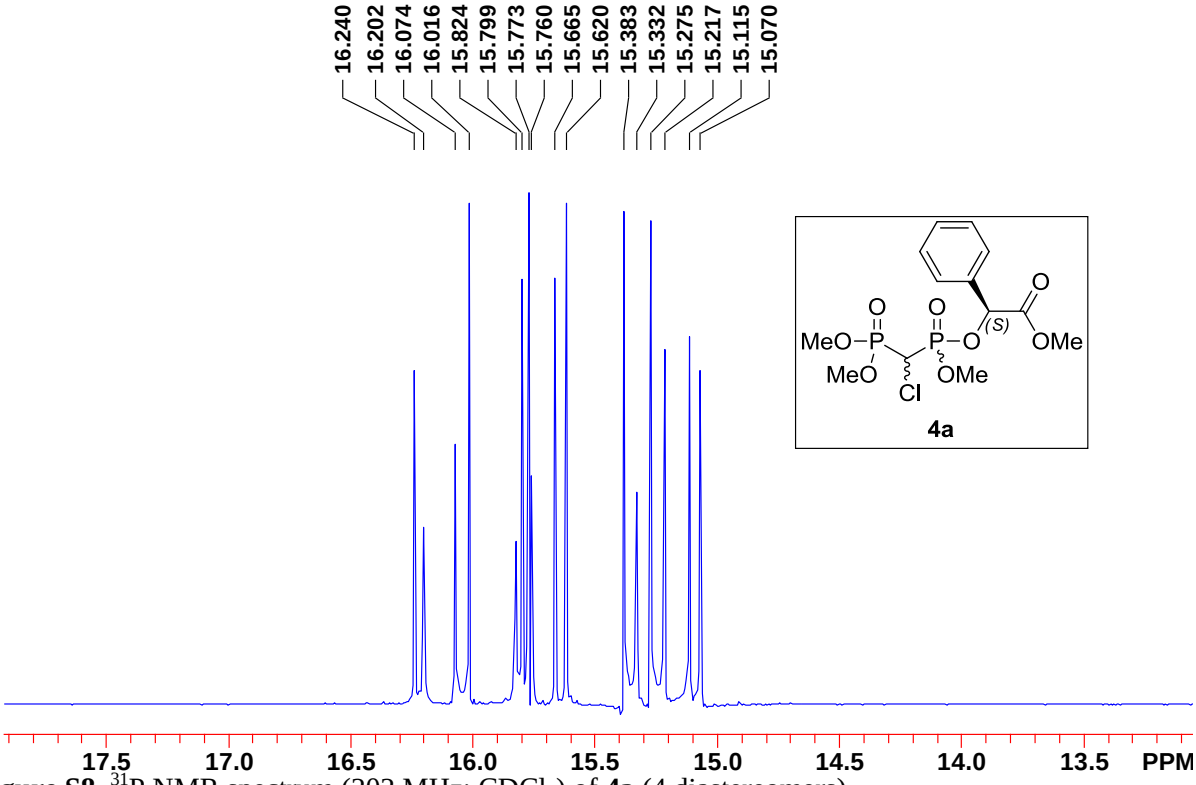

Figure S8. ^{31}P NMR spectrum (202 MHz; $CDCl_3$) of **4a** (4 diastereomers).

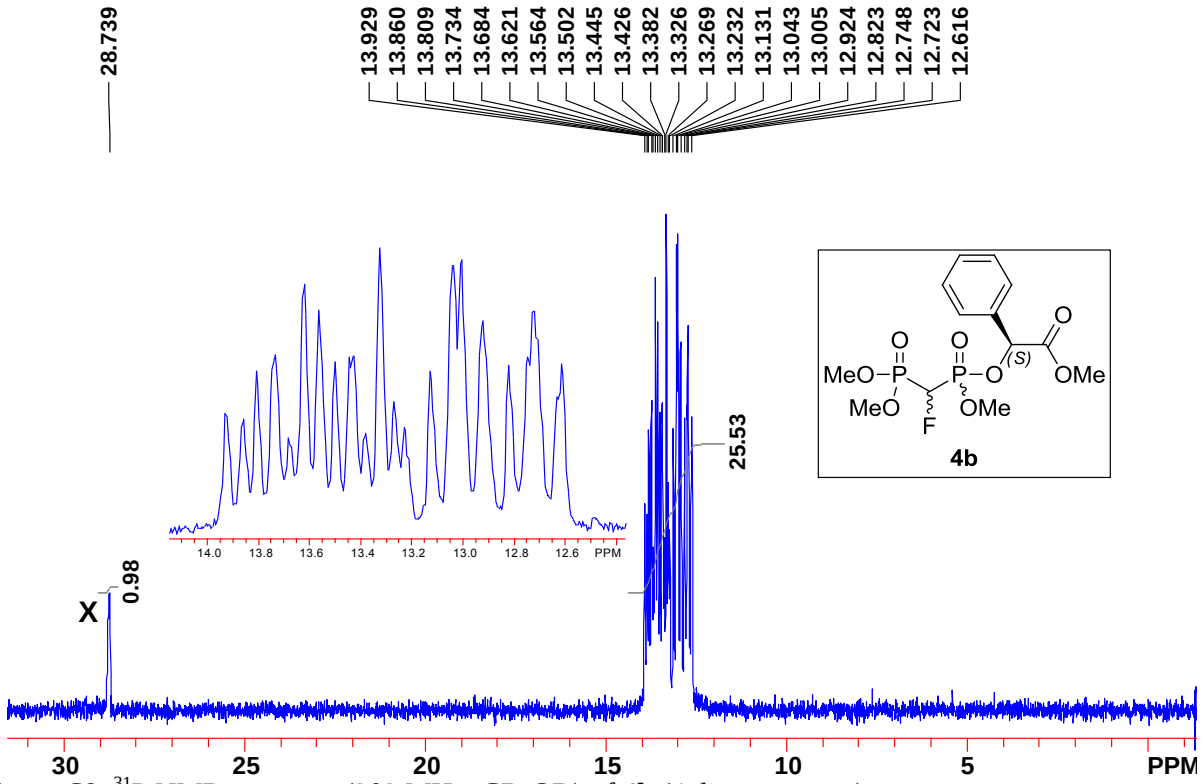

Figure S9. ^{31}P NMR spectrum (202 MHz; CD_3OD) of **4b** (4 diastereomers).
X = PPh_3O

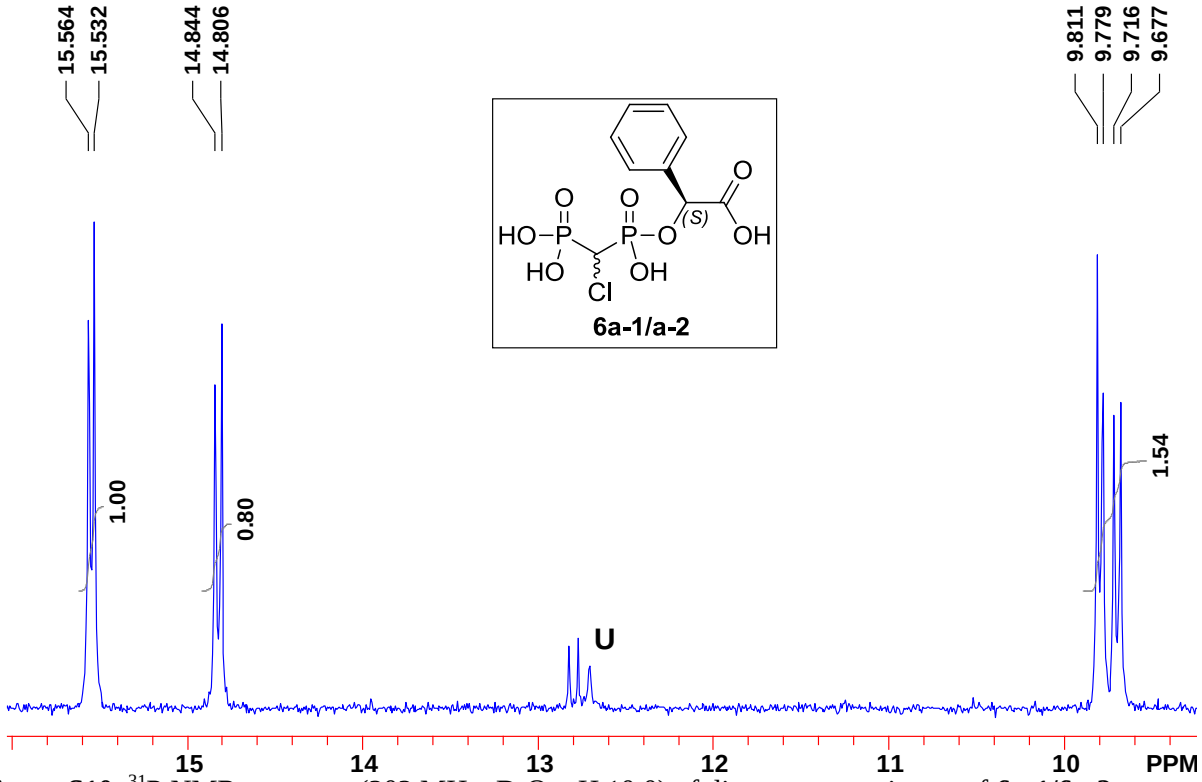

Figure S10. ^{31}P NMR spectrum (202 MHz; D_2O; pH 10.0) of diastereomer mixture of **6a-1/6a-2**.
U = unidentified byproducts

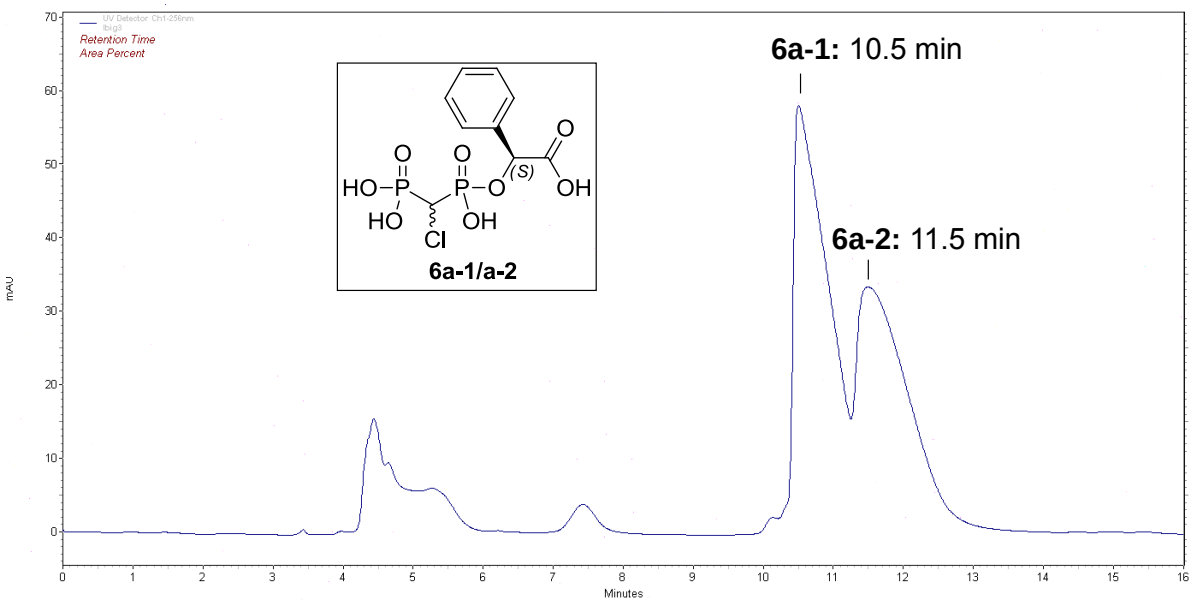

Figure S11. Preparative HPLC separation of diastereomers **6a-1** and **6a-2**.
For conditions see **Table S1**.

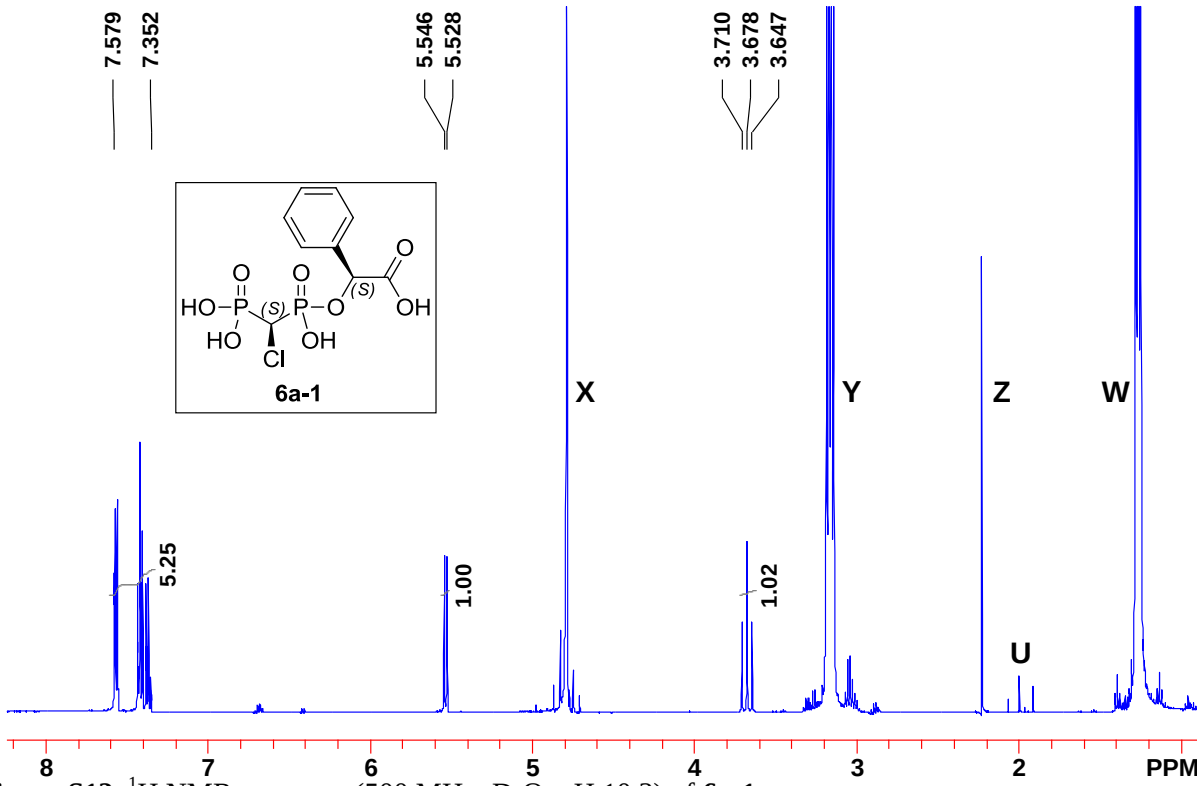

Figure S12. ^{1}H NMR spectrum (500 MHz; D_2O; pH 10.3) of **6a-1**.
U = unidentified impurities; X = HDO; Y, W = Et_3N; Z = acetone.

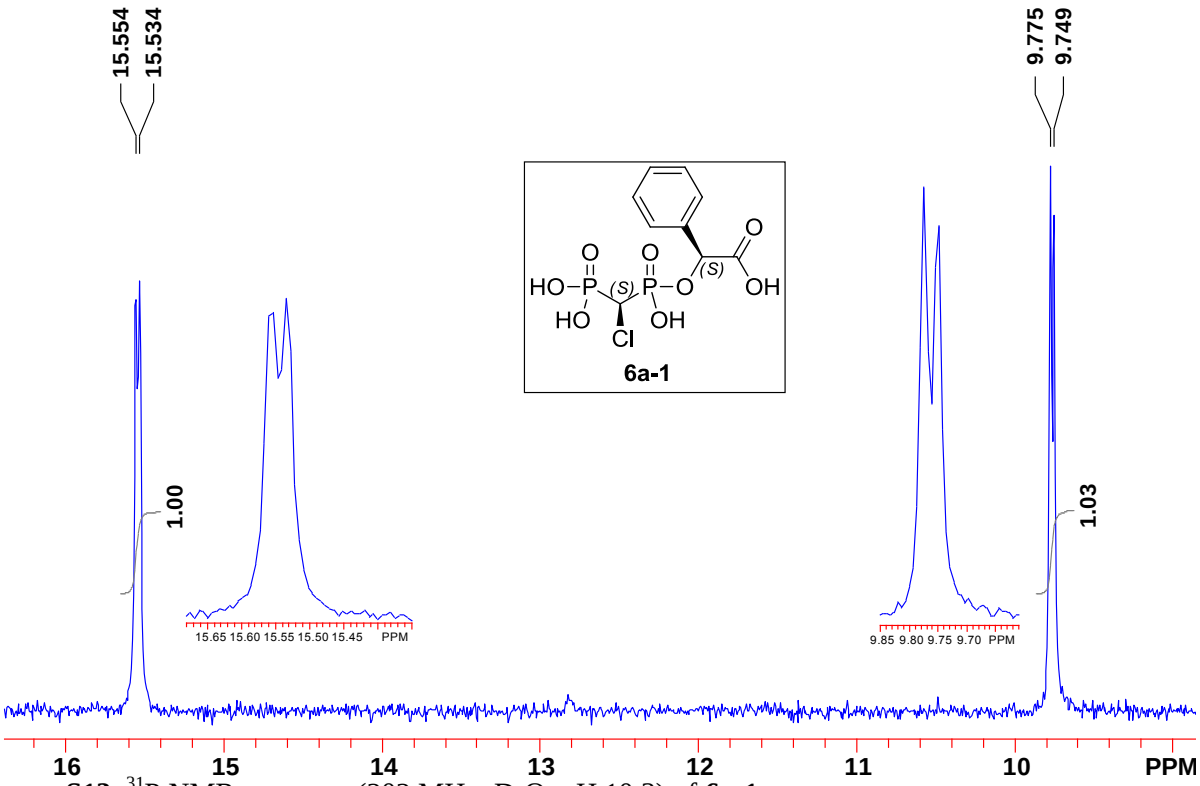

Figure S13. ^{31}P NMR spectrum (202 MHz; D_2O; pH 10.3) of **6a-1**.

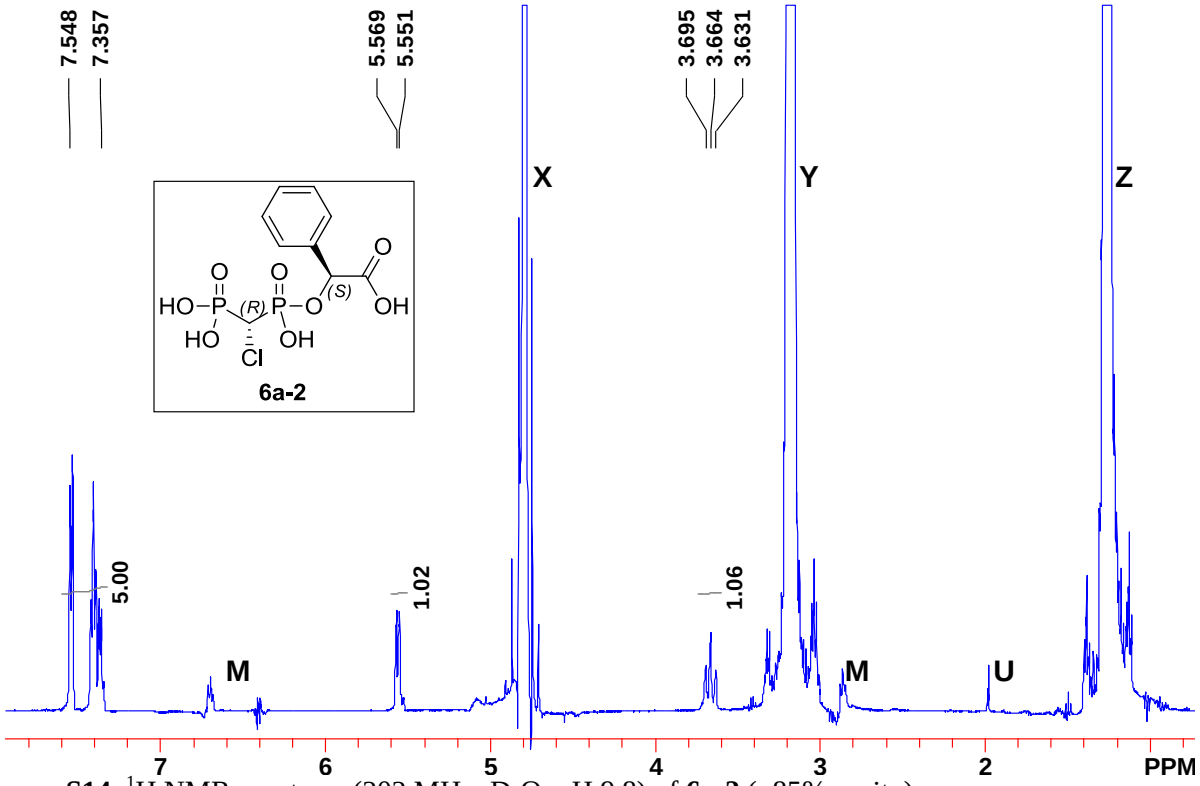

Figure S14. ^{1}H NMR spectrum (202 MHz; D_2O; pH 9.8) of **6a-2** (~85% purity).
There is some impurity of **6a-1**.
M = machine artifact; U = unidentified impurities; X = HDO; Y, Z = Et_3N.

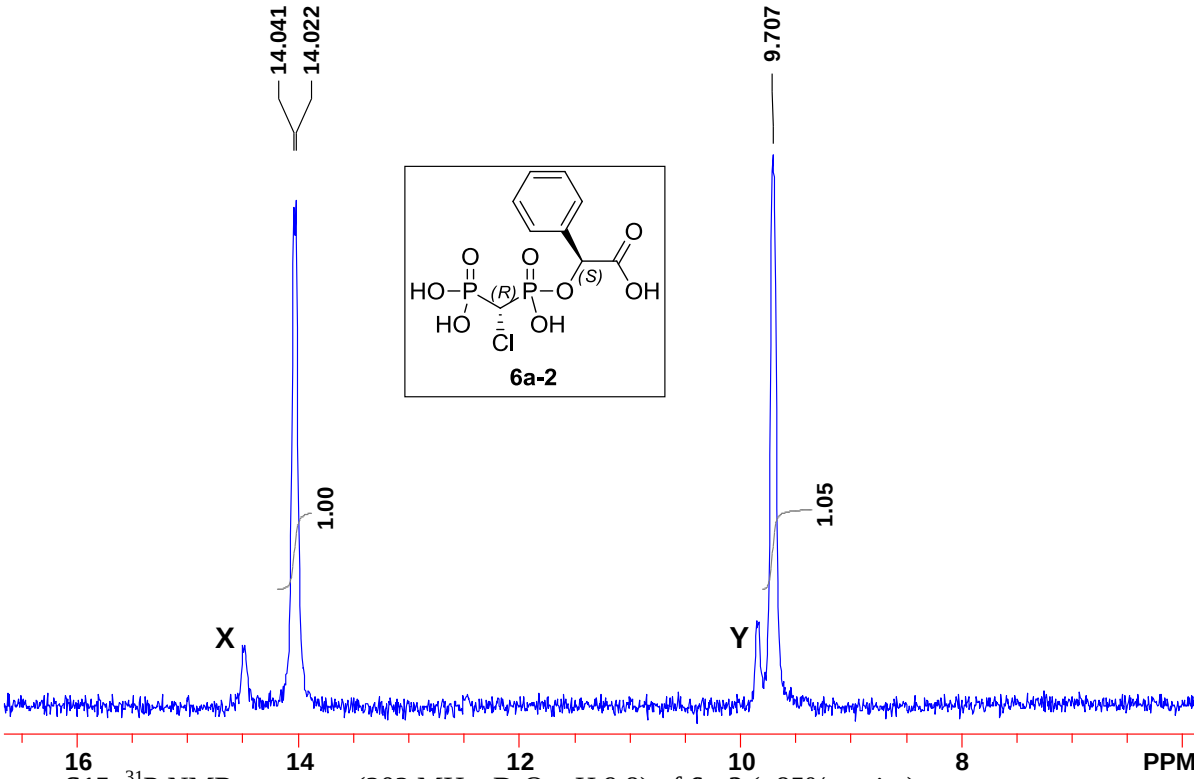

Figure S15. ^{31}P NMR spectrum (202 MHz; D_2O; pH 9.8) of **6a-2** (~85% purity). X, Y = **6a-1**.

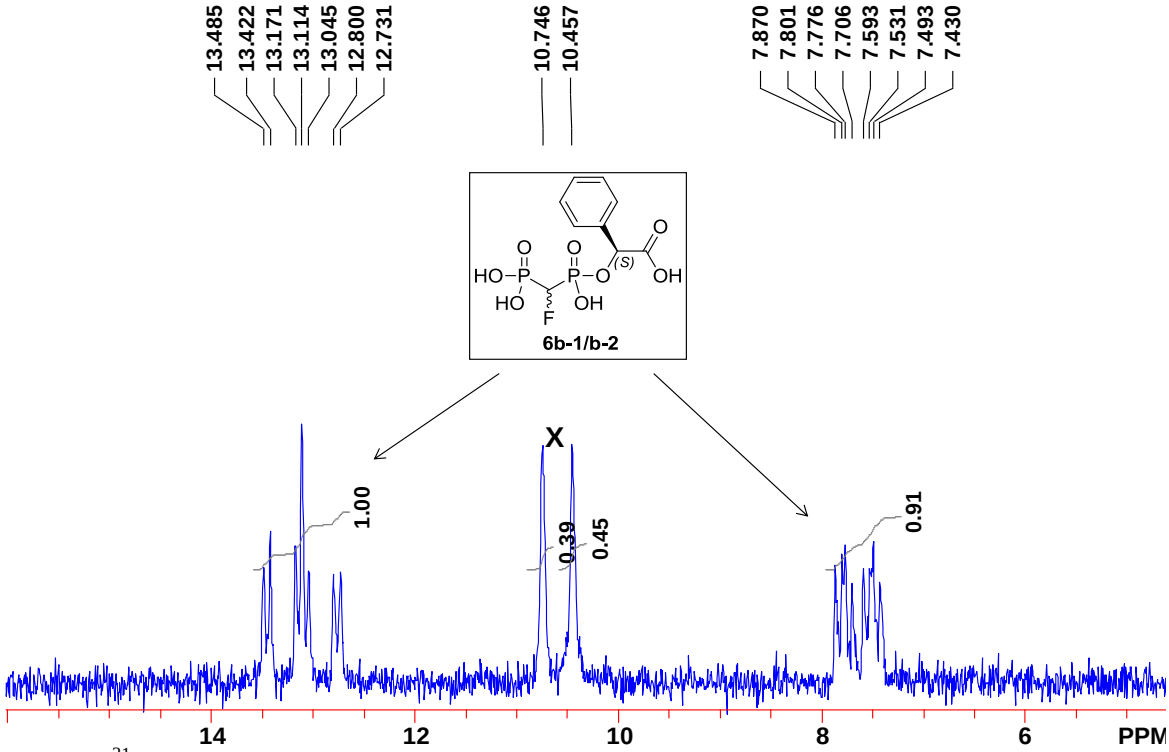

Figure S16. ^{31}P NMR spectrum (202 MHz; CD_3OD) of diastereomer mixture of **6b-1/6b-2** in reaction mixture. X = excess **1b**.

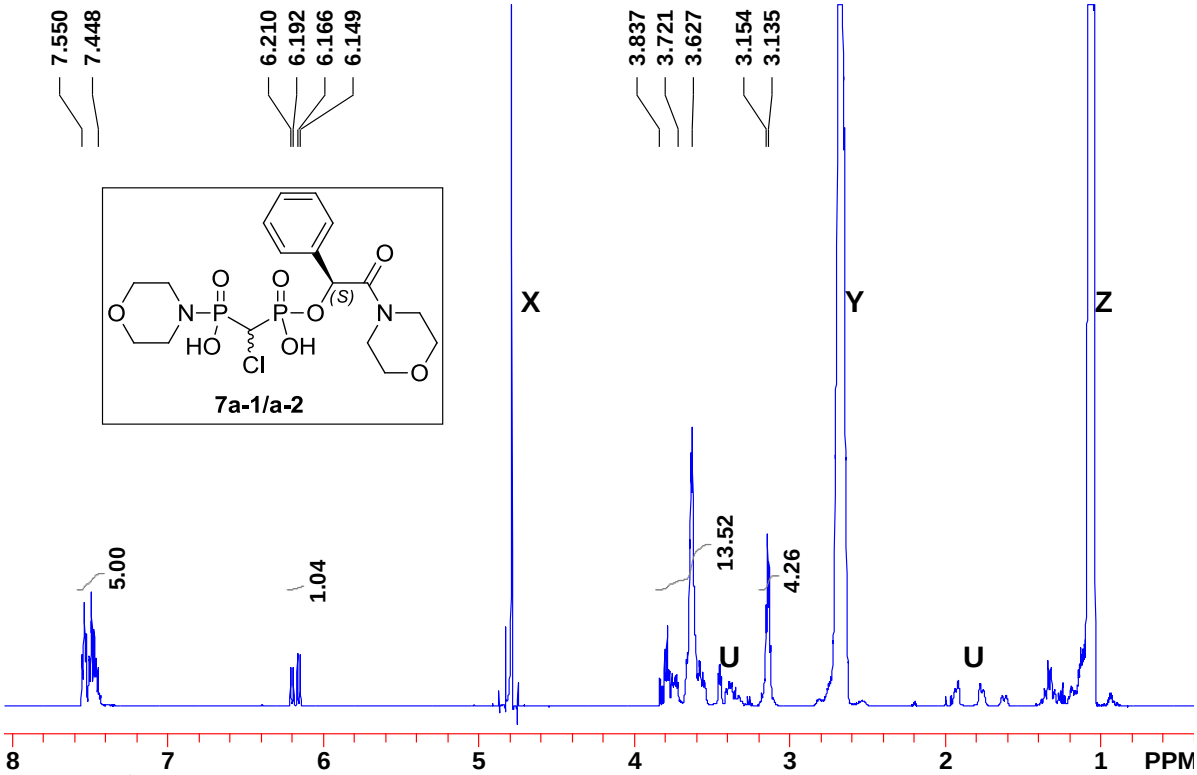

Figure S17. ^{1}H NMR spectrum (500 MHz; D_2O; pH 9.8) of diastereomer mixture of **7a-1/7a-2**. U = unidentified impurities; X = HDO; Y, Z = Et_3N.

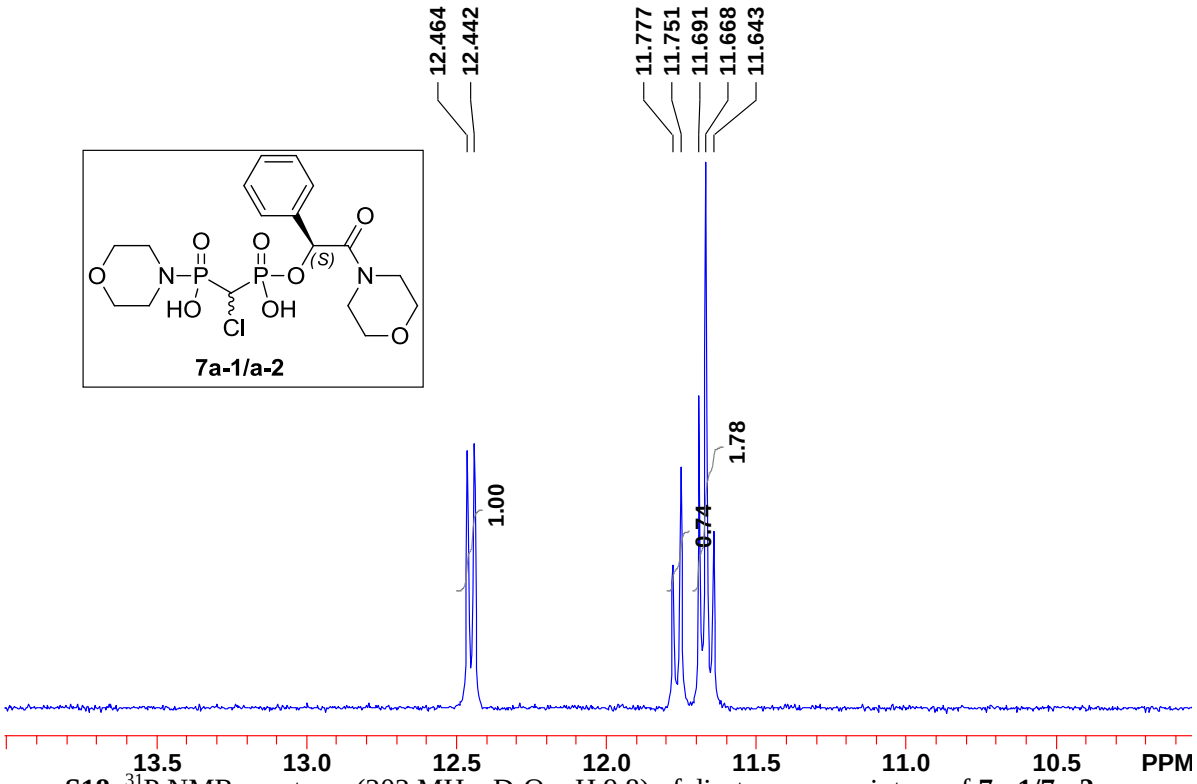

Figure S18. ^{31}P NMR spectrum (202 MHz; D_2O; pH 9.8) of diastereomer mixture of **7a-1/7a-2**.

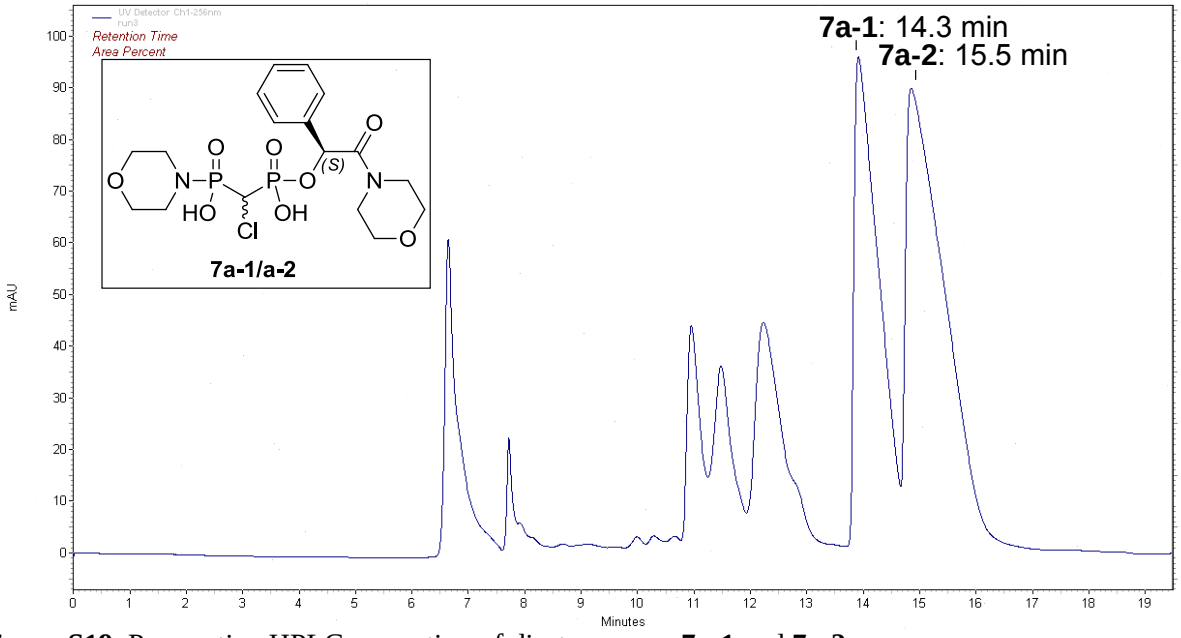

Figure S19. Preparative HPLC separation of diastereomers **7a-1** and **7a-2**.
For conditions see **Table S1**.

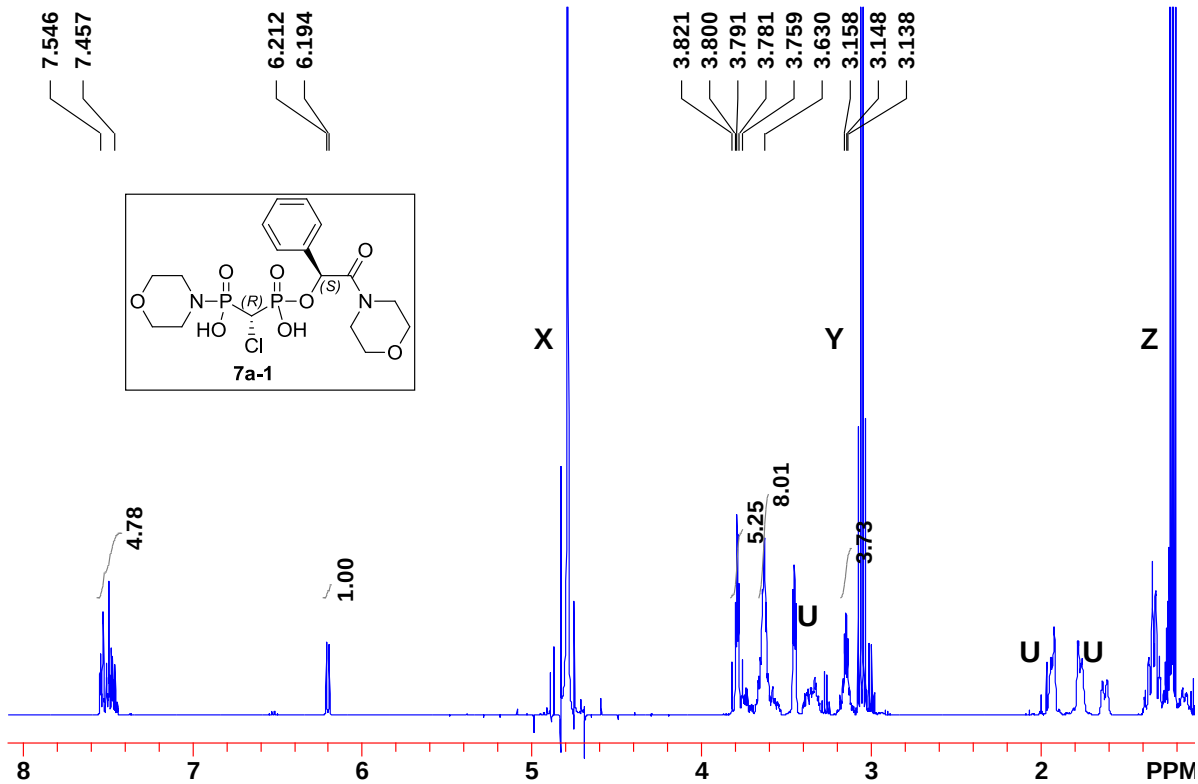

Figure S20. ^{1}H NMR spectrum (500 MHz; D_2O; pH 9.8) of **7a-1**.
U = unidentified impurities; X = HDO; Y, Z = Et_3N.

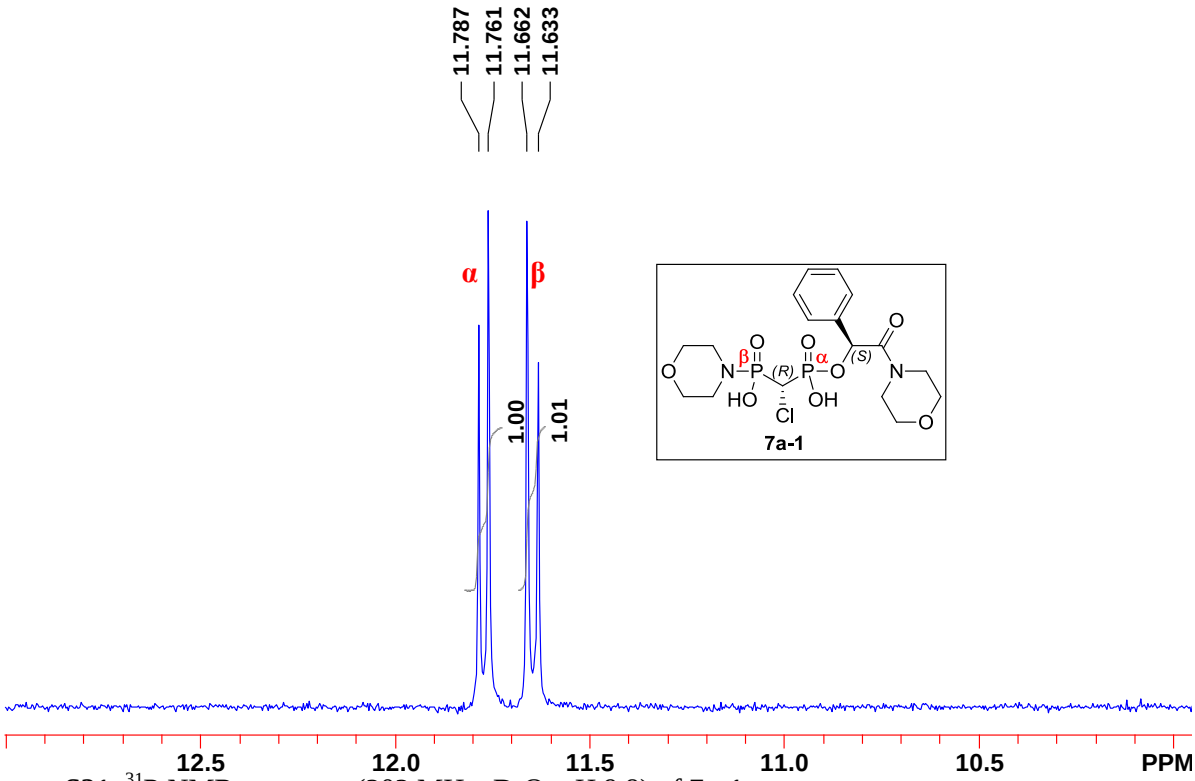

Figure S21. ^{31}P NMR spectrum (202 MHz; D_2O; pH 9.8) of **7a-1**.
Assignment of phosphorus signals is based on ^{1}H-^{31}P gHMBC spectrum.

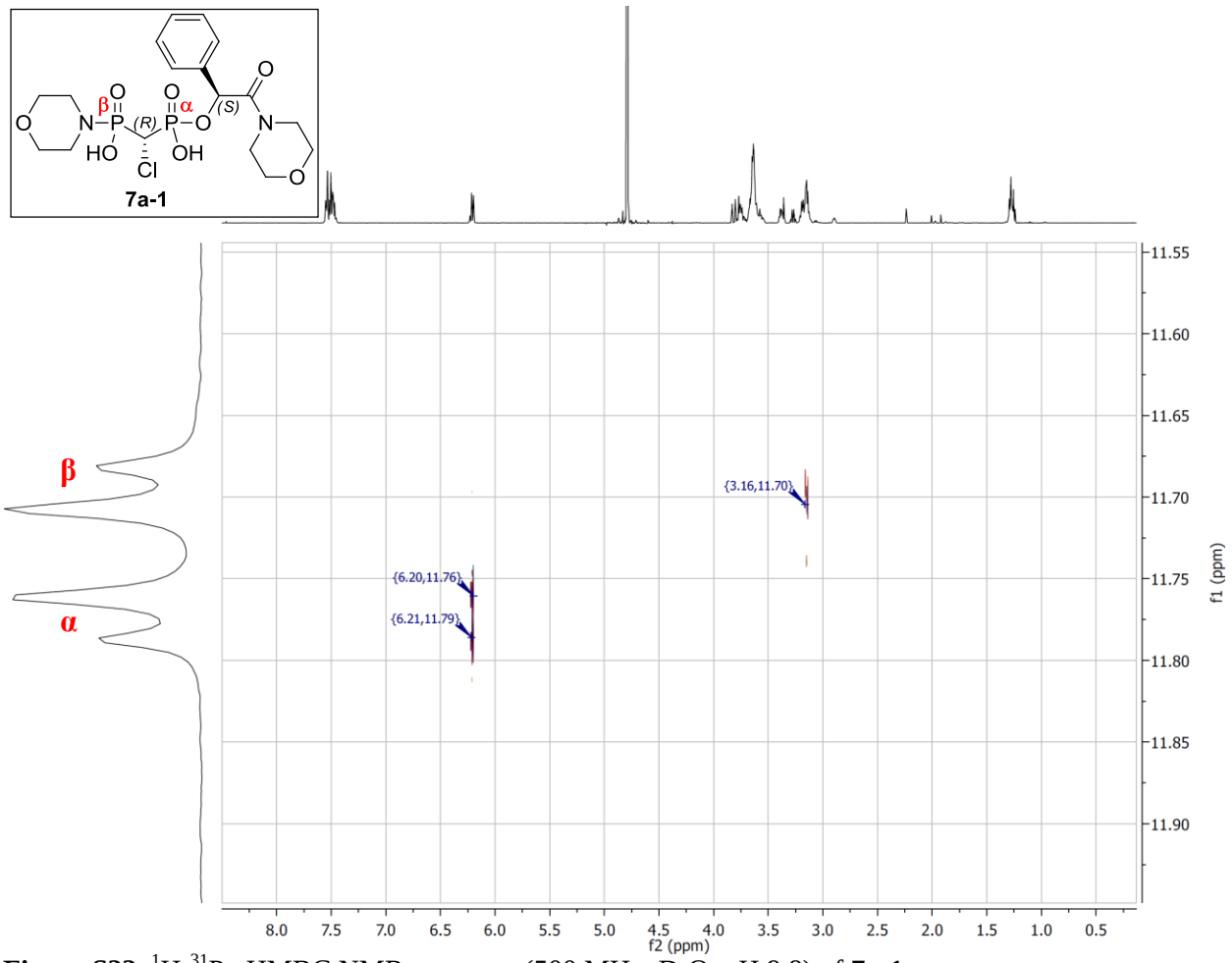

Figure S22. ^{1}H-^{31}P gHMBC NMR spectrum (500 MHz; D_2O; pH 9.8) of **7a-1**.

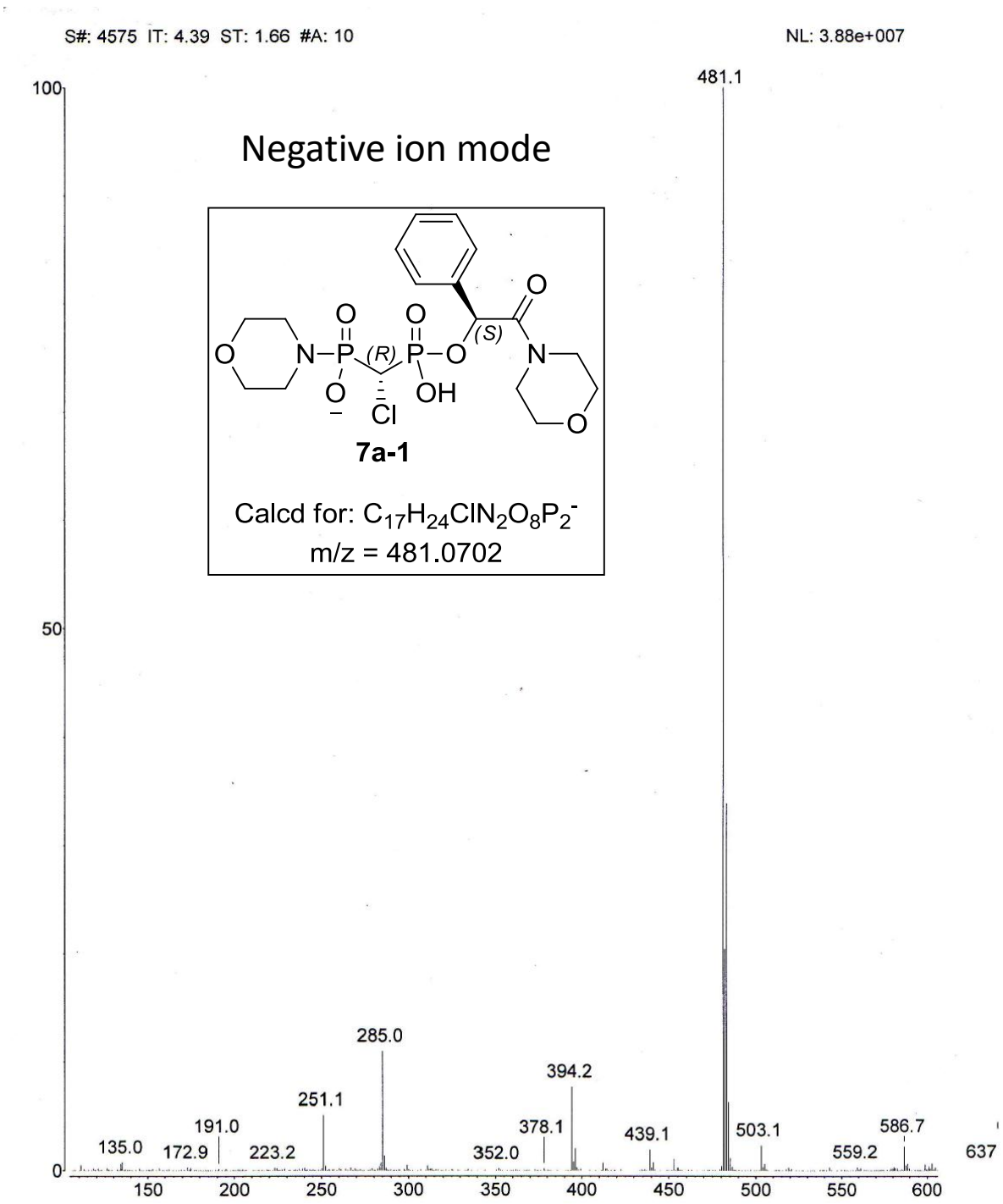

Figure S23. MS (ESI) $[M-1]^-$ spectrum of **7a-1**.

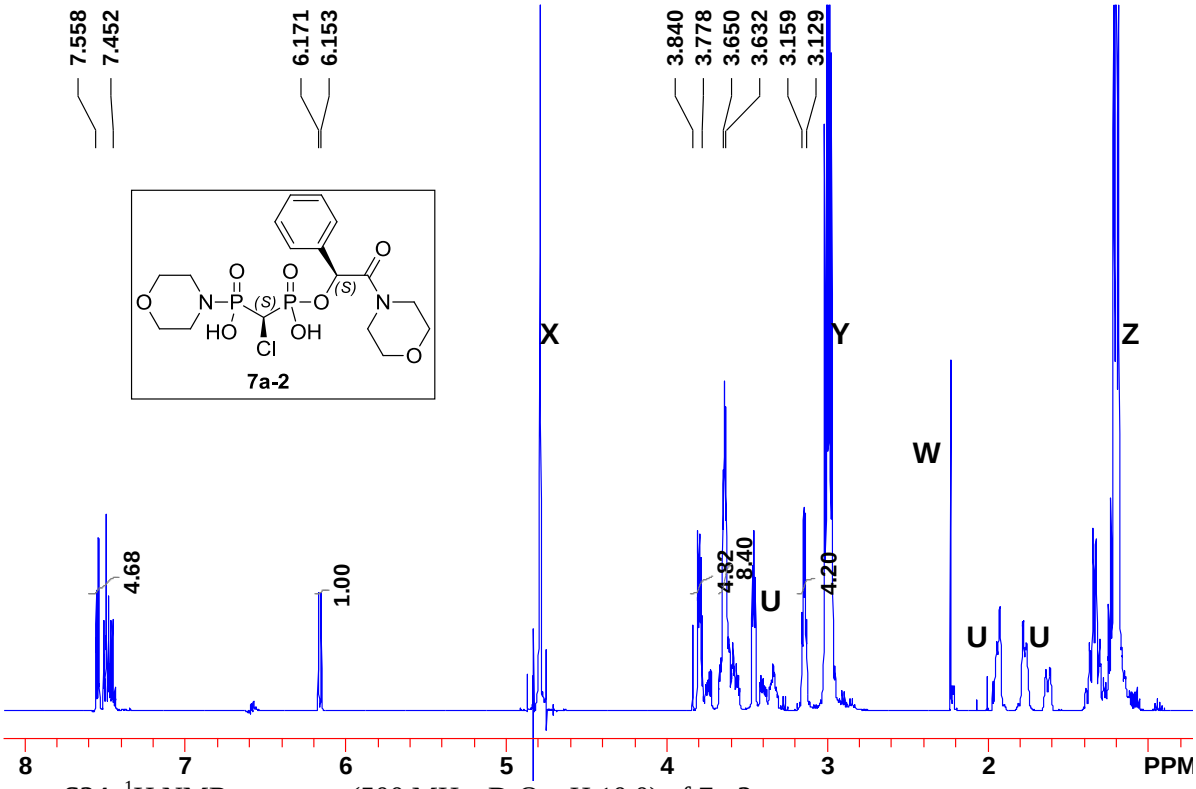

Figure S24. ^{1}H NMR spectrum (500 MHz; D_2O; pH 10.0) of **7a-2**.
U = unidentified impurities; W = acetone; X = HDO; Y, Z = Et_3N.

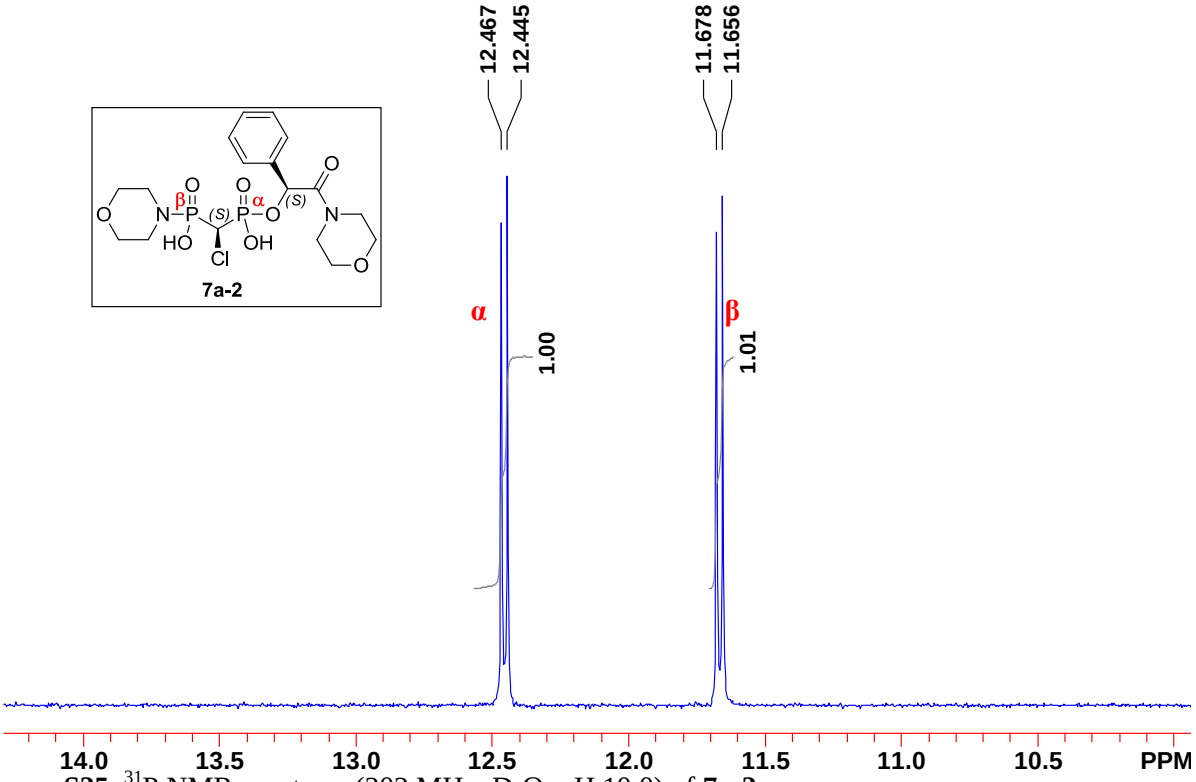

Figure S25. ^{31}P NMR spectrum (202 MHz; D_2O; pH 10.0) of **7a-2**.
Assignment of phosphorus signals is based on ^{1}H-^{31}P gHMBC spectrum.

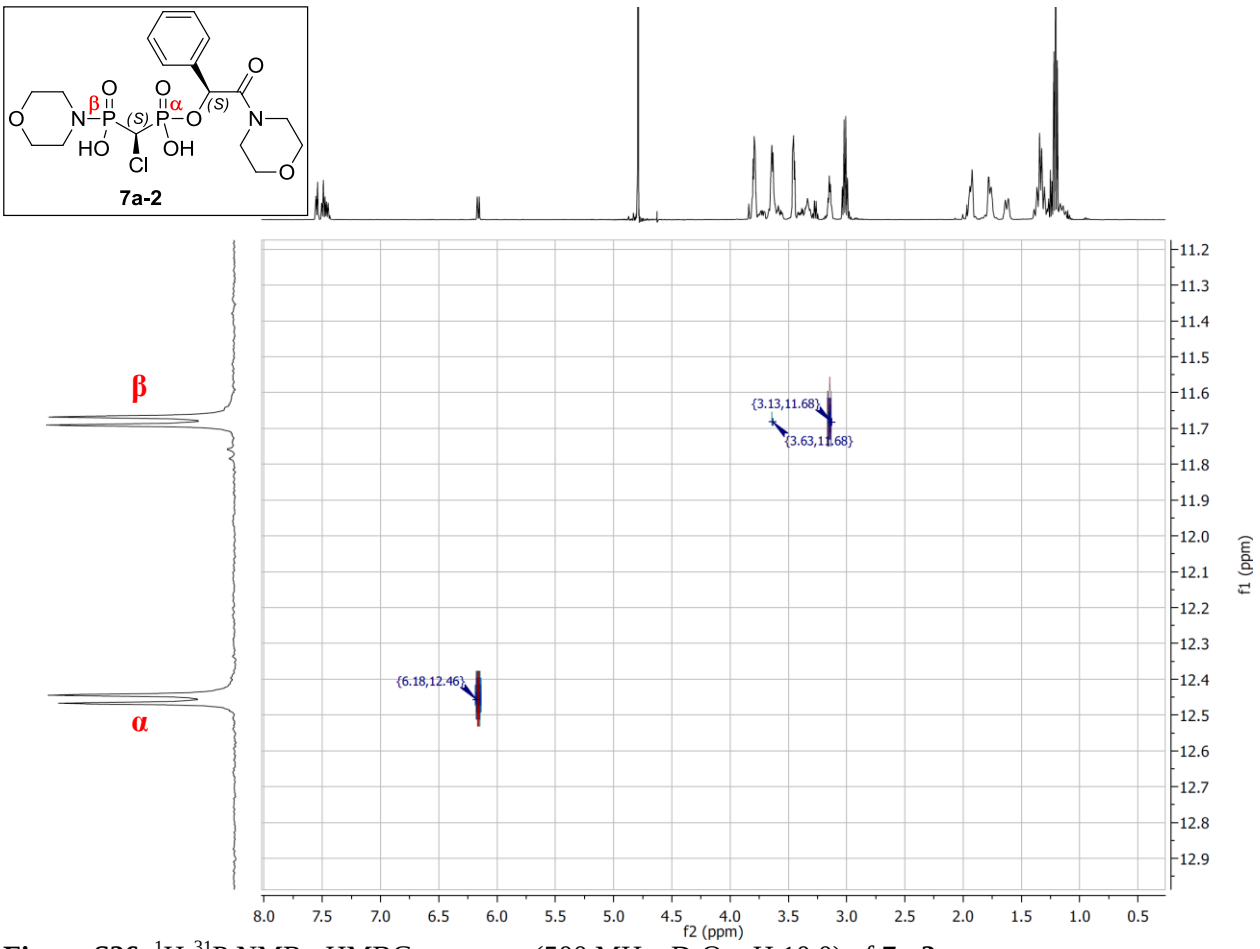

Figure S26. ^{1}H-^{31}P NMR gHMBC spectrum (500 MHz; D_2O; pH 10.0) of **7a-2**.

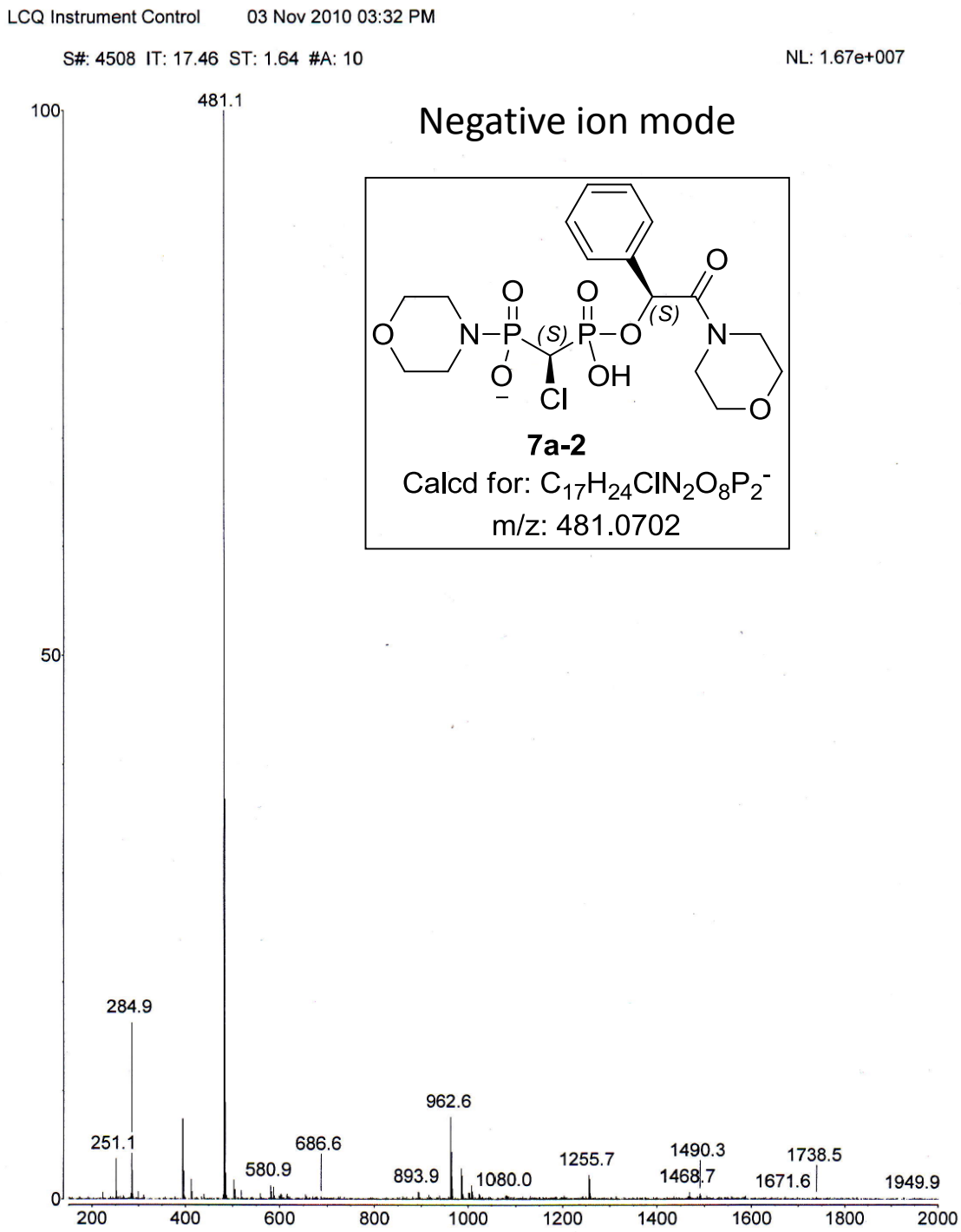

Figure S27. MS (ESI) $[M-1]^-$ spectrum of **7a-2**.

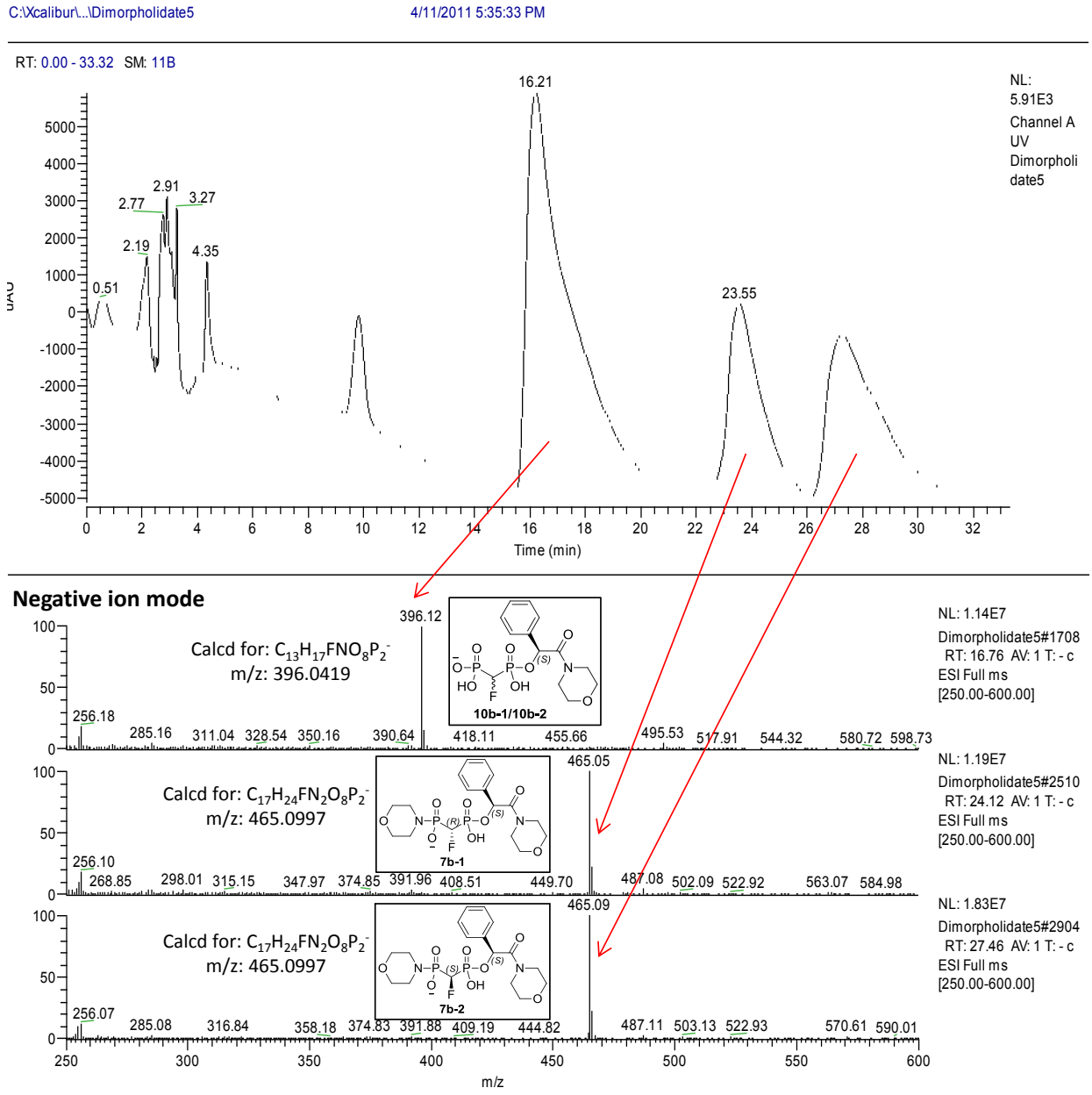

Figure S28. Analytical LC-MS (ESI) $[M-1]^-$ spectra of incomplete reaction mixture of dimorpholidation, compounds **7b-1** and **7b-2**.

For analytical HPLC conditions see **Table 1**.

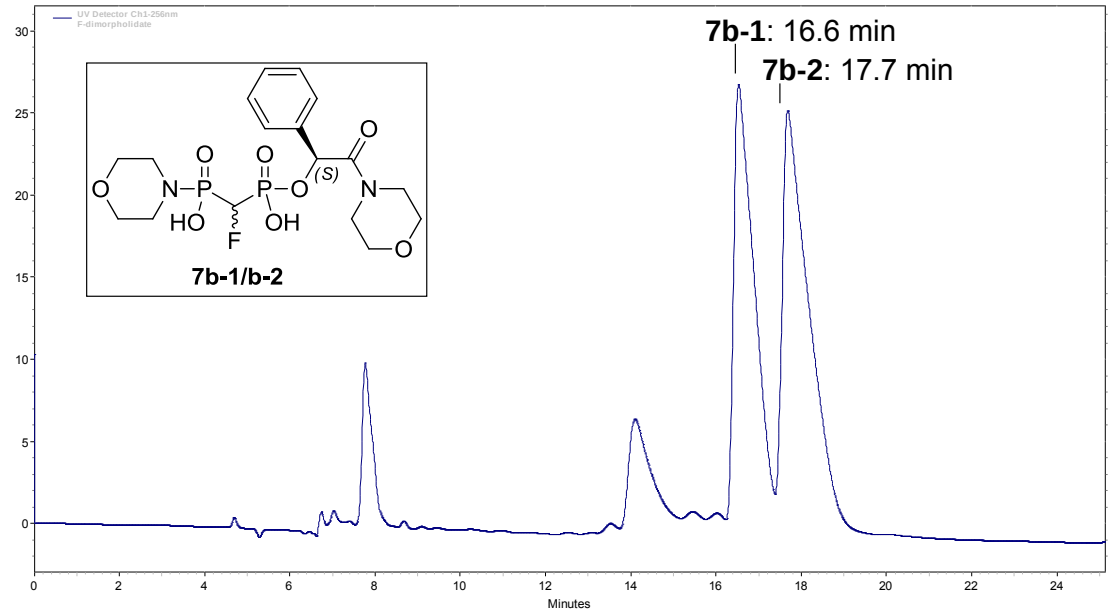

Figure S29. Preparative HPLC separation of diastereomers **7b-1** and **7b-2**.
For conditions see **Table S1**.

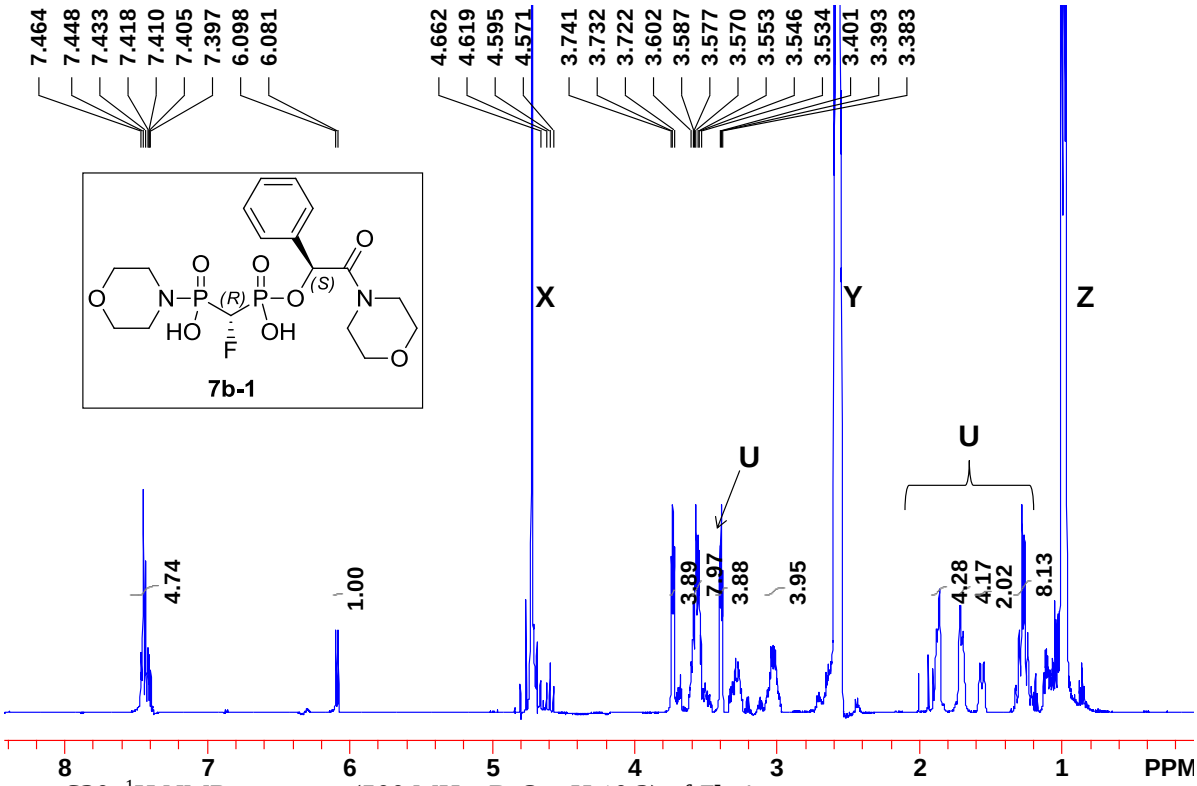

Figure S30. ^{1}H NMR spectrum (500 MHz; D_2O; pH 10.3) of **7b-1**.
X = HDO; Y, Z = Et_3N; U = Unidentified peaks.

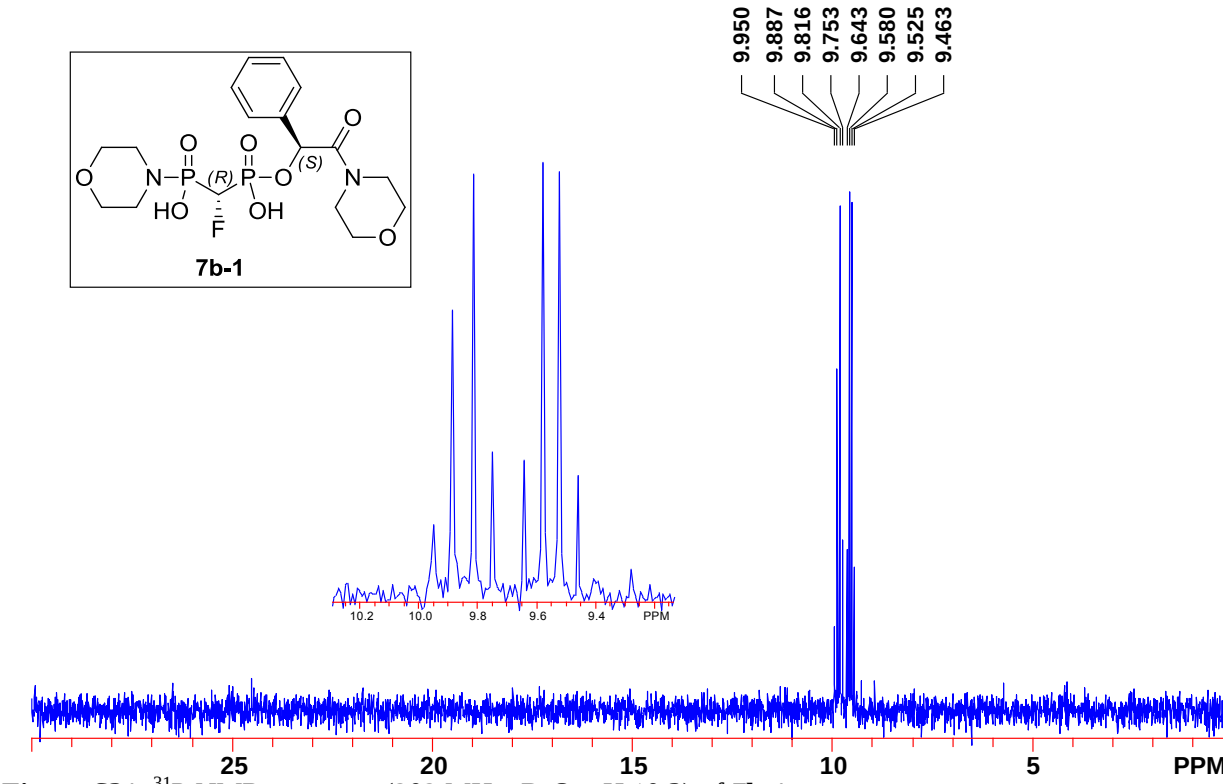

Figure S31. ^{31}P NMR spectrum (202 MHz; D_2O; pH 10.3) of **7b-1**.

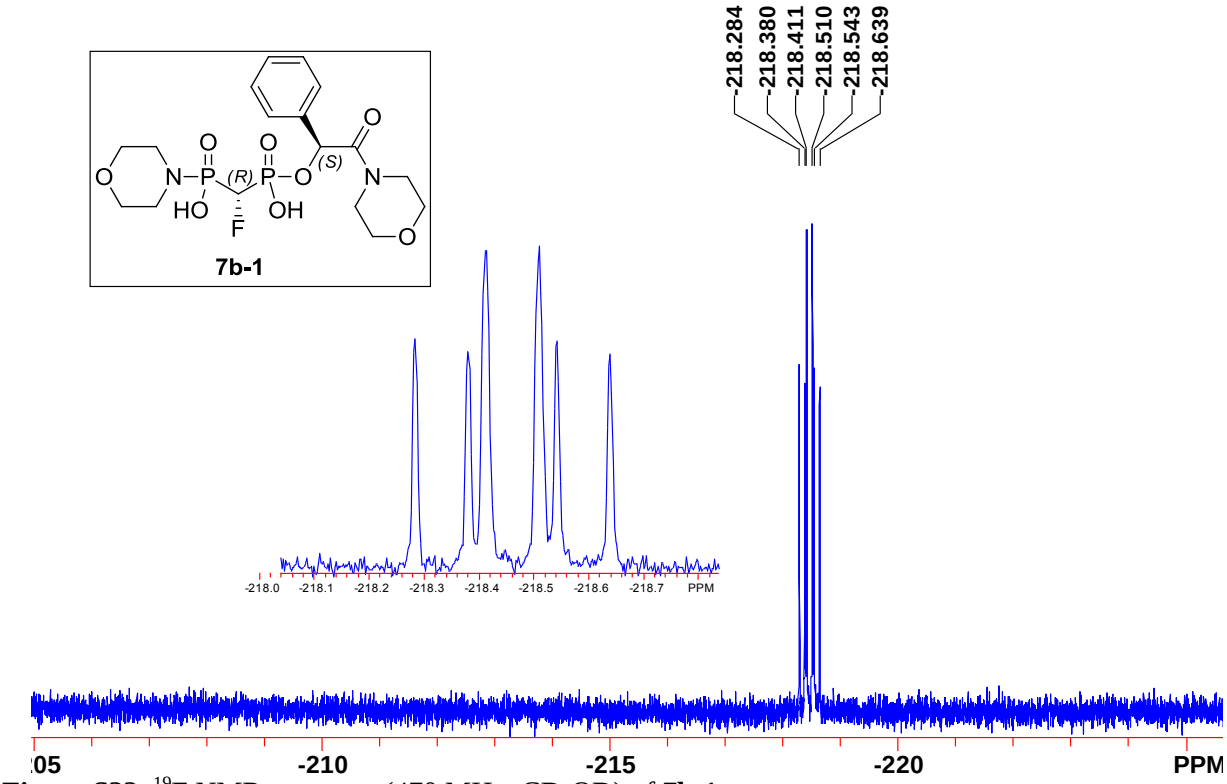

Figure S32. ^{19}F NMR spectrum (470 MHz; CD_3OD) of **7b-1**.

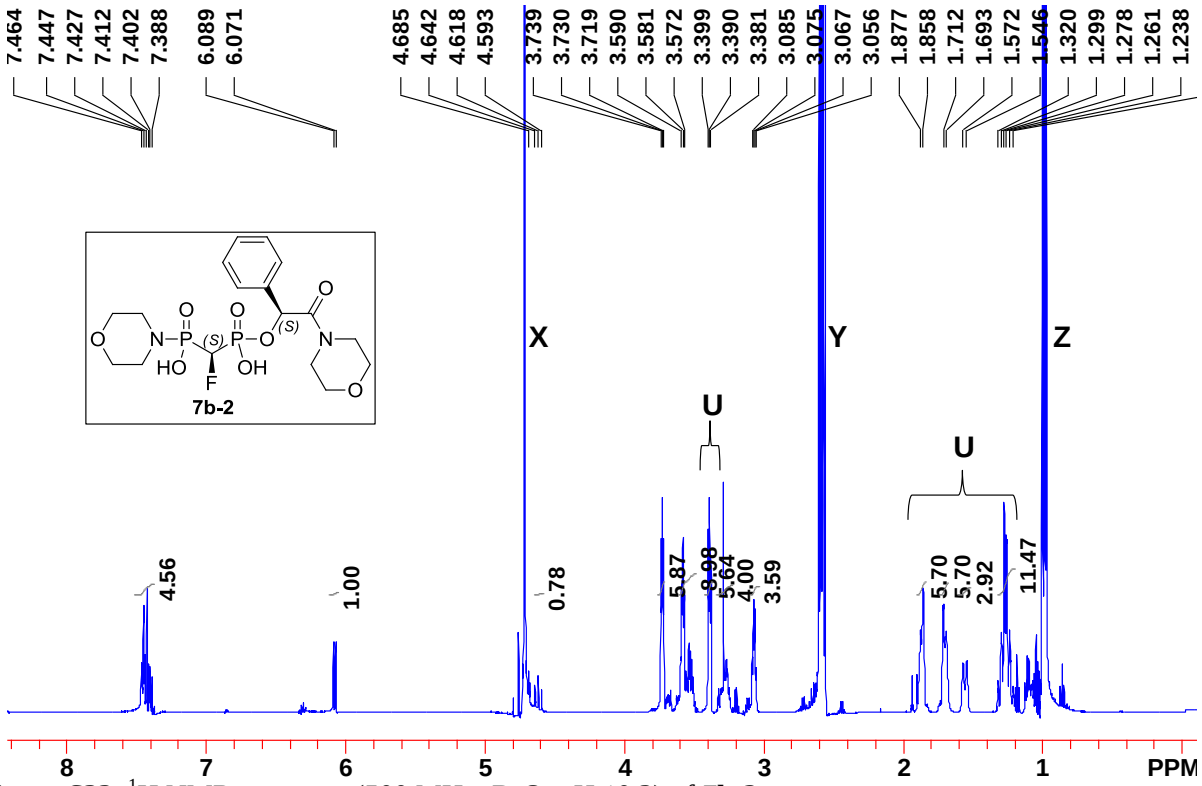

Figure S33. ^{1}H NMR spectrum (500 MHz; D_2O; pH 10.3) of **7b-2**.
X = HDO; Y, Z = Et_3N; U = Unidentified peaks.

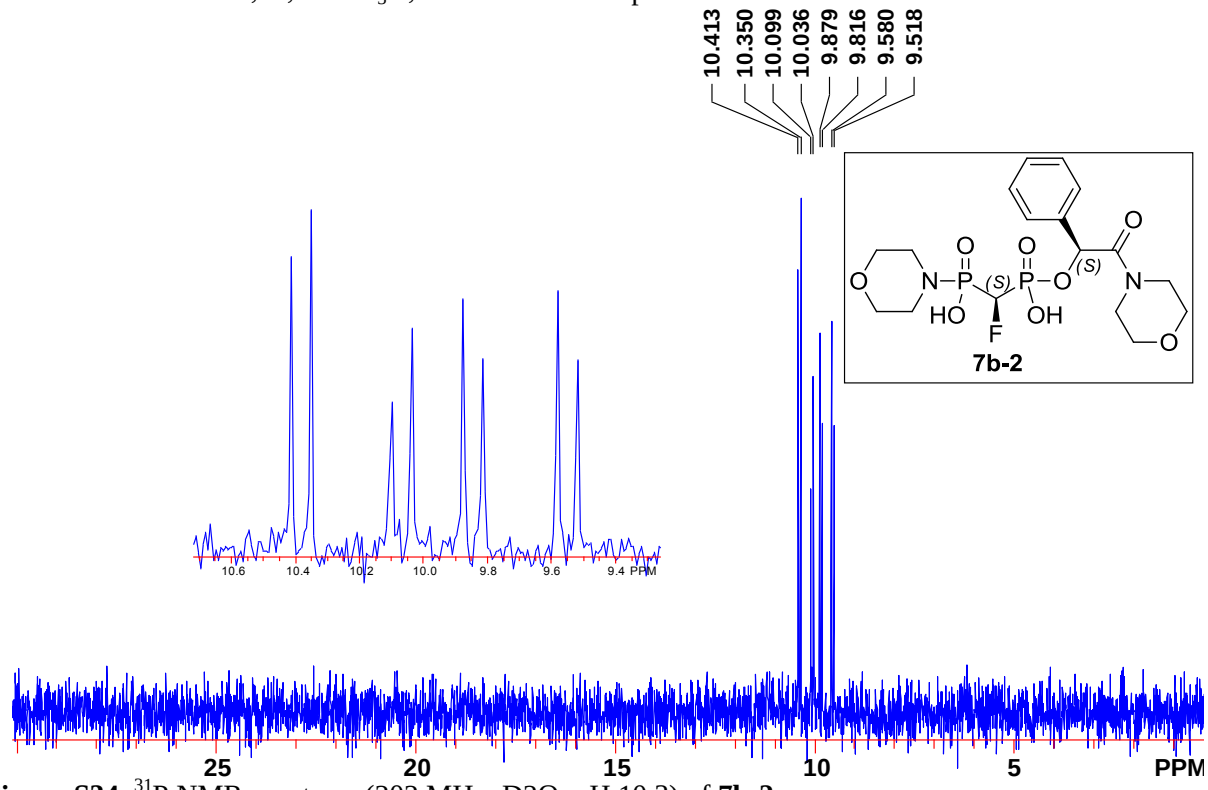

Figure S34. ^{31}P NMR spectrum (202 MHz; D2O; pH 10.3) of **7b-2**.

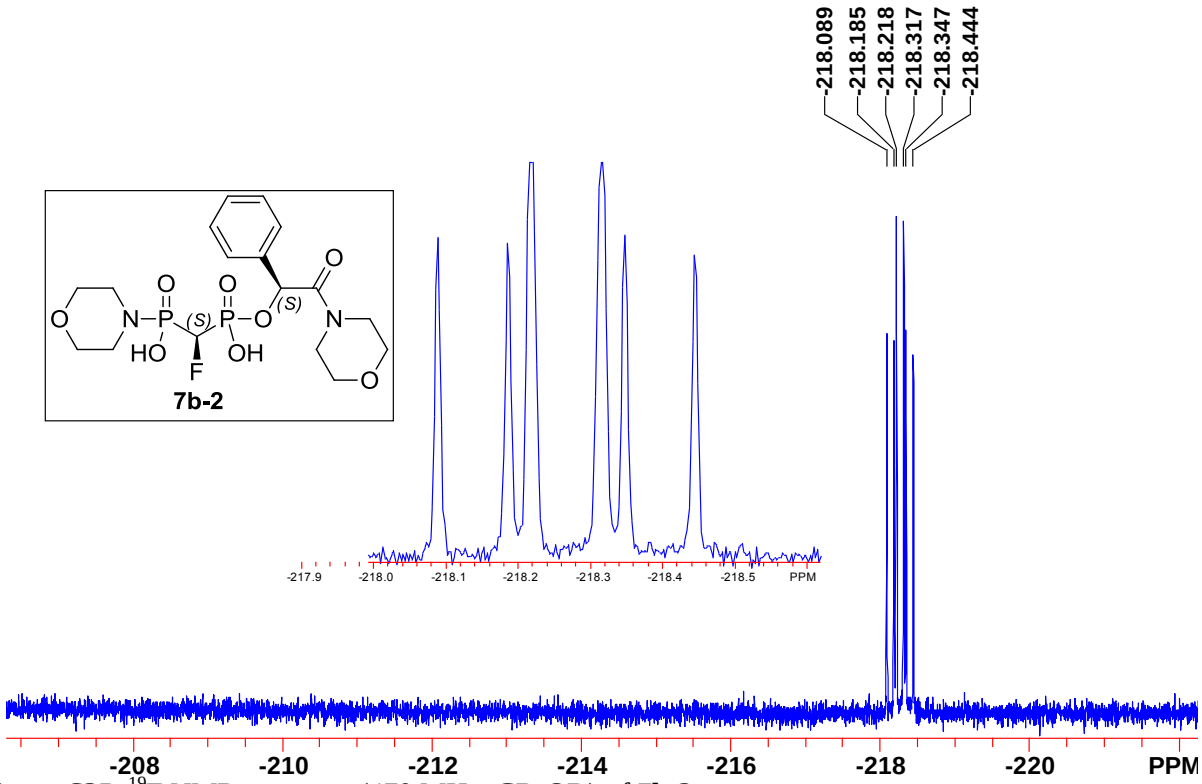

Figure S35. ^{19}F NMR spectrum (470 MHz; CD_3OD) of **7b-2**.

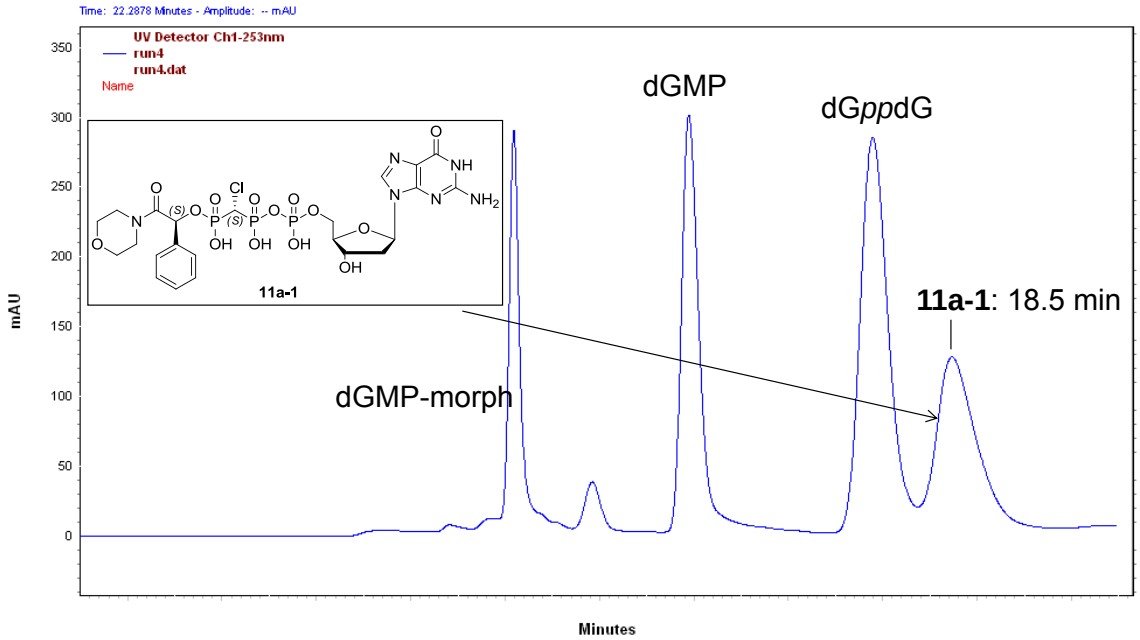

Figure S36. Preparative HPLC purification of **11a-1**.
For conditions see **Table S1**.

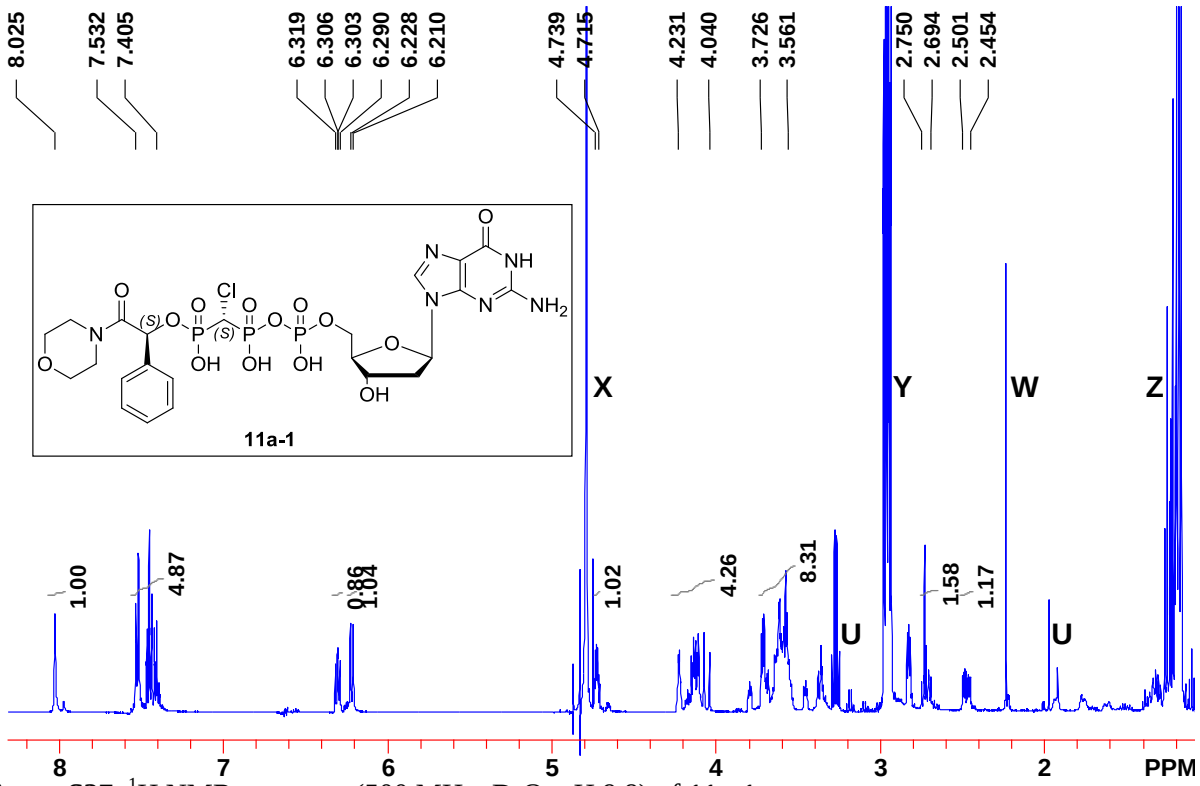

Figure S37. ^{1}H NMR spectrum (500 MHz; D_2O; pH 9.8) of **11a-1**.
U = unidentified impurities; X = HDO; Y, Z = Et_3N; W = acetone.

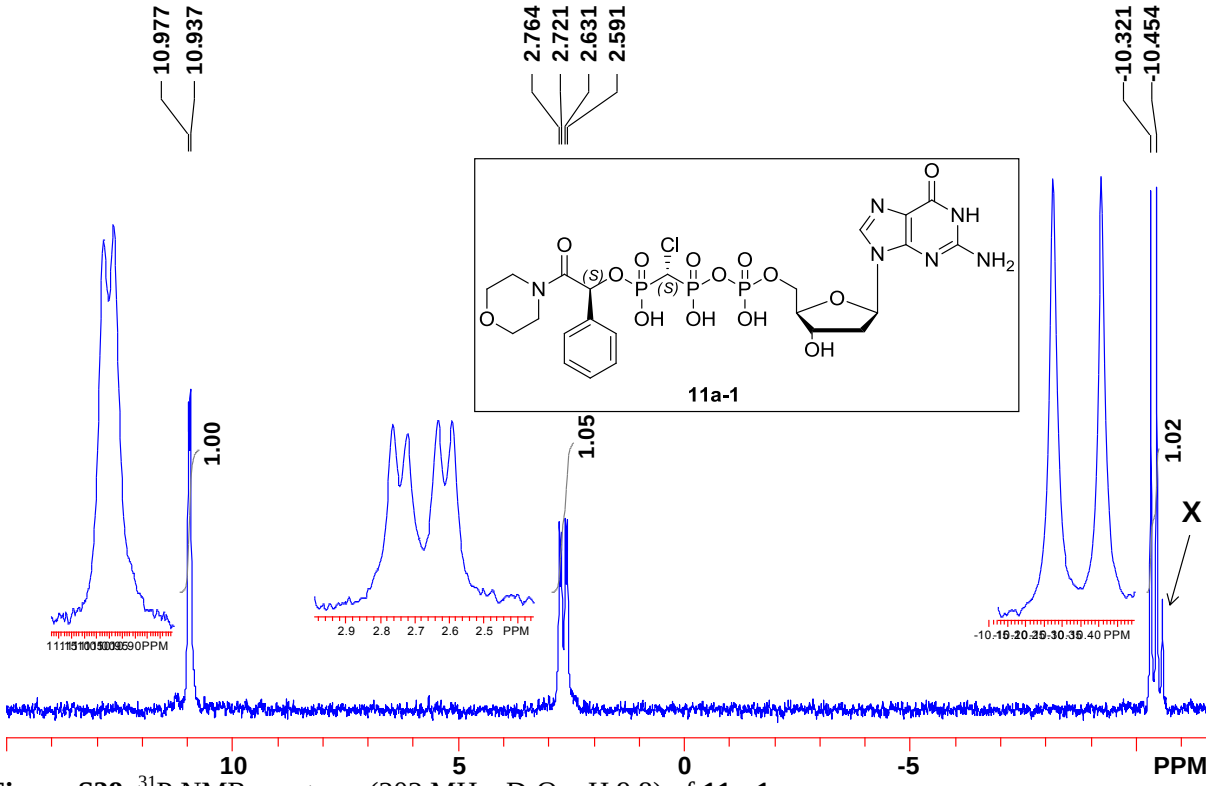

Figure S38. ^{31}P NMR spectrum (202 MHz; D_2O; pH 9.8) of **11a-1**.
X = dG*pp*dG

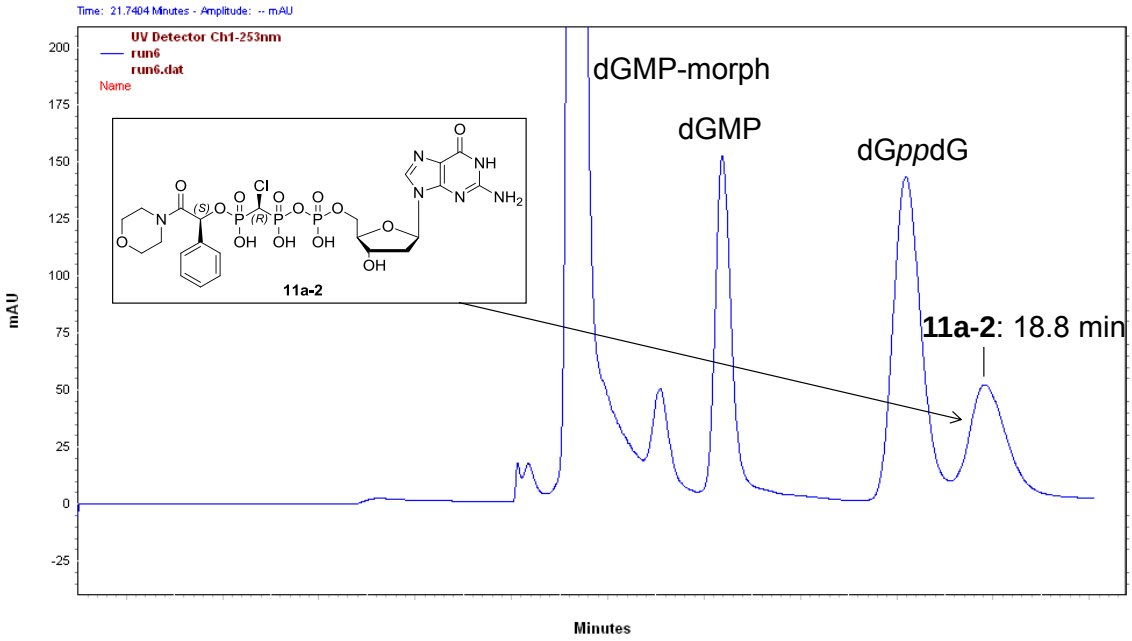

Figure S39. Preparative HPLC purification of **11a-2**.
For conditions see **Table S1**.

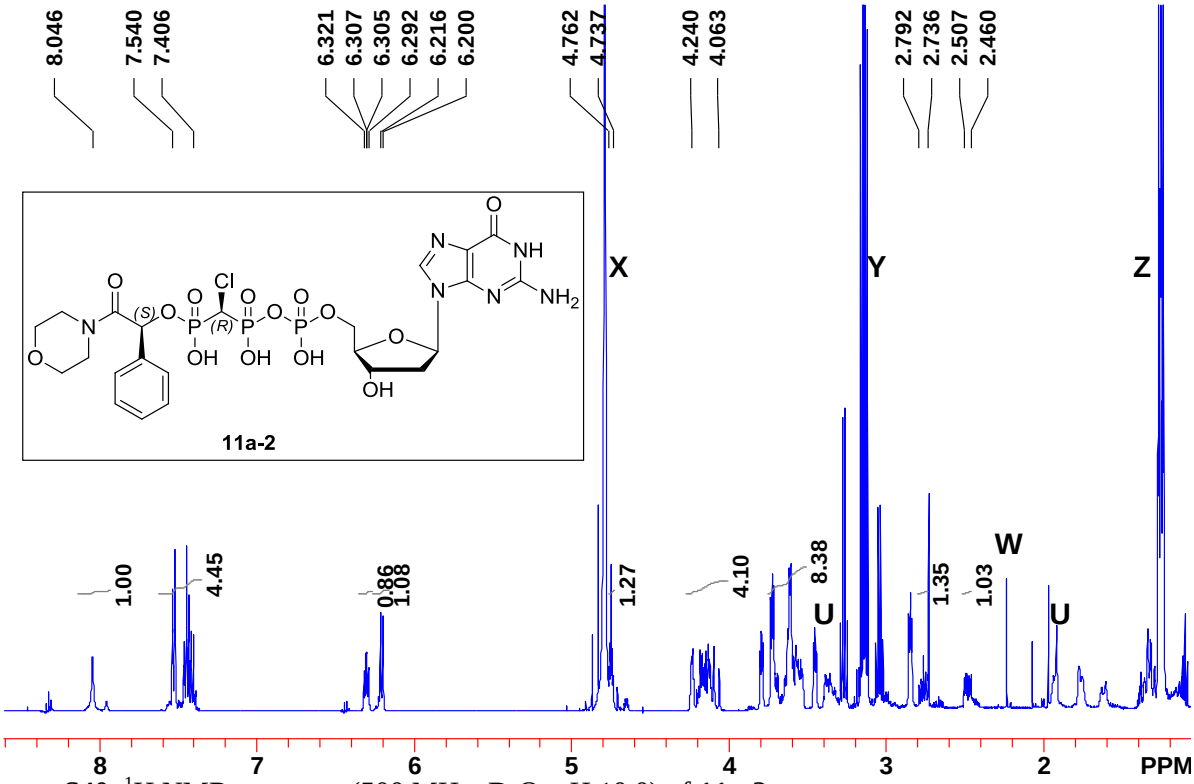

Figure S40. ^{1}H NMR spectrum (500 MHz; D_2O; pH 10.0) of **11a-2**.
U = unidentified impurities; X = HDO; Y, Z = Et_3N; W = acetone.

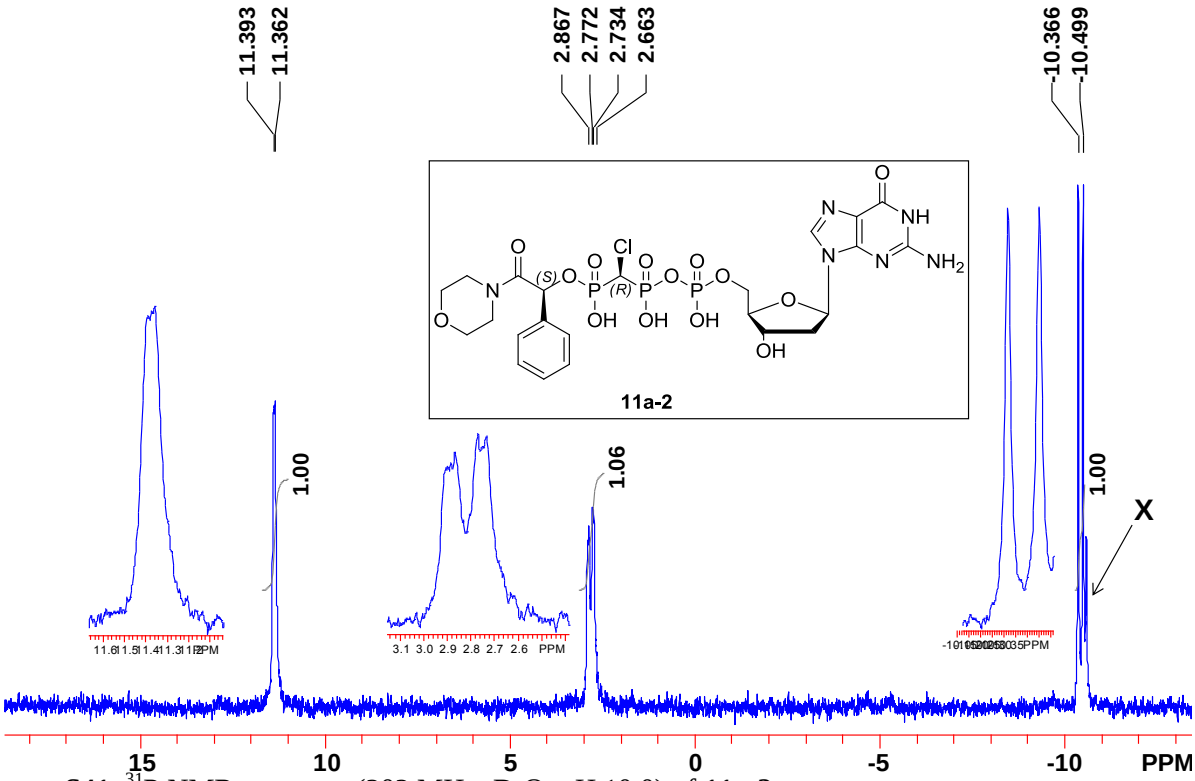

Figure S41. ^{31}P NMR spectrum (202 MHz; D_2O; pH 10.0) of **11a-2**.
X = dG*pp*dG

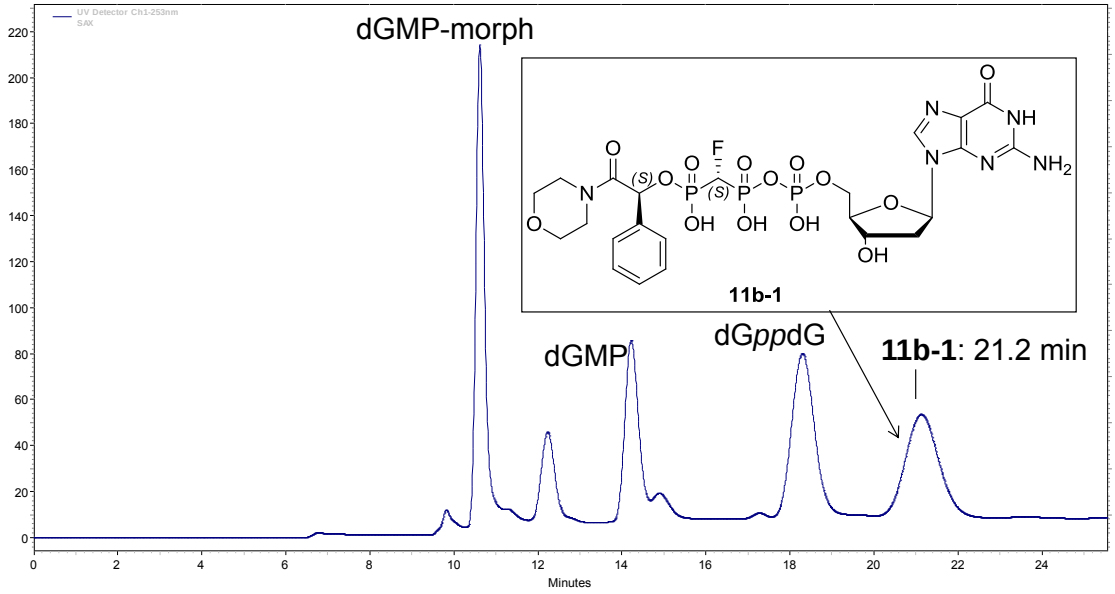

Figure S42. Preparative HPLC purification of **11b-1**.
For conditions see **Table S1**.

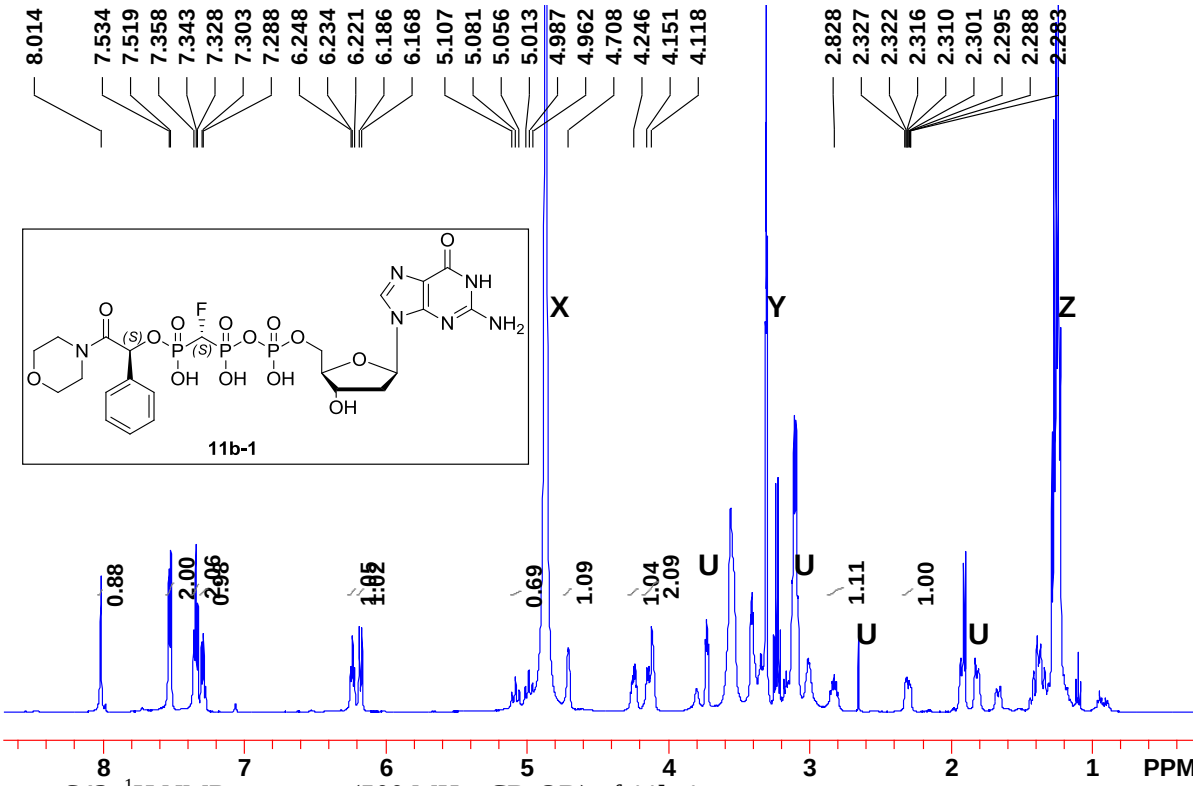

Figure S43. ^{1}H NMR spectrum (500 MHz; CD_3OD) of **11b-1**.
U = unidentified peaks; X = HDO; Y, Z = Et_3N.

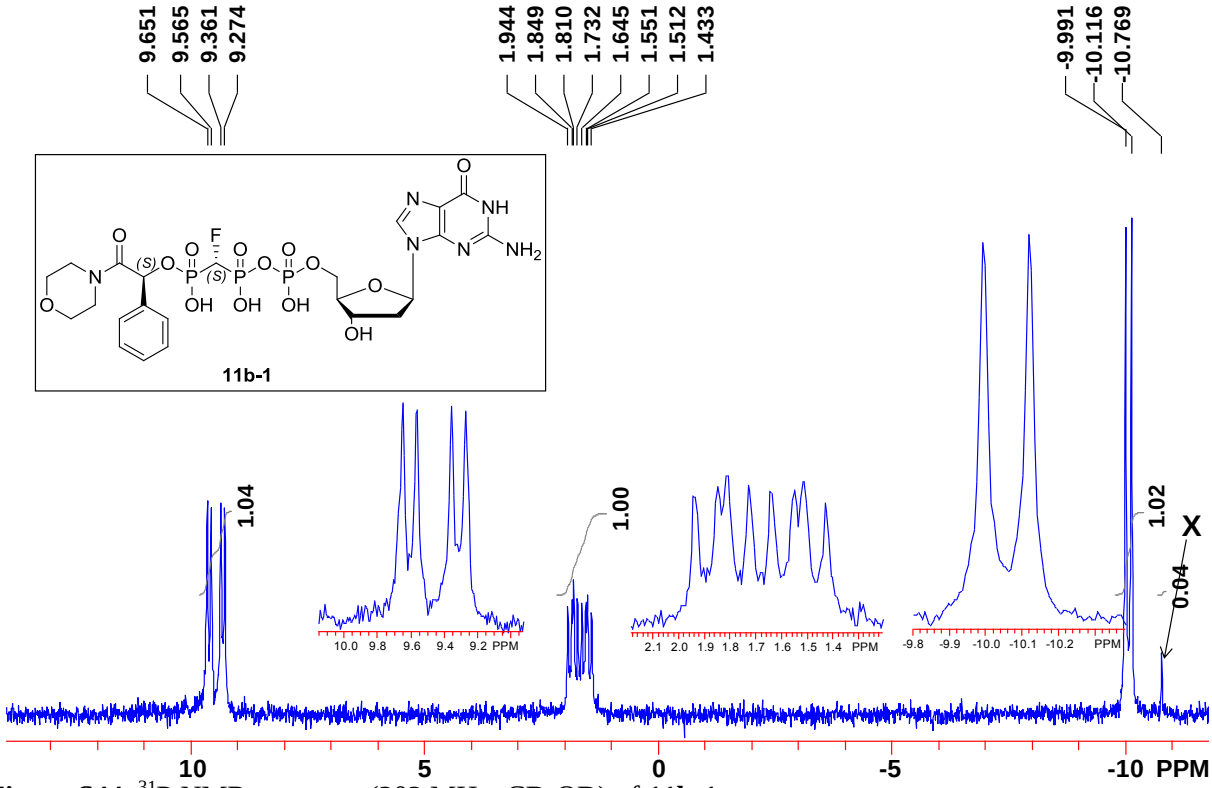

Figure S44. ^{31}P NMR spectrum (202 MHz; CD_3OD) of **11b-1**.
X = dG*pp*dG

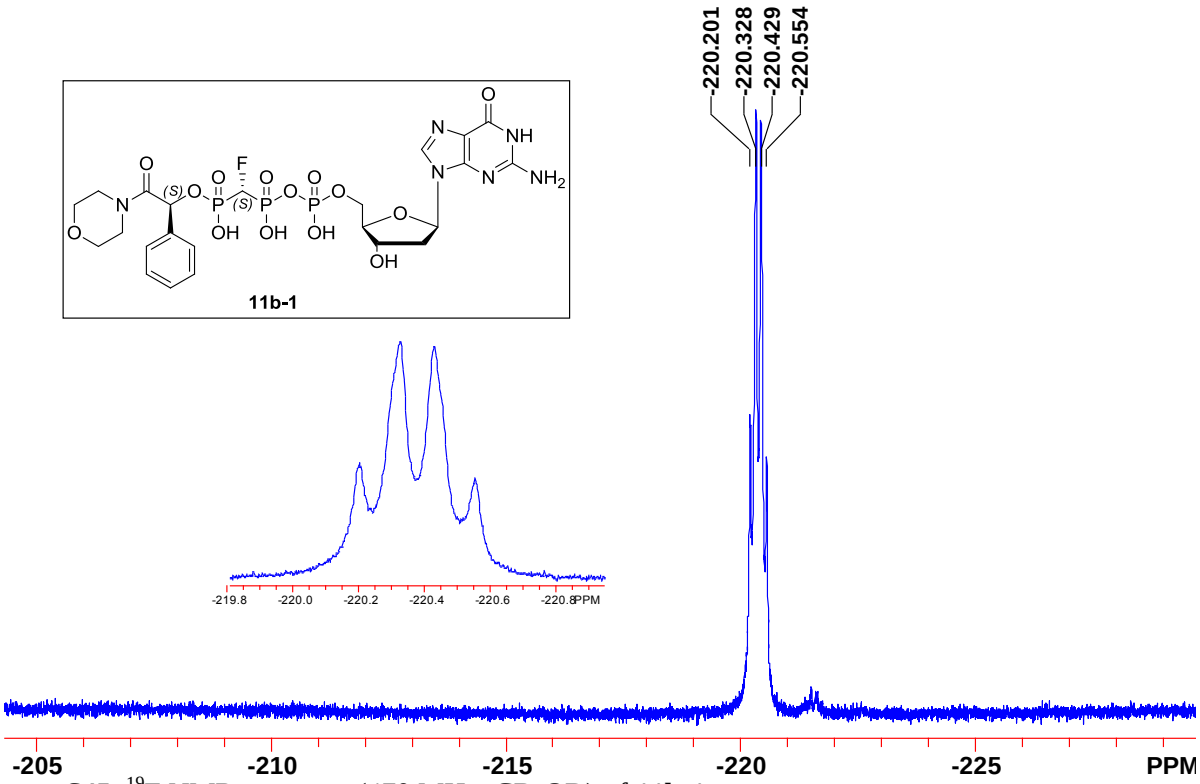

Figure S45. ^{19}F NMR spectrum (470 MHz; CD_3OD) of **11b-1**.

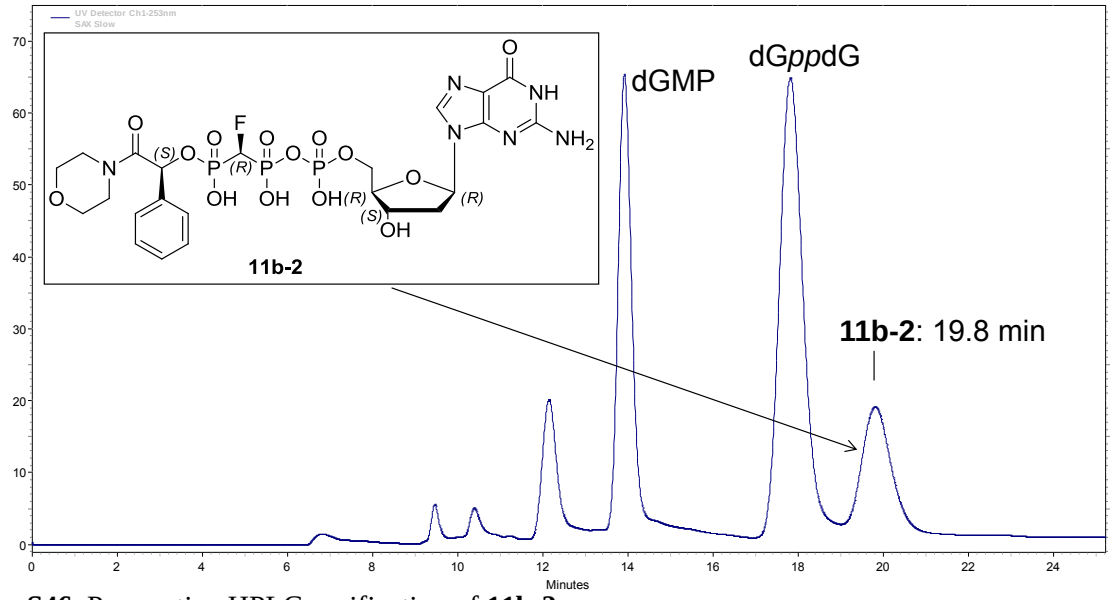

Figure S46. Preparative HPLC purification of **11b-2**.
For conditions see **Table S1**.

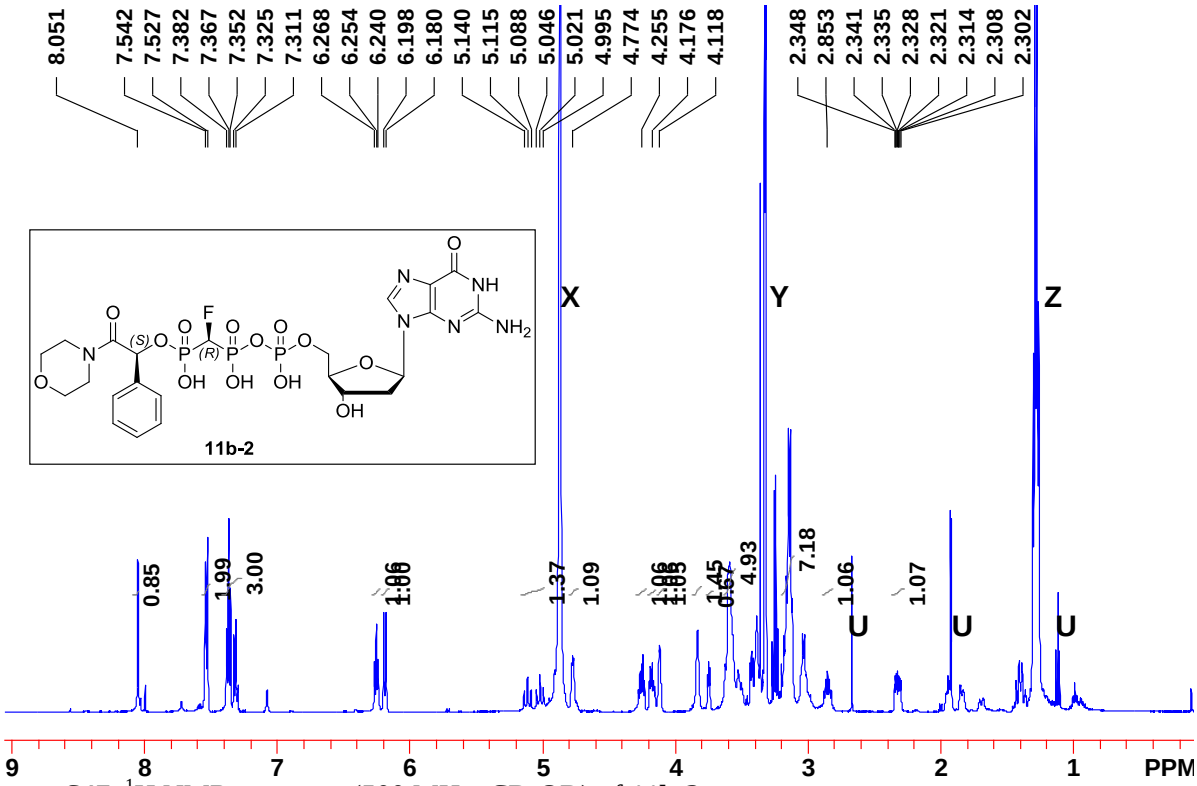

Figure S47. ^{1}H NMR spectrum (500 MHz; CD_3OD) of **11b-2**.
U = unidentified peaks; X = HDO; Y, Z = Et_3N.

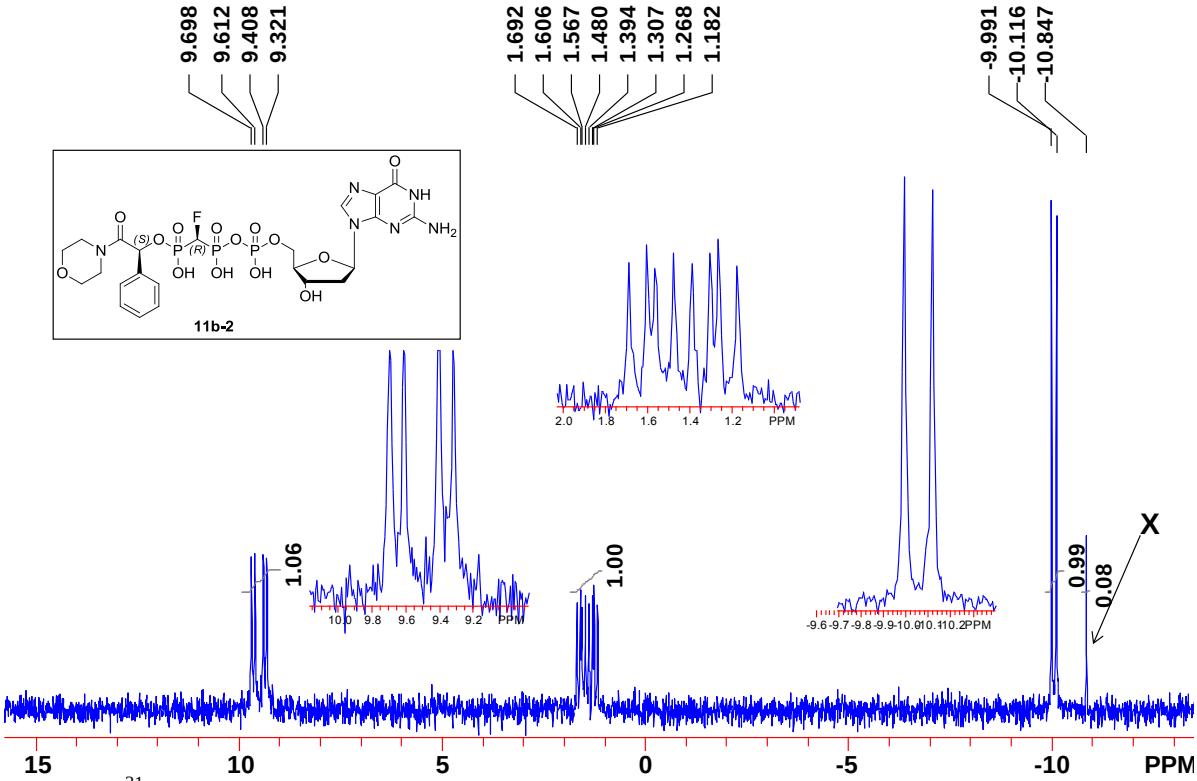

Figure S48. ^{31}P NMR spectrum (202 MHz; CD_3OD) of **11b-2**.
X = dG*pp*dG

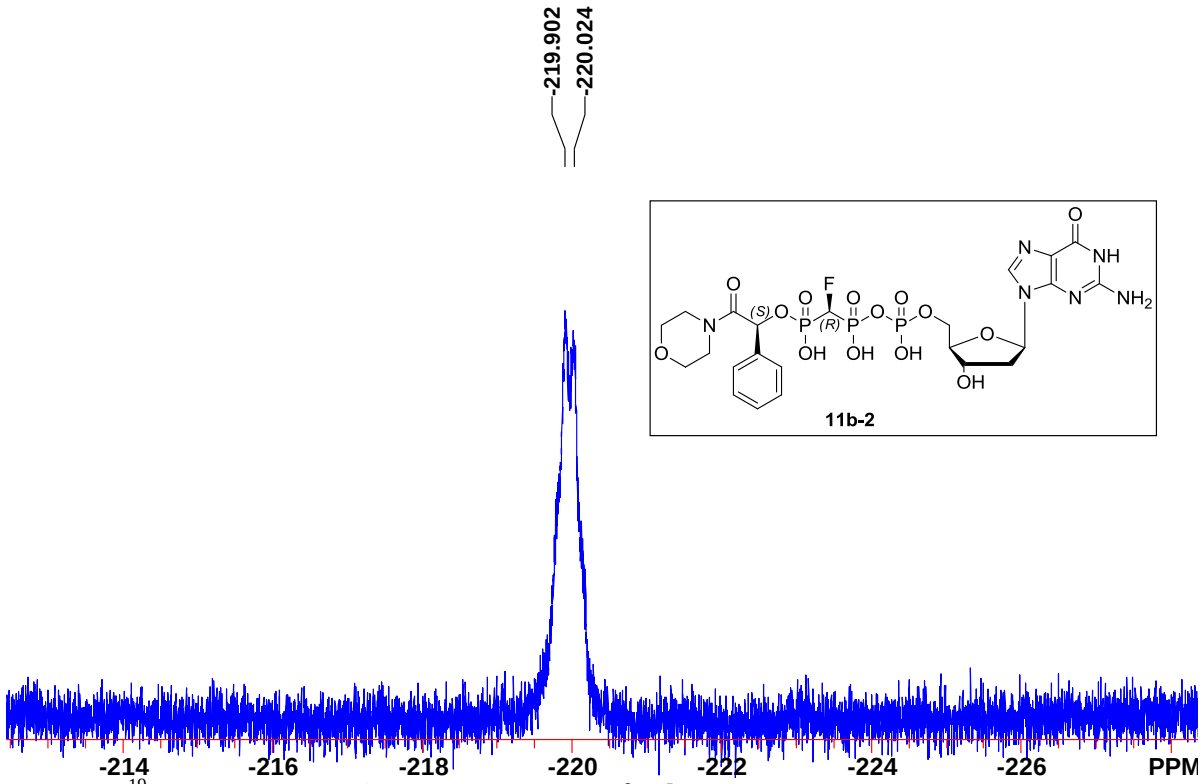

Figure S49. ^{19}F NMR spectrum (470 MHz; CD_3OD) of **11b-2**.

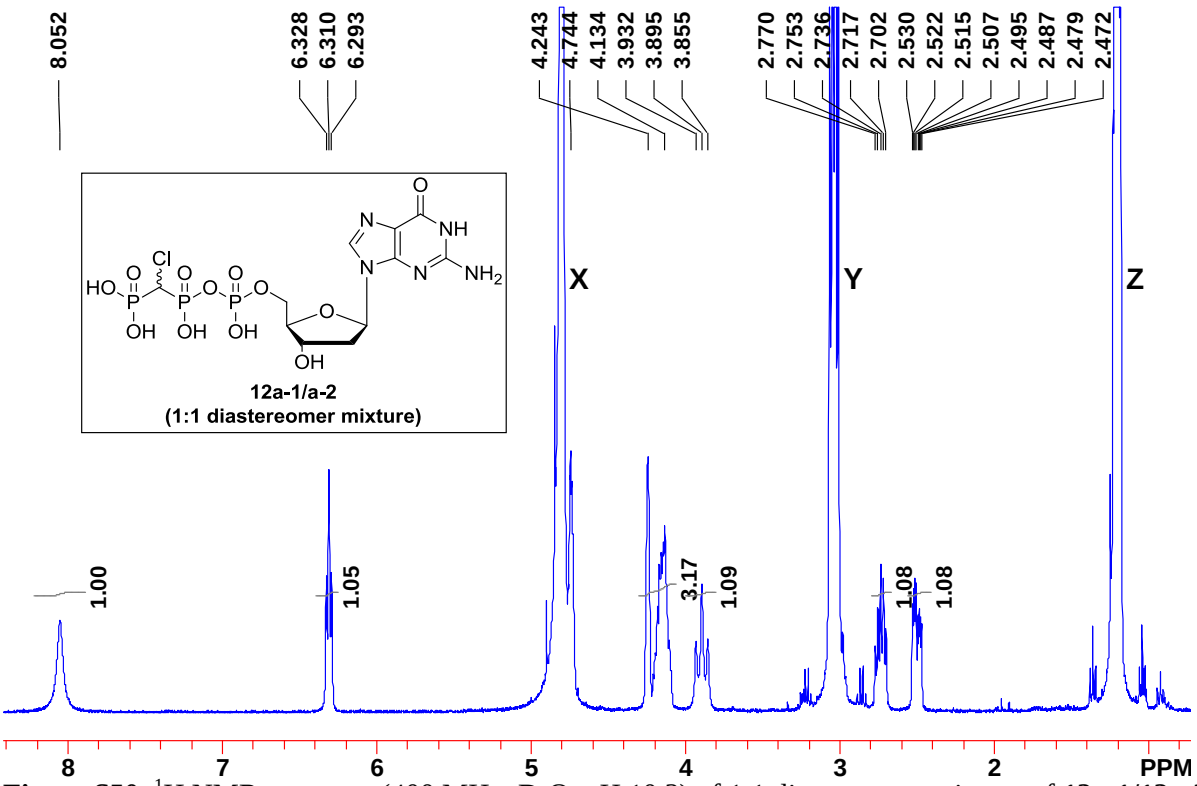

Figure S50. ^{1}H NMR spectrum (400 MHz; D_2O; pH 10.3) of 1:1 diastereomer mixture of **12a-1/12a-2**. X = HDO; Y, Z = Et_3N.

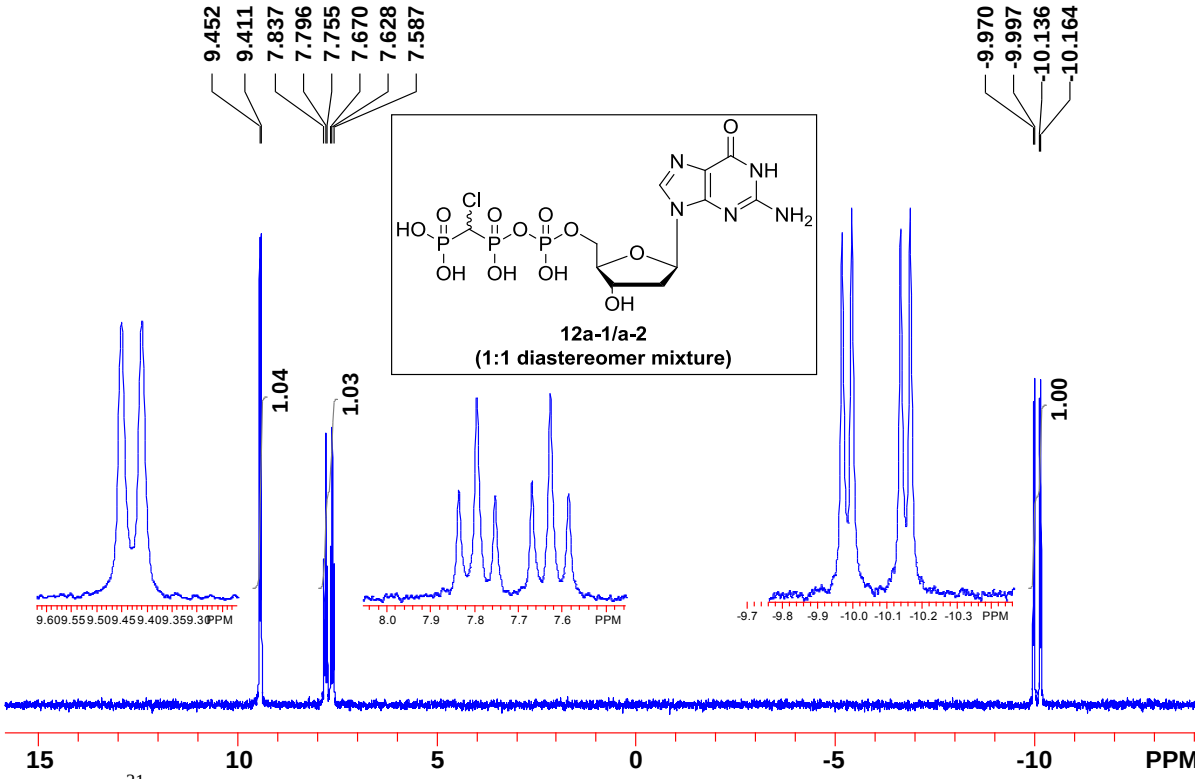

Figure S51. ^{31}P NMR spectrum (202 MHz; D_2O; pH 10.3) of 1:1 diastereomer mixture of **12a-1/12a-2**.

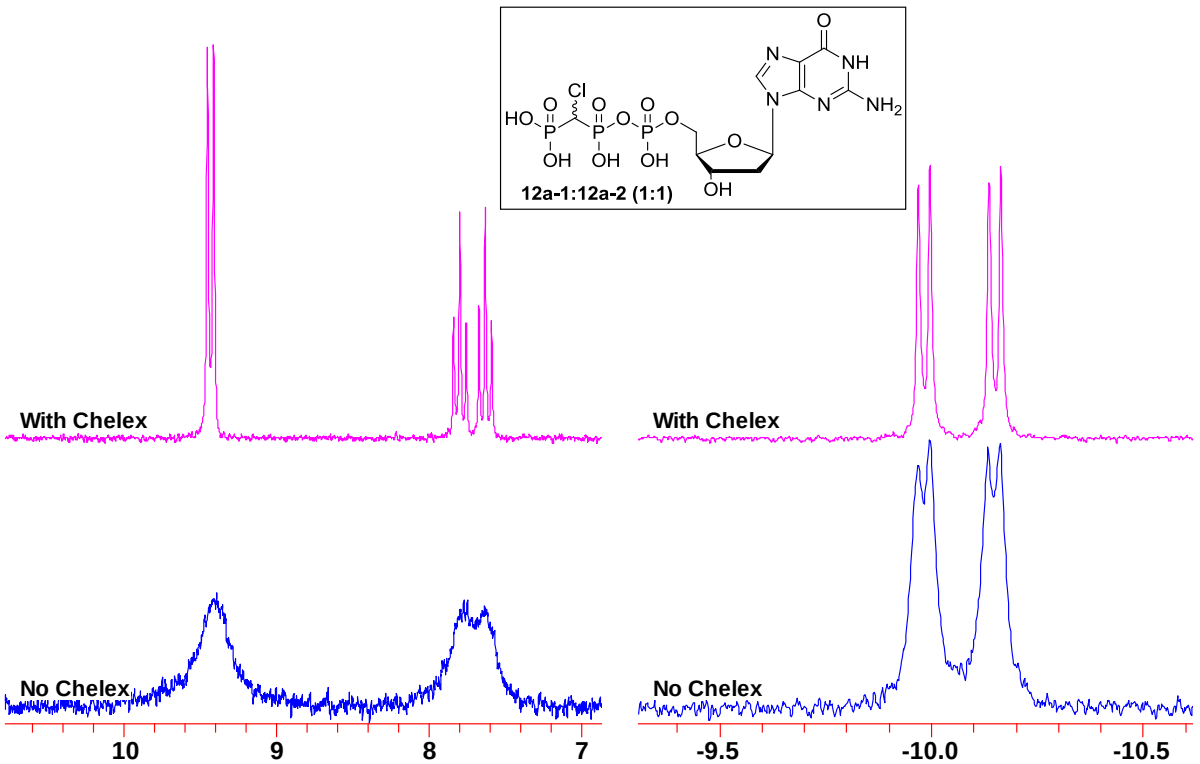

Figure S52. Comparison of ^{31}P NMR spectra (202 MHz; D_2O; pH 10) of 1:1 diastereomer mixture of **12a-1/12a-2** with and without addition of Chelex®-100.

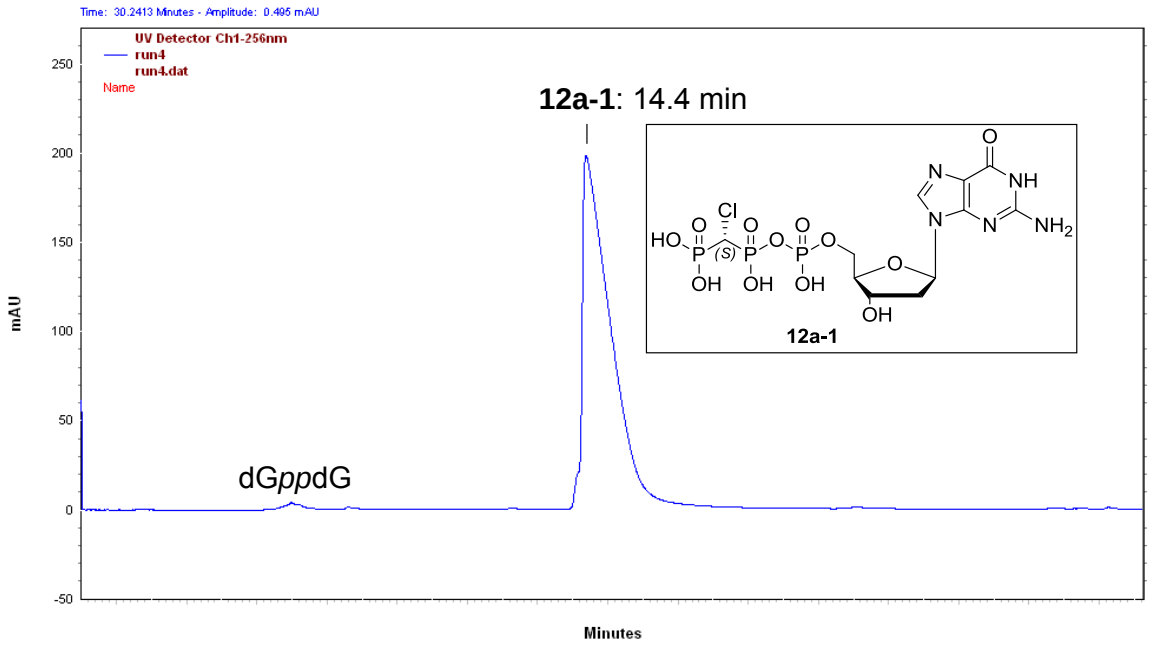

Figure S53. Preparative HPLC purification of **12a-1**.

For conditions see **Table S1**.

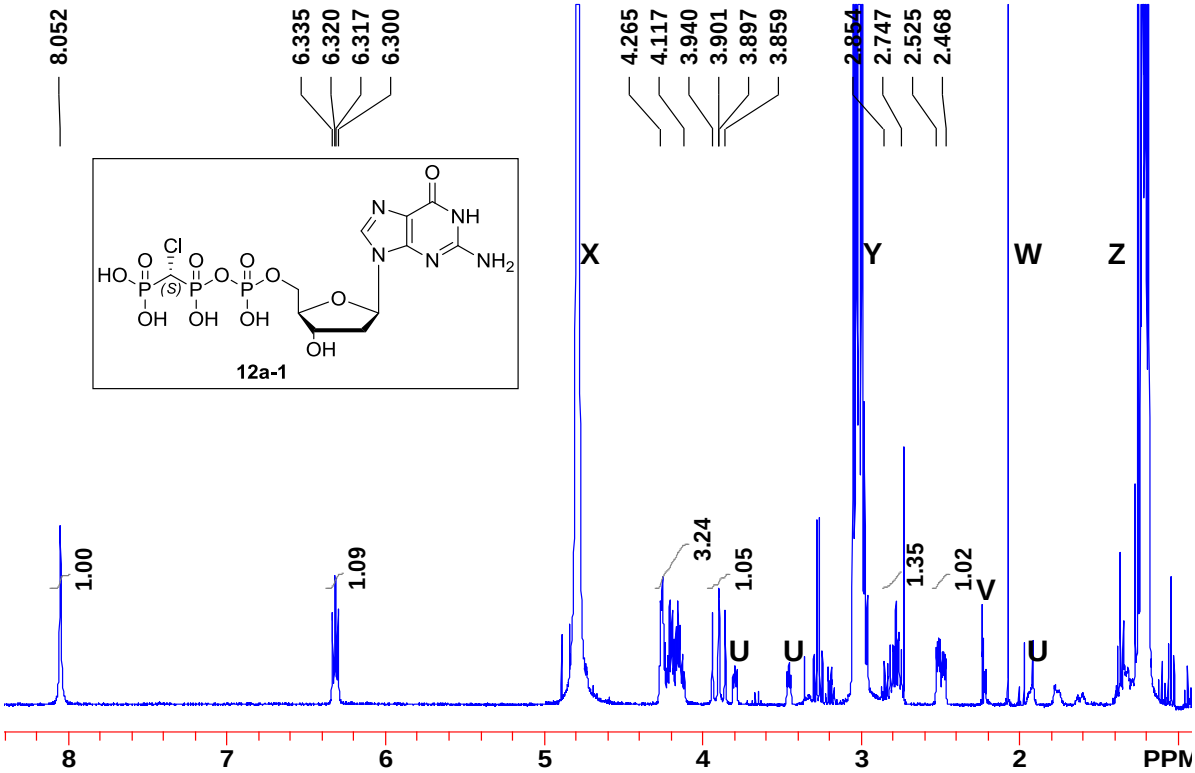

Figure S54. ^{1}H NMR spectrum (400 MHz; D_2O; pH 10.6) of **12a-1**.
U = unidentified impurities; V = acetone; W = CH_3CN; X = HDO; Y, Z = Et_3N.

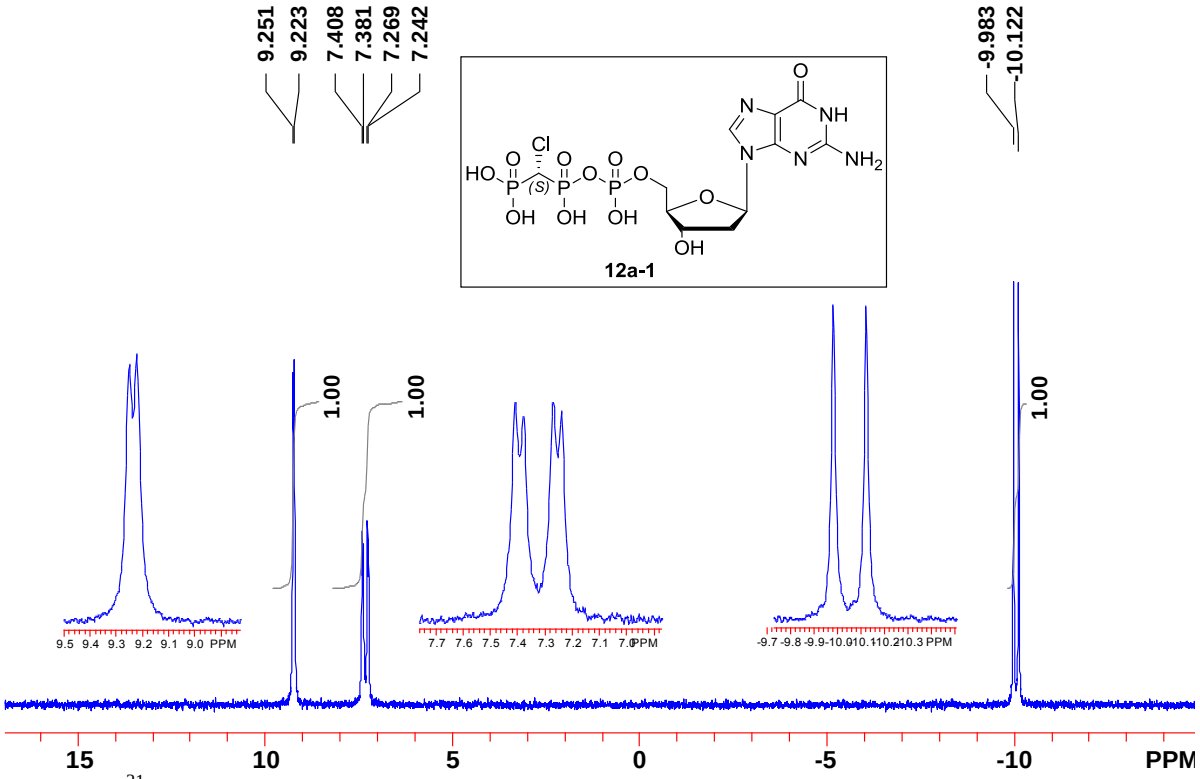

Figure S55. ^{31}P NMR spectrum (202 MHz; D_2O; pH 10.6) of **12a-1**.

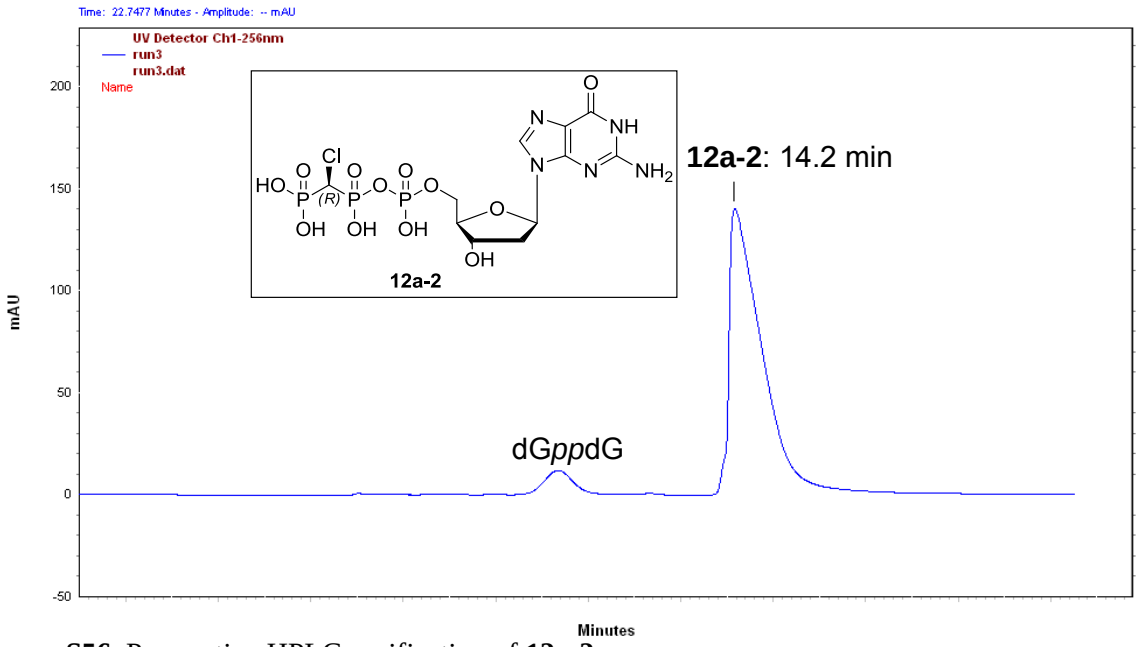

Figure S56. Preparative HPLC purification of **12a-2**.
For conditions see **Table S1**.

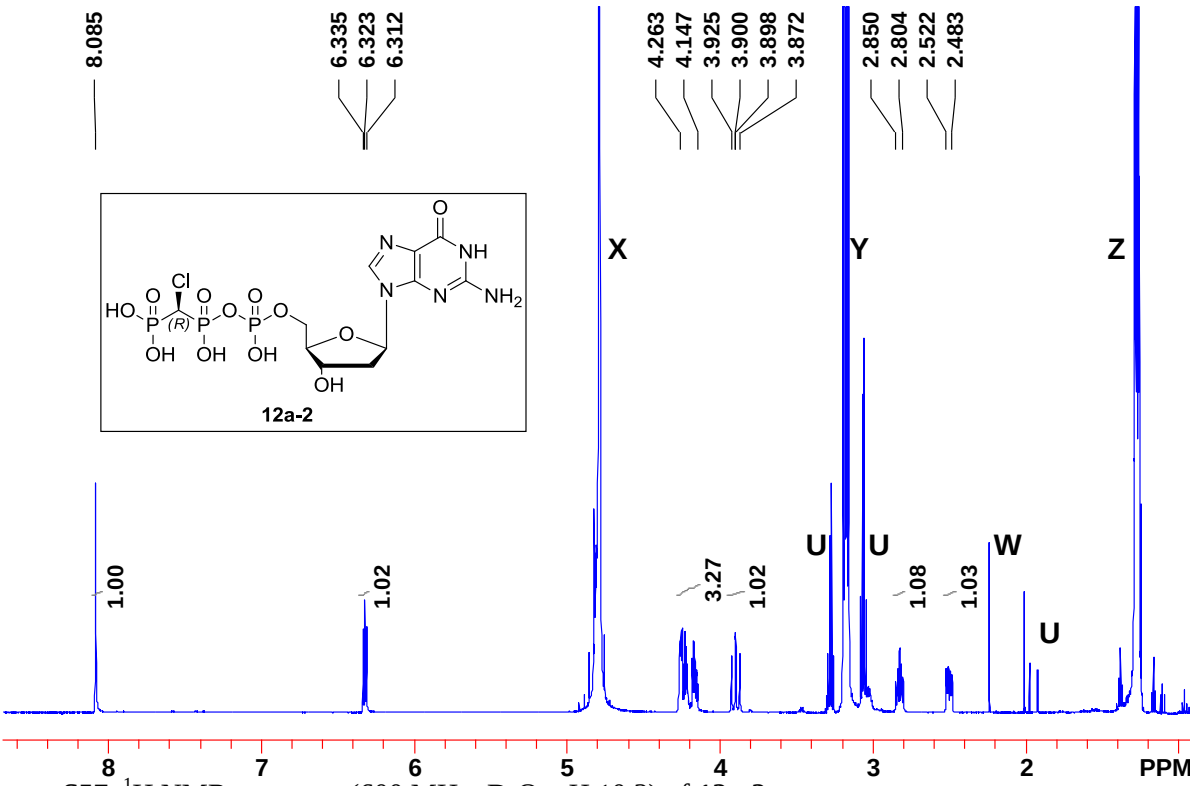

Figure S57. ^{1}H NMR spectrum (600 MHz; D_2O; pH 10.3) of **12a-2**.
U = unidentified impurities; W = acetone; X = HDO; Y, Z = Et_3N.

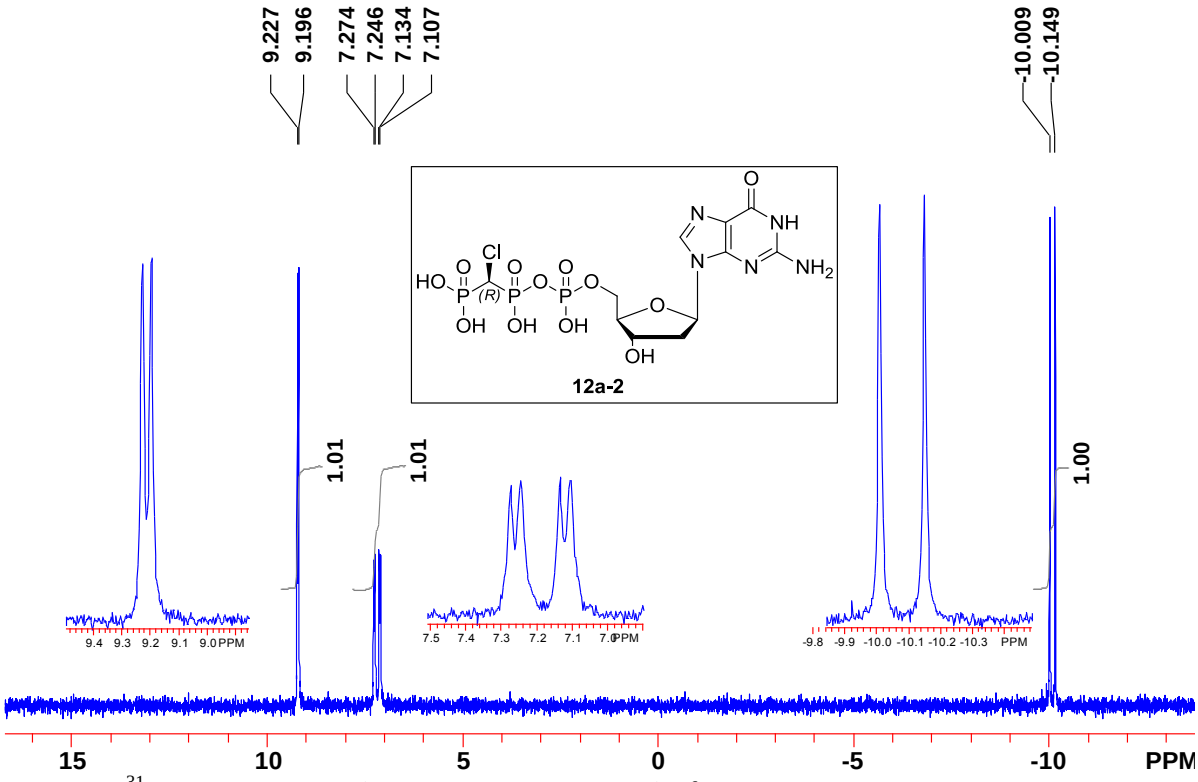

Figure S58. ^{31}P NMR spectrum (202 MHz; D_2O; pH 10.3) of **12a-2**.

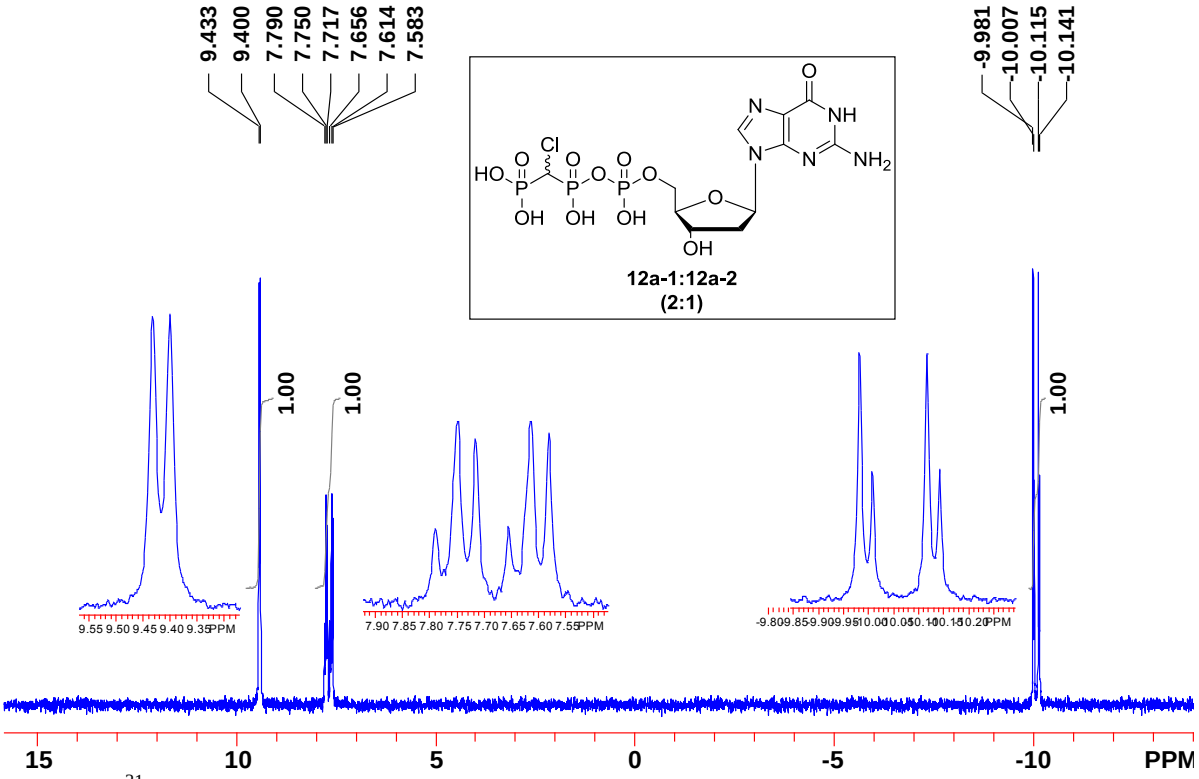

Figure S59. ^{31}P NMR spectrum (202 MHz; D_2O; pH 10.2) of mixture of **12a-1:12a-2** (2:1).

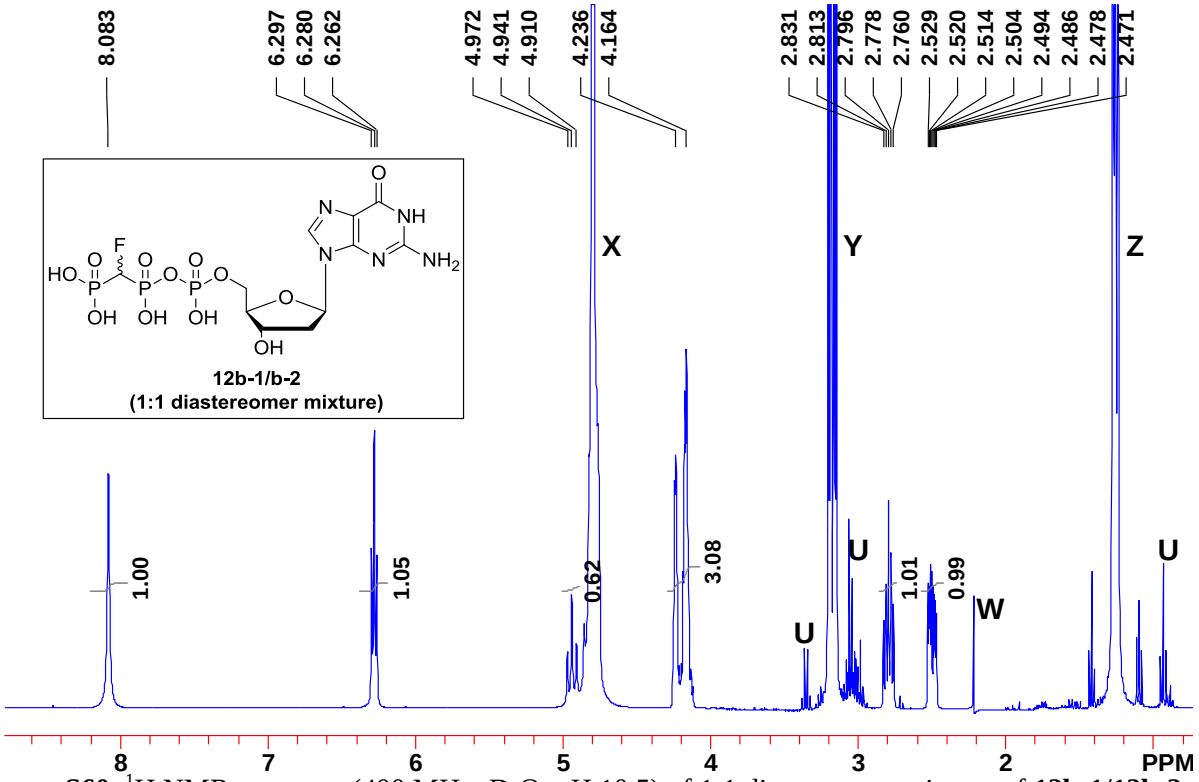

Figure S60. ^{1}H NMR spectrum (400 MHz; D_2O; pH 10.5) of 1:1 diastereomer mixture of **12b-1/12b-2**. U = unidentified impurities; W = acetone; X = HDO; Y, Z = Et_3N.

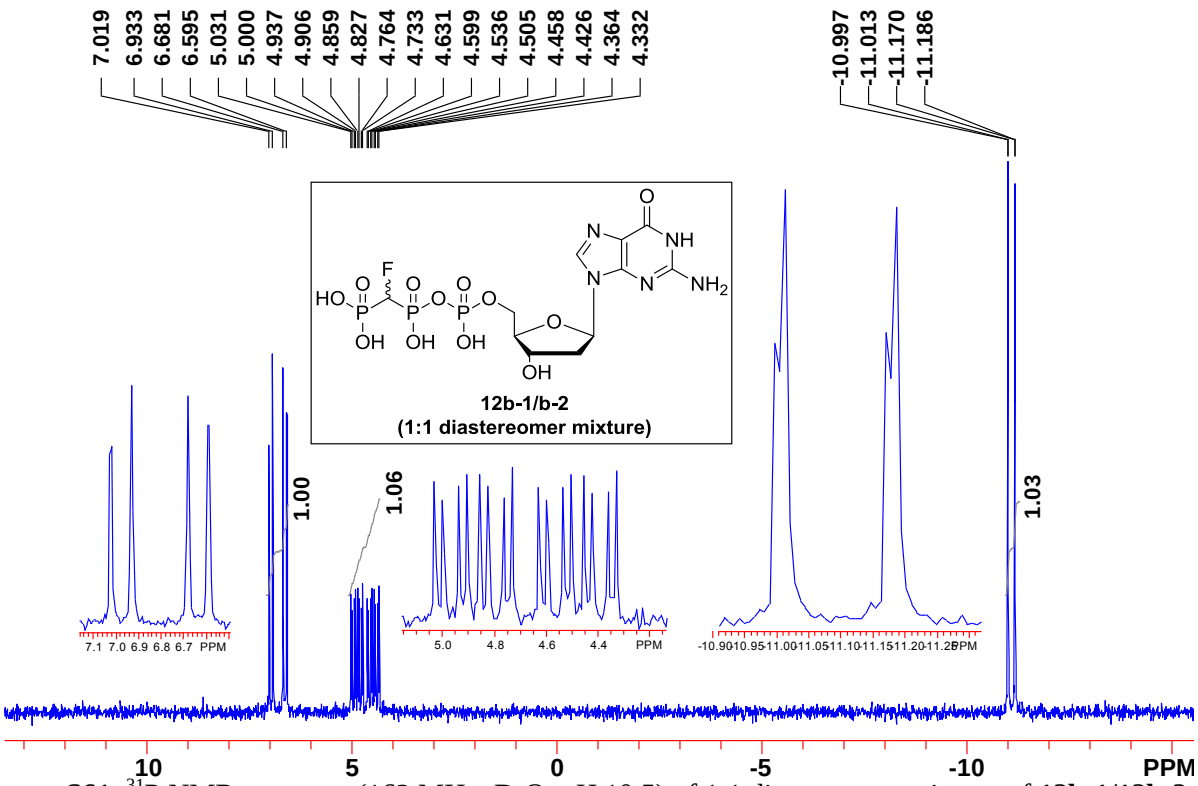

Figure S61. ^{31}P NMR spectrum (162 MHz; D_2O; pH 10.5) of 1:1 diastereomer mixture of **12b-1/12b-2**.

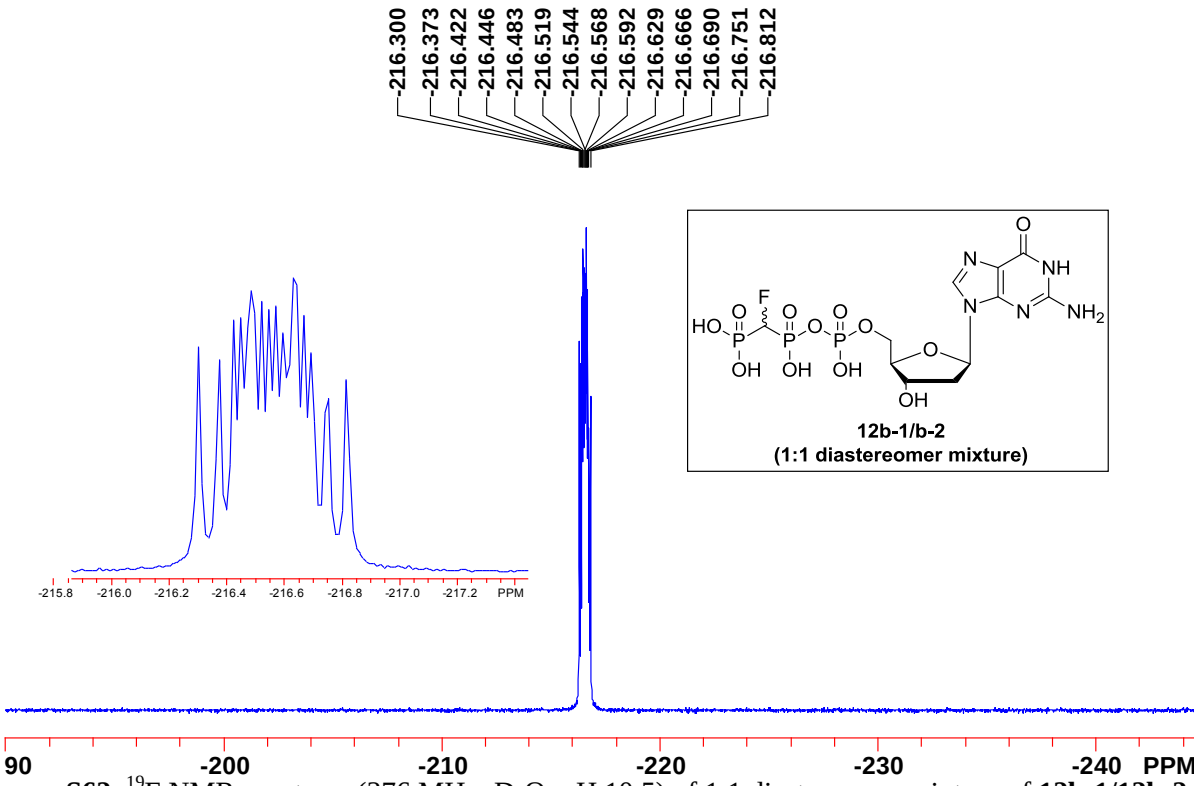

Figure S62. ^{19}F NMR spectrum (376 MHz; D_2O; pH 10.5) of 1:1 diastereomer mixture of **12b-1/12b-2**.

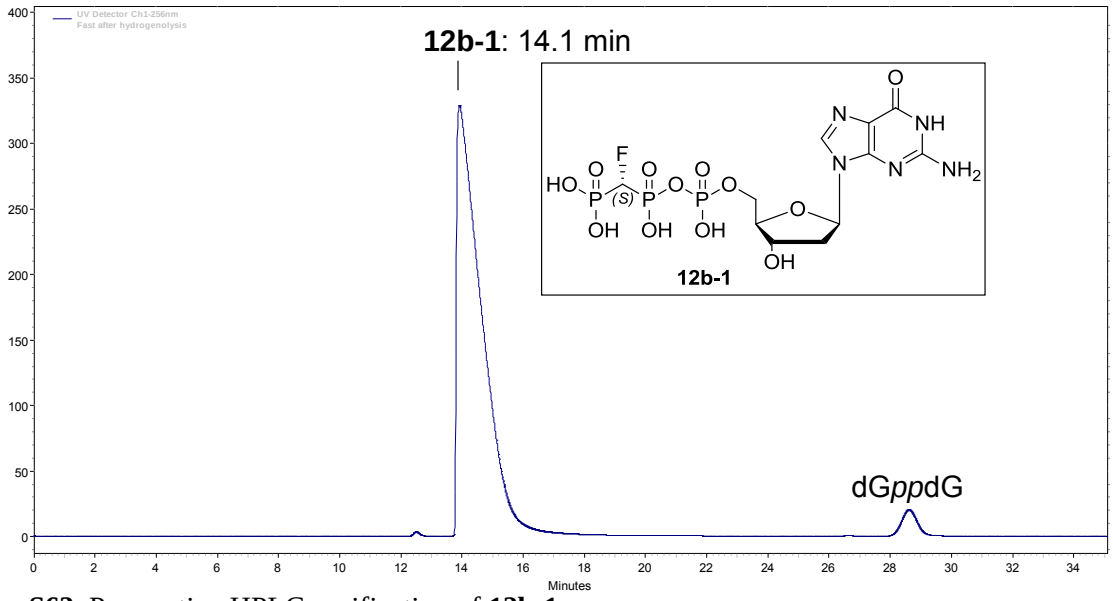

Figure S63. Preparative HPLC purification of **12b-1**.
For conditions see **Table S1**.

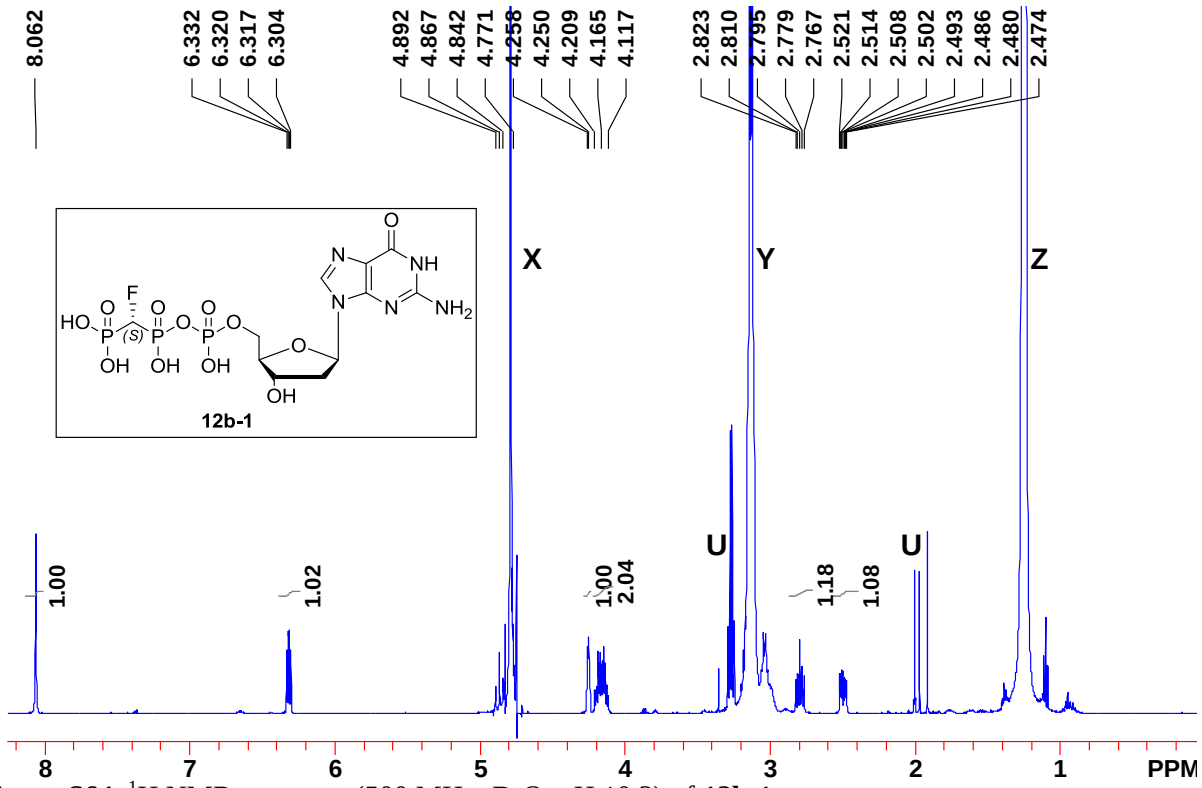

Figure S64. ^{1}H NMR spectrum (500 MHz; D_2O; pH 10.3) of **12b-1**.
U = unidentified impurities; X = HDO; Y, Z = Et_3N.

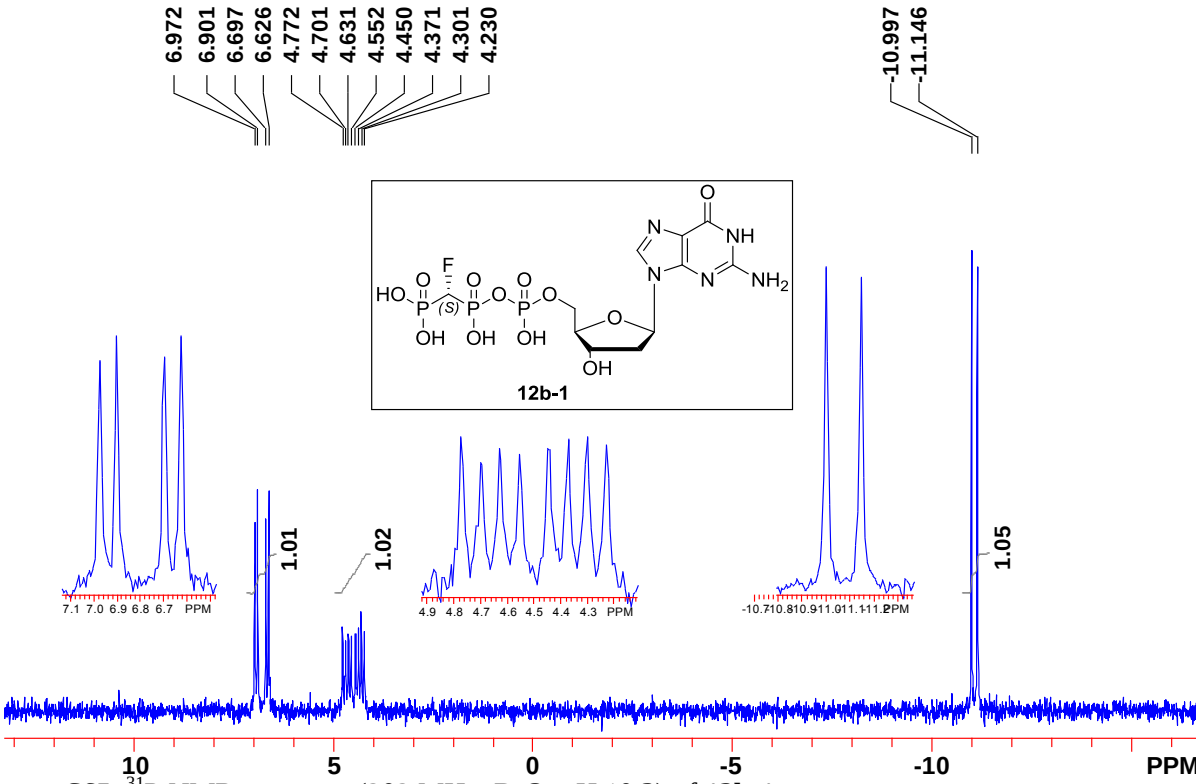

Figure S65. ^{31}P NMR spectrum (202 MHz; D_2O; pH 10.3) of **12b-1**.

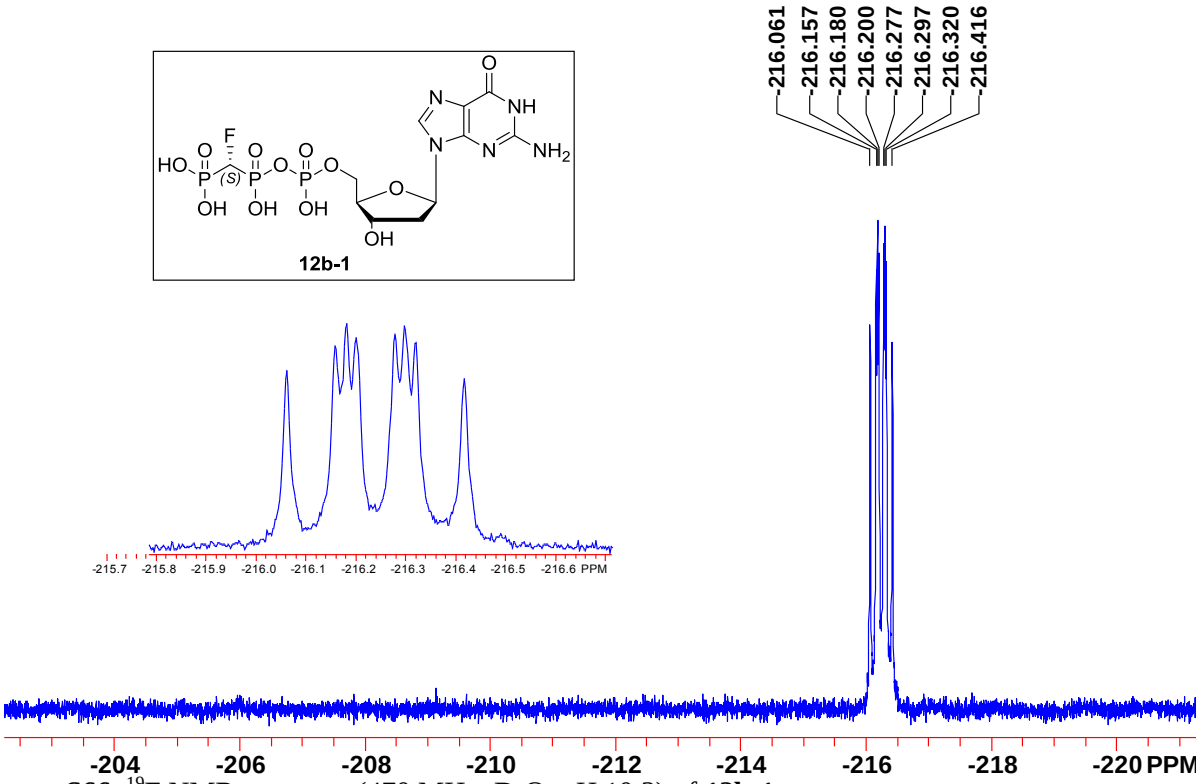

Figure S66. ^{19}F NMR spectrum (470 MHz; D_2O; pH 10.3) of **12b-1**.

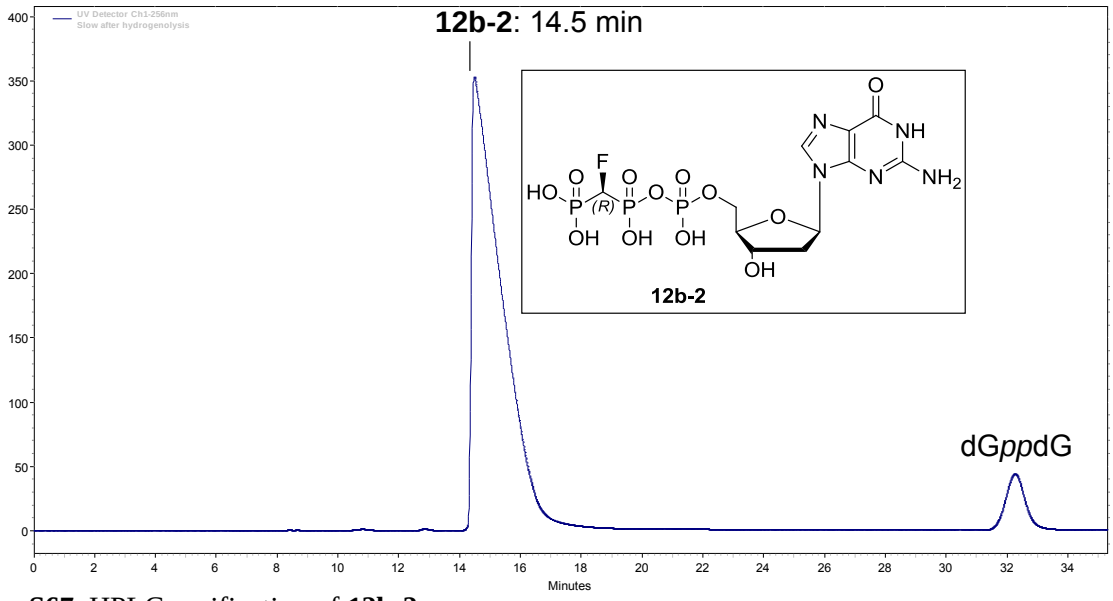

Figure S67. HPLC purification of **12b-2**.
For conditions see **Table S1**.

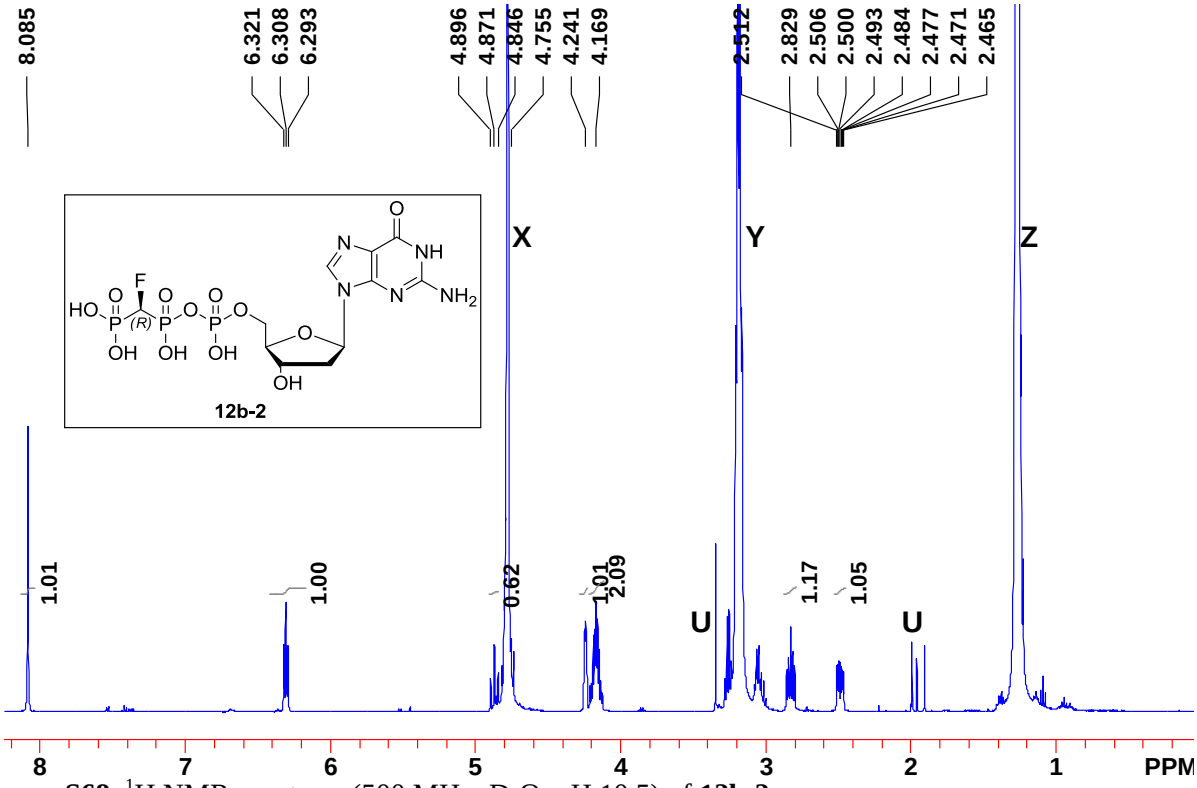

Figure S68. ^{1}H NMR spectrum (500 MHz; D_2O; pH 10.5) of **12b-2**.
U = unidentified impurities; X = HDO; Y, Z = Et_3N.

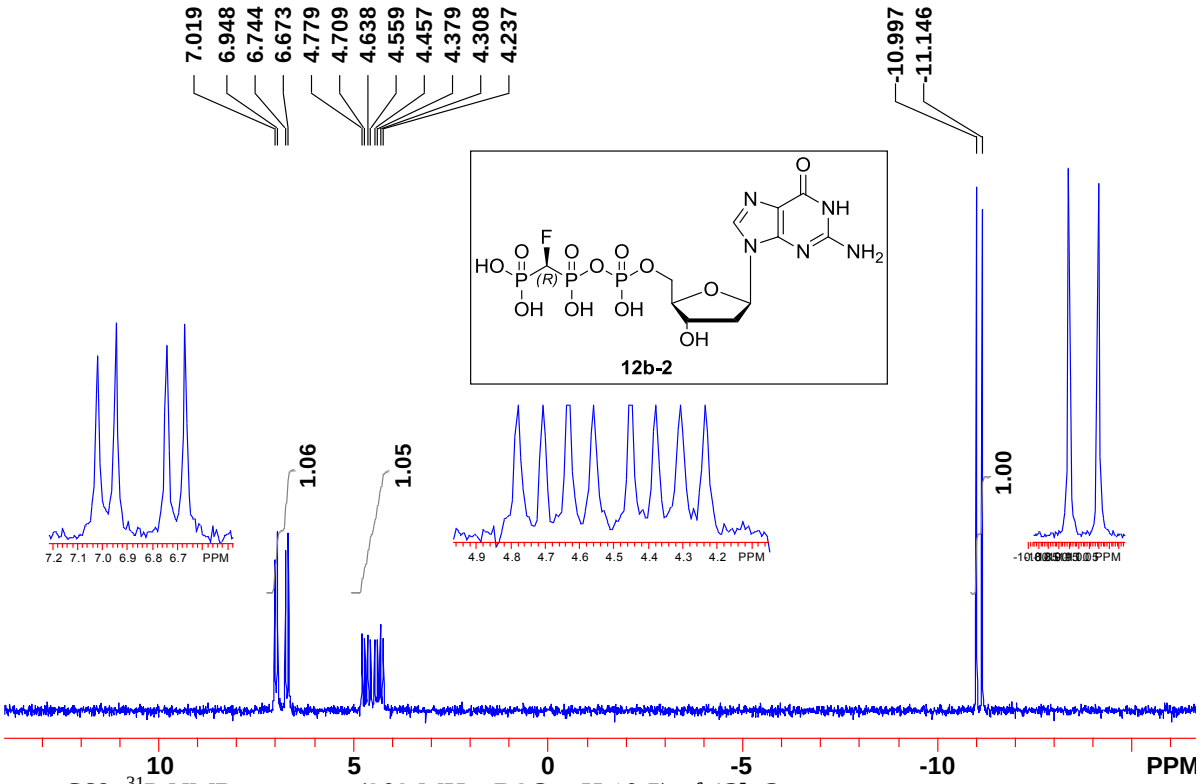

Figure S69. ^{31}P NMR spectrum (202 MHz; D2O; pH 10.5) of **12b-2**.

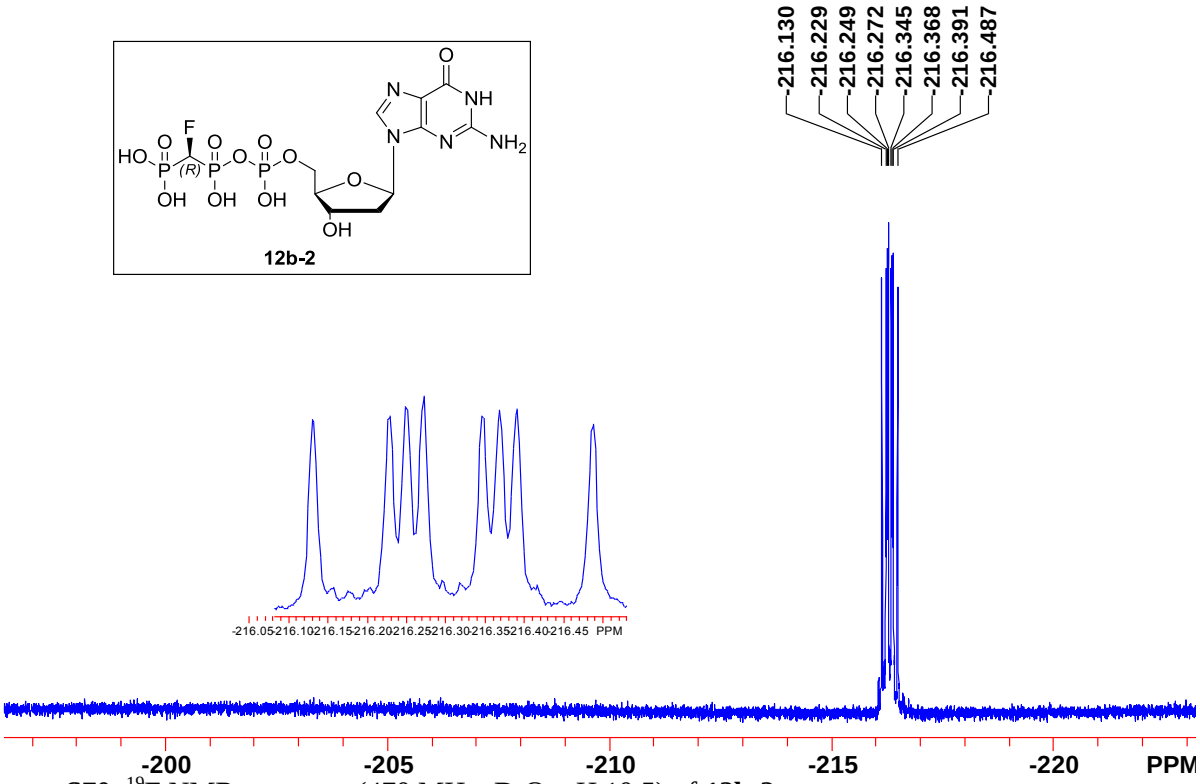

Figure S70. ^{19}F NMR spectrum (470 MHz; D_2O; pH 10.5) of **12b-2**.

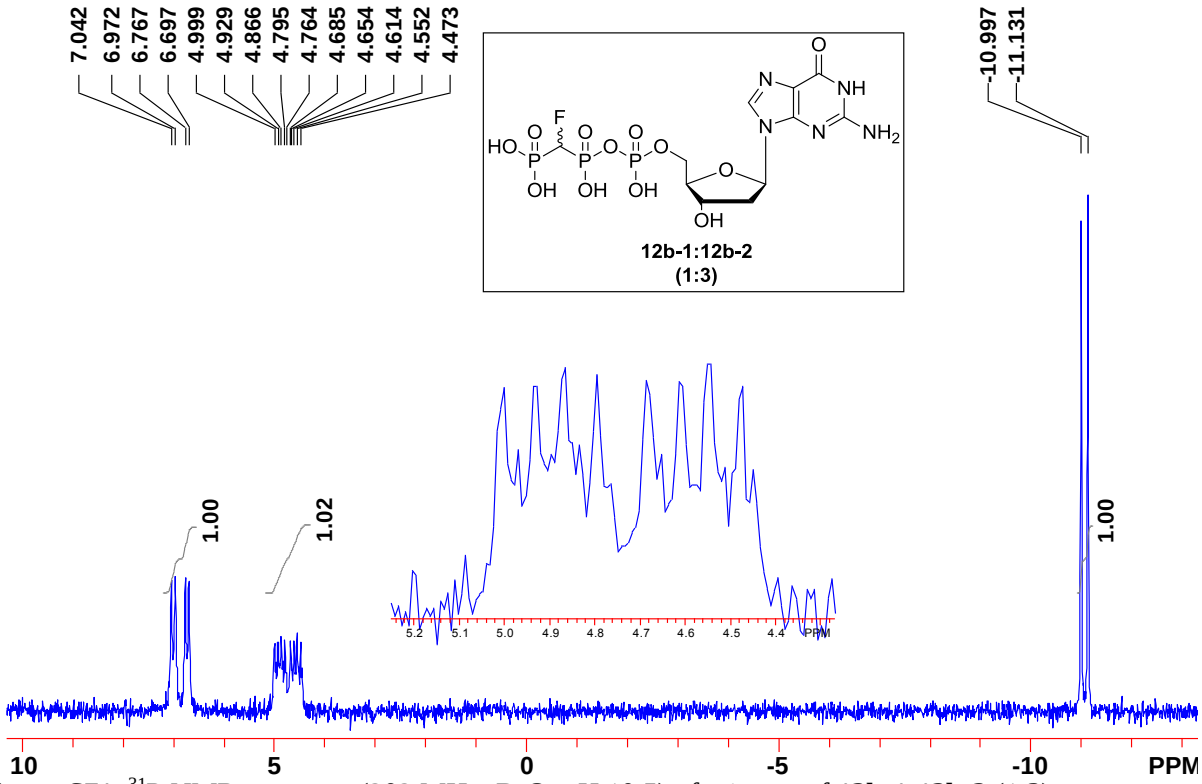

Figure S71. ^{31}P NMR spectrum (202 MHz; D_2O; pH 10.5) of mixture of **12b-1:12b-2** (1:3).

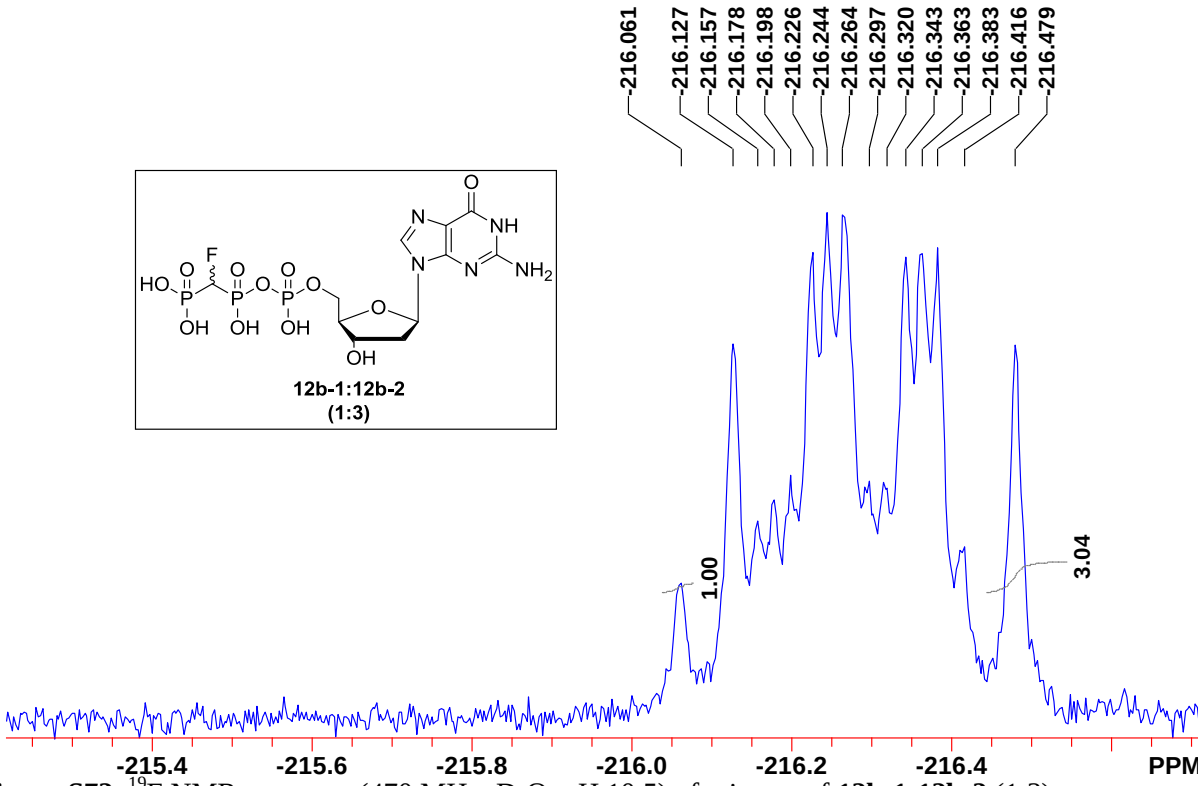

Figure S72. ^{19}F NMR spectrum (470 MHz; D_2O; pH 10.5) of mixture of **12b-1:12b-2** (1:3).

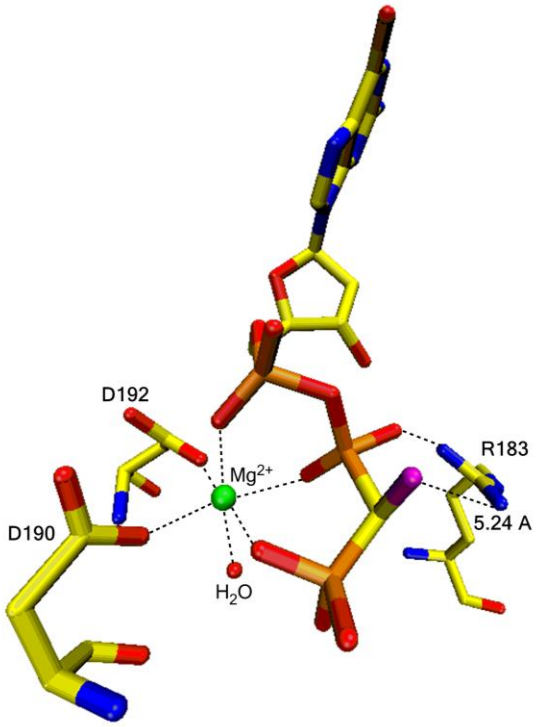

Figure S73. Detailed view of the incoming nucleotide **12a-1**, (*S*)-*β,γ*-CHCl-dGTP in the active site of the X-ray crystal structure of its ternary DNA pol β:DNA complex (PDB ID: 4DOC). The interatomic distance between the Cl (magenta) and Nη2 of Arg183 is 5.24 Å.

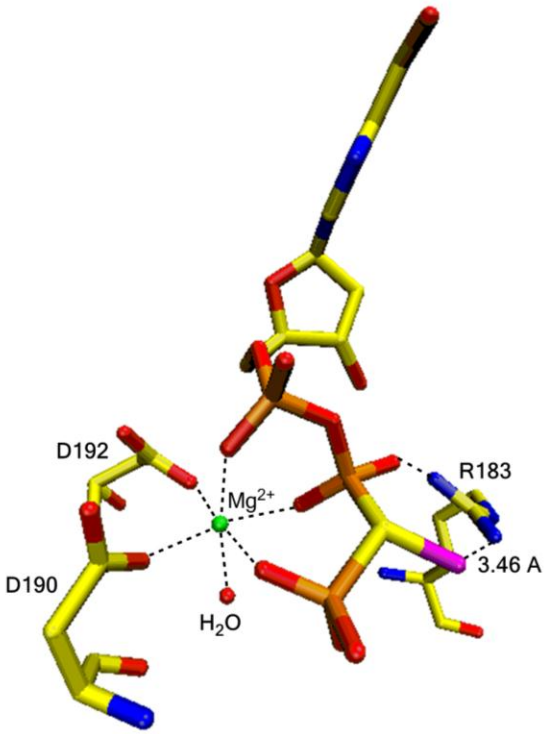

Figure S74. Detailed view of the incoming nucleotide **12a-2**, (*R*)-*β,γ*-CHCl-dGTP in the active site of the X-ray crystal structure of its ternary DNA pol β:DNA complex (PDB ID: 4DOB). The interatomic distance between the Cl (magenta) and Nη2 of Arg183 is 3.46 Å.

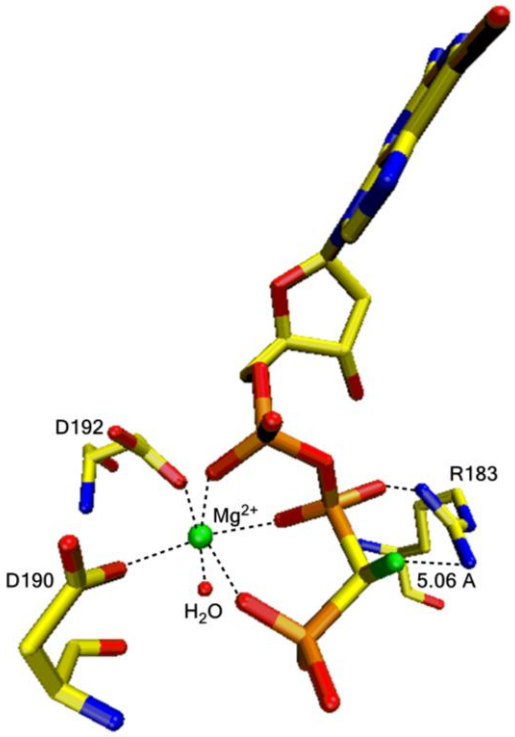

Figure S75. Detailed view of the incoming nucleotide **12b-1**, (*S*)-*β,γ*-CHF-dGTP in the active site of the X-ray crystal structure of its ternary DNA pol β:DNA complex (PDB ID: 4DOA). The interatomic distance between the F (green) and Nη2 of Arg183 is 5.06 Å.

Table S1. HPLC conditions.[a]

Experiment	Column	Mobile phase	Retention time
Separation of diastereomers of 6a	Varian Microsorb C_{18} HPLC column (5 μm, 250 mm × 21.4 mm)	0.1 N Triethylammonium bicarbonate, 3.5% CH_3CN, pH 7.2, 15.0 mL/min	**6a-1** 10.5 min
			6a-2 11.5 min
Separation of diastereomers of 7a	Varian Microsorb C_{18} HPLC column (5 μm, 250 mm × 21.4 mm)	0.1 N Triethylammonium bicarbonate, 15% CH_3CN, pH 7.4, 8.0 mL/min	**7a-1** 14.2 min
			7a-2 15.2 min
Separation of diastereomers of 7b	Varian Microsorb C_{18} HPLC column (5 μm, 250 mm × 21.4 mm)	0.1 N Triethylammonium bicarbonate, 15% CH_3CN, pH 7.4, 8.0 mL/min	**7b-1** 14.3 min
			7b-2 15.5 min
Analytical HPLC for LC-MS analysis of reaction mixture of 7b-1/7b-2	Varian Microsorb C_{18} HPLC column (5 μm, 250 mm × 4.6 mm)	0.1 N Triethylammonium bicarbonate, 10% CH_3CN, pH 7, 1.0 mL/min	**7b-1** 23.6 min
			7b-2 27.2 min
Purification of 11a-1, 11a-2, 11b-1, 11b-2	Macherey-Nagel Nucleogel SAX 1000-10 (150 mm × 25 mm)	0.5 N Triethylammonium bicarbonate, pH 7.4. Gradient: (0-10 min, 55%; 10-16 min, 55%; 16-25 min, 100%)	**11a-1** 18.5 min (9 mL/min)
			11-2 18.8 min (9 mL/min)
			11b-1 21.2 min (8 mL/min)
			11b-2 19.8 min (8 mL/min)
Purification of 12a-1, 12a-2, 12b-1, 12b-2	Varian Microsorb C_{18} HPLC column (5 μm, 250 mm × 21.4 mm)	0.1 N Triethylammonium bicarbonate, 3.5% CH_3CN, pH 7.4	**12a-1** 14.4 min (9 mL/min)
			12a-2 14.2 min (9 mL/min)
			12b-1 14.1 min (8 mL/min)
			12b-2 14.5 min (8 mL/min)

a. Detection wavelength 256 nm.

Table S2. Crystallographic statistics of **12b-2**.

Data Collection	
Space Group	$P2_1$
a (Å)	50.69
b (Å)	79.91
c (Å)	55.61
β ( )	107.66
d_{min} (Å)	2.05
R_{merge} (%)[a, b]	0.102 (0.427)
Completeness (%)	94.5 (68.3)
Unique Reflections	25195 (1817)
Total Reflections	89573
I/σ	11.1 (2.0)
Refinement	
r.m.s. deviations	
Bond lengths (Å)	0.007
Bond angles ( )	1.131
R_{work} (%)[c]	18.84
R_{free} (%)	24.31
Average B Factors (Å)	
Protein	26.50
DNA	38.39
Analogue	16.26
Ramachandaran Analysis	
Favored	98.2
Allowed	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] $R_{work} = 100 \times \Sigma |F_{obs}|-|F_{calc}|/\Sigma|F_{obs}|$

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