SUPPORTING INFORMATION

Remarkably stereospecific utilization of ATP α,β-halomethylene analogues by protein kinases

Feng Ni,[#,†,‡] Alvin Kung,[#,†,‡] Yankun Duan,[#,‖,§] Vivek Shah,[†] Carolina D. Amador,[†] Ming Guo,[⊥] Xuegong Fan,[‖] Lin Chen,[†,§] Yongheng Chen,[*,⊥] Charles E. McKenna,[*,†] and Chao Zhang[*,†,‡]

[†]Department of Chemistry, University of Southern California, Los Angeles, California 90089, United States; [‡]Loker Hydrocarbon Research Institute, University of Southern California, Los Angeles, California 90089, United States; [‖]Department of Infectious Diseases & Hunan Key Laboratory of Viral Hepatitis, XiangYa Hospital, Central South University, Changsha, Hunan 410008, China; [§]Molecular & Computational Biology Program, Department of Biological Sciences, University of Southern California, Los Angeles, California 90089, United States; [⊥]Key Laboratory of Cancer Proteomics of Chinese Ministry of Health, XiangYa Hospital, Central South University, Changsha, Hunan 410008, China

Materials and Methods

D-(-)-α-Phenylglycine methyl ester hydrochloride (Alfa Aesar, Lot No: 10171582, 96%, Optical rotation: -116°, c = 1 in water) and α,β-CH_2-ATP (lithium salt, Sigma-Aldrich) were purchased from commercial sources. Starting compounds 2',3'-*O*-isopropylidene-adenosine-5'-tosylate and methylene bisphosphonates for synthesis of **1** were prepared according to literature procedures.[1,2] Compounds **1**, **2**, **3** and **4** 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 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 are given on the δ–scale in parts per million (ppm) relative to internal D_2O (δ 4.79, ^{1}H NMR), external 85% H_3PO_4 (δ 0.00, ^{31}P NMR) and $CFCl_3$ (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 concentrations of the NMR samples were in the range of 1-5 mg/ml.

Protein Expression and Purification

6xHis tagged c-Src (residues 251-533) was coexpressed with a tyrosine phosphatase YopH in *Escherichia coli* BL21(DE3) cells.[3] Briefly, Src protein was purified by Ni-NTA immobilized metal ion affinity chromatography. The 6x-His was removed by TEV cleavage to yield the liberated kinase domain. After cleavage, an anion exchange chromatography and size-exclusion chromatography were utilized to purify the protein further. Protein in 50 mM Tris (pH 8.0), 100 mM NaCl, 5% Glycerol, 1 mM DTT was concentrated to 10 mg/ml and flash frozen for storage at -80 °C. Yields of purified c-Src protein per liter of bacteria culture ranged from 1 to 2 mg.

Crystallization and Structure Determination

For crystallization, c-Src and ATP analogue were mixed at a molar ratio of 1:2 on ice for 30 min before crystallization screen. The crystals were obtained within 2 days (mostly overnight) at 18 °C using the hanging drop vapor diffusion method. The reservoir solution contained 0.1 M MES (pH 6.4), 4% glycerol, 8% PEG 4000, 50 mM sodium acetate, 10 mM $MgCl_2$. Crystals were cryoprotected in reservoir solution plus 20% glycerol.

Diffraction data were collected at the Advanced Light Source (Lawrence Berkeley National Laboratory) beamline 502 and Shanghai Synchrotron Radiation Facility (SSRF) beamline BL17U. Data were reduced using HKL2000. The structures were solved by

molecular replacement by using the kinase domain of human c-Src (PDB code 2SRC) as the template with Phaser. The structures were refined and rebuilt using Phenix and Coot.

Peptide Synthesis

The peptides were manually synthesized on a Rink Amide MBHA resin (100-200 mesh, Novabiochem) with an approximated loading of 0.5 mmol/g used per reaction. The activation of N-Fmoc and side chain protected amino acids (Novabiochem) were conducted by HCTU (Novabiochem) for 15 min, and DIEA (EMD) followed by coupling with the resin for 1 h with N_2 line bubbling into the mixture. The Fmoc group was removed by incubating with 5% w/v piperazine in DMF for 15 min. The process was repeated until reaching desired peptide sequence. The peptide was then cleaved off by incubating it with a cleavage cocktail (5.2:1:2.6:5.2:86 H_2O/Triisopropylsilane/ethane dithiol/thioanisole/TFA) for 3 h at room temperature with gentle stirring. The solubilized cleaved peptide was precipitated out with 40 mL of cold diethyl ether at room temperature. Centrifugation collected the precipitant (5,000 rcf, 10 min, 4°C) and then the pellet was re-washed with 40 mL of cold diethyl ether follow by centrifugation once more. The crude pellet was re-dissolved with 20% acetonitrile, 0.5% DMSO, in H_2O and purified by RP-HPLC onto a C18 preparative column (Phenomenex). The fractions were collected (0-60% B gradient over 40 min) and characterized by MALDI (Voyager) in α-cyano-4-hydroxycinnamic acid. Desired fractions were flash-frozen and lyophilized overnight. Peptide was re-dissolved in 20 mM Tris, pH 8.0 to yield a stock solution of 2 mM that was used for the kinase assays.

Omnia Kinase Assay for K_M and k_{cat} Determination

For the kinetic studies, the Omnia Kinase Assay Kit (KNZ3021) reaction was run on 384-well plate (Corning) and the detailed protocols can be found on ThermoFisher Scientific. Specifically, a chelation-enhanced fluorophore-labeled peptide substrate [Ac-(Sox)PGIYGELEA-NH_2] that incorporates a fluorescence tag SOX was used to measure the phosphorylation level. Varying concentrations of nucleotides were used to determine K_M and k_{cat}. Briefly, the reaction was initiated by the addition of the master mix (10 μM Y2 Sox-based Src peptide, 200 μM DTT, 10 nM Src, 1x Src kinase buffer, pH 7.5) to the ATP analogue concentrations varied from 1 μM to 1 mM. The assay was performed at 30 ^{o}C, and the fluorescence measurement was collected every 30 s for 30 min using an excitation wavelength of 360 nm and the emission wavelength of 485 nm by a microplate reader (Synergy H4). The reaction rates were calculated based on the change in fluorescence intensity over time (RFU/sec).

Phosphocellulose-based Src Kinase Assay

To determine K_i values, the phosphocellulose disk assay was used.[4] Specifically, the assay was performed in 50 mM Tris (pH 8.0), 10 mM $MgCl_2$, 10 nM Src, 5.5 nM [γ-^{32}P]-ATP (PerkinElmer), 1 mg/mL BSA, 0.5 mM peptide substrate (IYGEFKKK, Src-tide) and various concentration of a nucleotide in 30 μL reaction. The kinase reaction was initiated by the addition of 0.5 μCi [γ^{32}-P]-ATP and allowed to run at r.t. for 30 min. Upon completion, 27 μL of the reaction was spotted onto phosphocellulose paper disk and quenched with 10% acetic acid. This was followed by three washes with 0.5% phosphoric acid, one additional wash with acetone, and drying in the air. The radioactivity in the disks was measured on a scintillation counter (Beckman LC6500). The data were analyzed using nonlinear regression (GraphPad Prism) to obtain IC_{50} values, which equal K_i values under the assay settings.

Determination of kinetic parameters for ATP analogues (Nanosyn Caliper Assay)

The kinetic parameters of ATP, (*S*)-CHFATP and (*R*)-CHFATP were determined using an assay based on electrophoresis shift. The nucleotides were prepared as 10 mM stocks in water and assayed at 12 concentrations (a 2-fold dilution series with 4 mM as the highest concentration). The kinase reactions were assembled in 384-well plates (Greiner) in a total volume of 25 μL. The ATP analogues were serially pre-diluted in water. The serial dilutions (10 uL) were added to 10 uL of the kinase buffer comprising: 100 mM HEPES pH 7.5, 2 mM DTT, 0.2% BSA, 0.02% Triton X-100, 25 mM of either $MgCl_2$ or $MnCl_2$ and the respective kinase protein (please refer to **Table S4** below for enzyme concentrations, peptide substrate sequences, and choice of divalent metal for each kinase). The reactions were initiated by addition of the 5 uL of FAM-labeled substrate peptide (prepared in 100 mM HEPES, pH 7.5) to a final concentration of 1 μM and incubated for 3 h at room temperature. Following incubation, the reactions were quenched by addition of 45 μL of the termination buffer (100 mM HEPES, pH 7.5, 0.01% Triton X-100, 50 mM EDTA). The terminated plates were analyzed on a microfluidic electrophoresis instrument (Caliper LabChip® 3000, Caliper Life Sciences/Perkin Elmer). Enzymatic phosphorylation of the substrate peptide results in a change of the net charge, enabling electrophoretic separation of product from substrate. As substrate and product are separated by electrophoresis, two peaks of fluorescence can be observed. Activity in each test sample was determined as %-conversion of the substrate peptide: P/(S+P), where P is the peak height of the product, and S is the peak height of the peptide substrate. The control samples (0%-activity, in the absence of ATP or ATP analogues) were assembled

in replicates of four and were used to calculate the %-activity values in the presence of compounds. The reaction rates were derived from the %-conversion values and then plotted against the concentration of the ATP or ATP analogs. Finally, the data were fitted with Michaelis-Menten steady state model: $V_0 = (V_{max}/(1+(K_m/[ATP])))$ to determine the K_m, V_{max} and k_{cat} values.

Labeling of Kinase Substrates with γ-S-ATP Analogues in Cell Lysates

HEK293 cells were lysed and reactions were set up containing 0.25 mM ATPγS analogs, 0.25 mM ATP, 3 mM GTP, 1 mM DTT, 0.5 mg whole-cell lysate, 20 mM Tris-HCl at pH 7.6, 20 mM $MgCl_2$ with a total volume of 20 μL at 30 ^oC for 30 min. Upon completion, the reaction was quenched with 20 mM EDTA and alkylated 2.5 mM PNBM for 1 h at 30 ^oC. Subsequently, the samples were resolved on an SDS-PAGE gel (4-20%, Bio-Rad) and transferred onto PVDF membrane (TurboTransfer, Bio-Rad), following standard western blot protocols. Briefly, the membrane was blocked for 2 h with 5% non-fat milk, washed three times with TBS-T (0.1% Tween 20, 150 mM NaCl, 10 mM Tris at pH 8.0), and incubated with the thiophosphate ester specific monoclonal antibody (Ab92570; Abcam) at 1:1,000 in 5% non-fat milk for overnight at 4 ^oC. The PVDF membrane was washed three times with TBS-T (0.1% Tween 20, 150 mM NaCl, 10 mM Tris pH 8.0) and incubated with a secondary anti-rabbit HRP conjugated antibody (1:10,000 in 5% non-fat milk, Jackson ImmunoResearch) for 1 h, washed three times, and developed using ECL (Bio-Rad) and imagined by ChemiDoc XRS+ molecular imager (Bio-Rad).

HPLC Methods for Nucleotide Purification

Preparative HPLC was performed using a Varian ProStar equipped with a Shimadzu SPD-10A UV detector (0.5 mm path length) with detection at wavelength of 254 nm. RP-HPLC and Ion-exchange HPLC separations use C_{18} column (Phenomenex, Luna, 250 × 21.2mm, 5 micron) and anion exchange columns (Macherey-Nagel, NUCLEOGEL SAX, 21.4 x 250 mm, SP15/25 and Agilent, PL-SAX, 25 x 150 mm, 1000A, 10 μM), respectively. Specific conditions for compounds in current report are summarized as follows.

Condition A for RP-HPLC, Mobile phase: solvent A, 0.1 N Triethylammonium bicarbonate, pH 7.6; solvent B, acetonitrile; elution gradient: 0-8 min, 20-35% B; 8-13 min, 35% B; flow-rate: 10 ml/min.

Condition B for Ion-exchange HPLC (column NUCLEOGEL SAX**),** Mobile phase: solvent A, HPLC water; solvent B, 0.5 N Triethylammonium bicarbonate, pH 7.8; elution

gradient: 0-5 min, 0-60% B; 5-10 min, 60% B; 10-15 min, 60-100% B, 15-20 min, 100% B; flow-rate: 8 ml/min.

Condition C for RP-HPLC, Mobile phase: solvent A, 0.25 N ammonium acetate; solvent B, acetonitrile; Isocratic: 13% B; flow-rate: 10 ml/min.

Condition D for RP-HPLC, Mobile phase: solvent A, 0.1 N Triethylammonium bicarbonate, pH 7.6; solvent B, acetonitrile; Isocratic: 5% B; flow-rate: 10 ml/min.

Condition E for RP-HPLC, Mobile phase: solvent A, 0.1 N Triethylammonium bicarbonate, pH 7.6; solvent B, acetonitrile; Isocratic: 15% B; flow-rate: 10 ml/min.

Condition F for Ion-exchange HPLC (column PL-SAX**),** Mobile phase: solvent A, HPLC water; solvent B, 0.5 N Triethylammonium bicarbonate, pH 7.8; elution gradient: 0-10 min, 100% B; 10-30 min, 100% B; flow-rate: 8 ml/min.

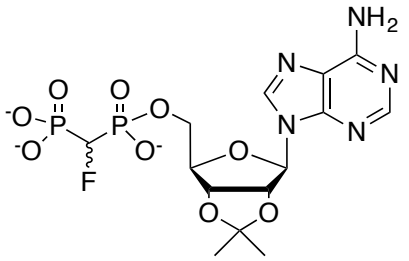
Synthesis of ATP analogues

Synthesis of 2',3'-O-isopropylideneadenosine-5'-(methylene)bisphosphonates 1

A general procedure for preparation of 2',3'-O-isopropylidene-adenosine-5'-(methylene)bisphosphonates (1)

Compound **1** was synthesized by following previously reported procedures with minor modifications.[5] The displacement reactions are performed in rubber septum sealed vials (1.5 ml) equipped for magnetic stirring under Ar atmosphere at room temperature. The well-dried tris(tetra-n-butylammonium) hydrogen (methylene)bisphosphonates (0.2 mmol) are dissolved in acetonitrile to a concentration of 1.0 to 1.5 M and 2',3'-*O*-isopropylidene-adenosine-5' tosylates (1.0 equiv.) is added to the stirred viscous solution. High concentrations of tosylate and bisphosphonates are necessary for the reactions to proceed at reasonable rates. The progress of each reaction is monitored by changes in the ^{31}P NMR. Upon completion, the reaction mixture is diluted with 5-10 volumes of water and extracted by equal volume of $CHCl_3$ (3x). The aqueous phase is loaded to RP-HPLC for purification (**HPLC Condition A**). The fractions containing product are pooled, and triethylammonium bicarbonate is removed by rotary evaporation. The resulting solid residue is dissolved in 1 ml water and then lyophilized to yield the phosphorylated nucleosides white hygroscopic solids.

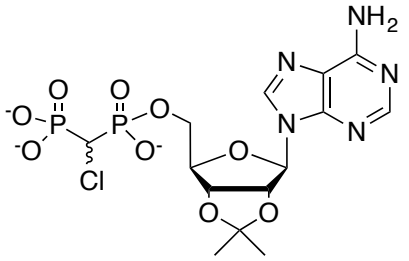
2',3'-isopropylidene-α,β-CHF-ADP (R/S-1a)



1a

Tris(tetra-n-butylammonium) hydrogen (monofluoromethylene)bisphosphonates (0.2 mmol) was dissolved in 0.1 mL of acetonitrile before addition of 2',3'-*O*-isopropylidene-adenosine-5'-tosylates (92 mg, 0.2 mmol). The solution was stirred for 3 h (~70% conversion as identified by ^{31}P NMR) before workup to yield 83.2 mg (61%, ditriethylammonium salt) of a white hygroscopic solid. HPLC: retention time: 9.0 minutes. ^{1}H NMR (400 MHz, Deuterium Oxide, pH = 9.5) δ 8.51 (s, 1H), 8.26 (s, 1H), 6.29 (d, *J* = 3.5 Hz, 1H), 5.43 (dt, *J* = 6.3, 3.2 Hz, 1H), 5.26 (dd, *J* = 6.1, 2.3 Hz, 1H), 4.67 (dd, *J* = 10.0, 2.6 Hz, 1H), 4.32 – 3.97 (m, 1H), 1.70 (s, 3H), 1.48 (s, 3H). ^{31}P NMR (162 MHz, Deuterium Oxide, pH = 9.5) δ 13.69 (dt, *J* = 63.4, 13.2 Hz), 6.96 (ddd, *J* = 55.8, 12.1, 1.5 Hz). ^{19}F NMR (376 MHz, Deuterium Oxide, pH = 9.5) δ -217.52 (ddd, *J* = 63.2, 56.6, 46.1 Hz). ESI-MS [M-H]$^{-}$: calcd. $C_{14}H_{19}FN_5O_9P_2^-$, Exact Mass: 482.1 found, 482.3 m/z.

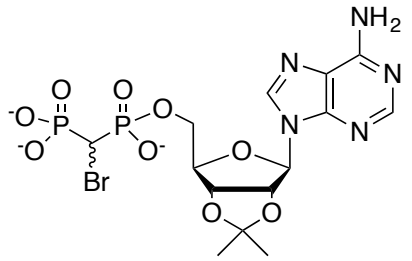
2',3'-isopropylidene-α,β-CHCl-ADP (R/S-1b)



1b

Tris(tetra-n-butylammonium) hydrogen (monochloromethylene)bisphosphonates (0.2 mrnol) was dissolved in 0.1 mL of acetonitrile before addition of 2',3'-*O*-isopropylidene-adenosine-5'-tosylates (92 mg, 0.2 mmol). The solution was stirred for overnight (~70% conversion as identified by ^{31}P NMR) before workup to yield 76.8 mg (55%, ditriethylammonium salt) of a white hygroscopic solid: HPLC: retention time: 7.5 minutes. ^{1}H NMR (400 MHz, Deuterium Oxide, pH = 9.5) δ 8.53 (s, 1H), 8.26 (s, 1H), 6.29 (d, *J* = 3.4 Hz, 2H), 5.52 – 5.35 (m, 3H), 5.27 (td, *J* = 5.9, 2.2 Hz, 3H), 4.70 – 4.63 (m, 1H), 4.32 – 4.05 (m, 2H). ^{31}P NMR (162 MHz, Deuterium Oxide, pH = 9.5) δ 15.19 (dd, *J* = 30.2, 4.0 Hz), 8.41 (t, *J* = 5.0 Hz). ESI-MS [M-H]$^{-}$: calcd. $C_{14}H_{19}ClN_5O_9P_2^-$, Exact Mass: 498.0; found, 498.3 m/z.

2',3'-isopropylidene-α,β-CHBr-ADP (R/S-1c)



1c

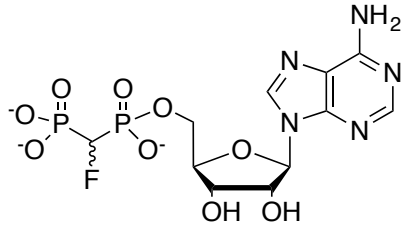
Tris(tetra-n-butylammonium) hydrogen (monobromomethylene)bisphosphonates (0.2 mmol) was dissolved in 0.1 mL of acetonitrile before addition of 2',3'-*O*-isopropylidene-adenosine-5'-tosylates (92 mg, 0.2 mmol). The solution was stirred for overnight (~70% conversion as identified by ^{31}P NMR) before workup to yield 67 mg (45%, ditriethylammonium salt) of a white hygroscopic solid: HPLC: retention time: 7.8 minutes. ^{1}H NMR (500 MHz, Deuterium Oxide, pH = 10.9) δ 8.38 (d, *J* = 6.3 Hz, 1H), 8.09 (s, 1H), 6.13 (t, *J* = 3.7 Hz, 1H), 5.91 (s, 0.2H), 5.30 (q, *J* = 5.1 Hz, 1H), 5.14 (d, *J* = 5.5 Hz, 1H), 4.89 (d, *J* = 5.8 Hz, 0.3H), 4.58 (s, 0.7H), 4.54 (t, *J* = 4.8 Hz, 1H), 4.17 – 3.94 (m, 2H), 1.56 (s, 3H), 1.33 (s, 3H). ^{31}P NMR (162 MHz, Deuterium Oxide, pH = 10.9) δ 14.91 , 14.67 , 7.52. ESI-MS $[M-H]^-$: calcd. $C_{14}H_{19}BrN_5O_9P_2^-$, Exact Mass: 542.0; found, 542.1 m/z.

Synthesis of α,β-methylene-adenosine triphosphate analogues (Enzymatic phosphorylation)

General procedure for preparation of α,β-methylene-adenosine bisphosphonates 4

10 μmol of 2',3'-*O*-isopropylideneadenosine-5'-(methylene)bisphosphonates **1a** and **1b** separately are added to 0.5 ml 1 N HCl and stirred to move the protecting group isopropylidene. After 1 h, the mixture is evaporated to remove excess of HCl followed by adding 1N Na_2CO_3 solution to adjust pH to neutral.

(R/S)-α,β-CHF-ADP R/S-4a

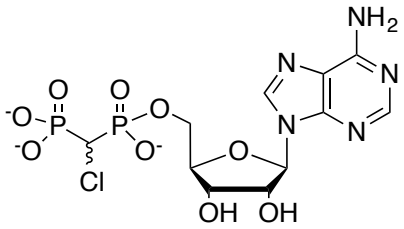


4a

Yield: quantitative; ^{1}H NMR (400 MHz, Deuterium Oxide, pH = 3) δ 8.51 (s, 1H), 8.33 (s, 1H), 6.04 (d, *J* = 5.1 Hz, 1H), 4.99 (td, *J* = 12.7, 5.7 Hz, 0.5H), 4.92 – 4.82 (m, 0.5H), 4.64 (q, *J* = 5.2 Hz, 1H), 4.42 (t, *J* = 4.7 Hz, 1H), 4.31 – 4.25 (m, 1H), 4.20 (ddq, *J* = 14.8,

8.7, 2.8 Hz, 1H). ^{31}P NMR (162 MHz, Deuterium Oxide, pH = 6.5) δ 10.53 (dd, *J* = 65.7, 12.5 Hz), 9.12 (dd, *J* = 60.2, 12.6 Hz). ^{19}F NMR (376 MHz, Deuterium Oxide, pH = 6.5) δ -224.22 (dddd, *J* = 65.5, 60.4, 45.2, 12.8 Hz). ESI-MS [M-H]$^-$: calcd. $C_{11}H_{15}FN_5O_9P_2^-$, 442.0; found, 442.3 m/z.

(R/S)-α,β-CHCl-ADP R-S-4b



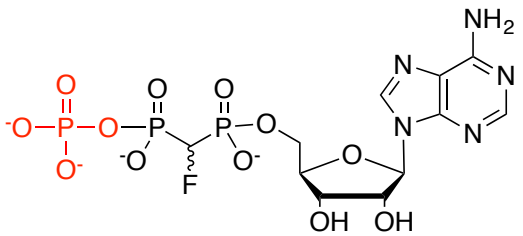
4b

Yield: quantitative. ^{1}H NMR (400 MHz, Deuterium Oxide, pH =10) δ 8.60 (s, 1H), 8.27 (s, 1H), 6.15 (d, *J* = 5.1 Hz, 1H), 4.67 – 4.60 (m, 1H), 4.39 (s, 1H), 4.35 – 4.06 (m, 2H). .^{31}P NMR (162 MHz, Deuterium Oxide, pH = 10) δ 15.53 (dd, *J* = 6.0, 3.6 Hz), 8.40 (dd, *J* = 8.4, 3.7 Hz). ESI-MS [M-H]$^-$: calcd. $C_{11}H_{15}ClN_5O_9P_2^-$, 458.0; found, 458.2 m/z.

General procedure for enzymatic phosphorylation of α,β-methylene-adenosine bisphosphonates 4 to α,β-methylene-adenosine triphosphates 3.

α,β-methylene-adenosine diphosphates **4** are added to eppendorf vials and dissolved in appropriate volume of freshly prepared reaction buffer containing 200 mM KCl, 80mM $MgCl_2$, 100 mM phosphoenol pyruvic acid (PEP), catalytic amount of UTP (1.0 mM), 10 units/ml nucleoside diphosphate kinase (NDPK), 12 units/ml pyruvate kinase (PK) and 100 mM HEPES (pH 7.5). The reaction is diluted by adding water and is monitored by changes in the ^{1}H NMR chemical shift for nucleotide pyrimidine proton. After approximately 24-72 hours, the enzymes are removed by 0.2 micro membrane filtration and the reaction mixture is quenched by EDTA. The products are purified via preparative ion-exchange HPLC (using **Condition B**)

(R/S)-α,β-CHFATP R/S-3a

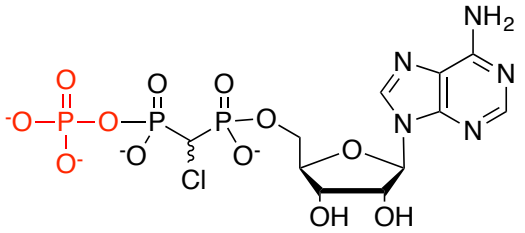


***R/S*-3a**

4a (5 mg, 10 μmol) is added to an eppendorf vial and dissolved in 0.25 ml freshly prepared reaction buffer. Reaction mixture is diluted by 0.75 ml water and stays at RT.

After 24 h, reaction is quenched by 0.4 ml 0.1 N EDTA before HPLC purification to give **3a**. (4.1mg, 82%). >97% purity. ^{1}H NMR (500 MHz, Deuterium Oxide, pH = 10.2) δ 8.58 (s, 1H), 8.26 (s, 2H), 6.15 (d, *J* = 5.9 Hz, 2H), 5.25 – 5.06 (m, 2H), 4.63 – 4.54 (m, 2H), 4.43 – 4.35 (m, 2H), 4.33 – 4.21 (m, 2H). ^{31}P NMR (202 MHz, D_2O, pH =10.2) δ 11.41 (ddd, *J* = 61.0, 26.5, 14.8 Hz), -0.16 (ddt, *J* = 61.2, 25.6, 13.3 Hz), -5.38 (d, *J* = 25.2 Hz). ^{19}F NMR (470 MHz, D_2O, pH =10.2) δ -219.64 (dtd, *J* = 222.2, 61.0, 45.3 Hz). ESI-MS [M-H]-: calcd. $C_{11}H_{16}FN_5O_{12}P_3^-$, 522.0; found, 522.2 m/z.

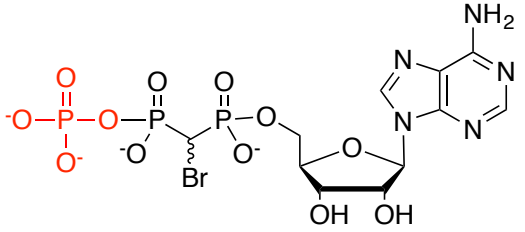
(R/S)-α,β-CHClATP R/S-3b



R/S*-3b** ***or 3b-1/2

4b (5.2 mg, 10 μmol) is added to an eppendorf vial and dissolved in 0.25 ml freshly prepared reaction buffer. Reaction mixture is diluted by 0.75 ml water and stays at RT. After 72 h, reaction is quenched by 0.4 ml 0.1 N EDTA before HPLC purification to give **3b**. (3.2 mg, 64%). >97% purity. ^{1}H NMR (500 MHz, Deuterium Oxide, pH = 10) δ 8.58 (s, 1H), 8.22 (s, 1H), 6.13 (d, *J* = 5.8 Hz, 1H), 4.64 – 4.54 (m, 1H), 4.42 – 4.34 (m, 1H), 4.34 – 4.13 (m, 3H). ^{31}P NMR (202 MHz, Deuterium Oxide, pH = 10) δ 13.00 (dd, *J* = 23.3, 6.5 Hz), 1.88 (dd, *J* = 24.9, 6.3 Hz), -5.23 (dd, *J* = 24.7, 2.7 Hz). ESI-MS [M-H]-: calcd. $C_{11}H_{16}ClN_5O_{12}P_3^-$, 538.0; found, 538.2 m/z.

(R/S)-α,β-CHBr-ATP R/S-3c



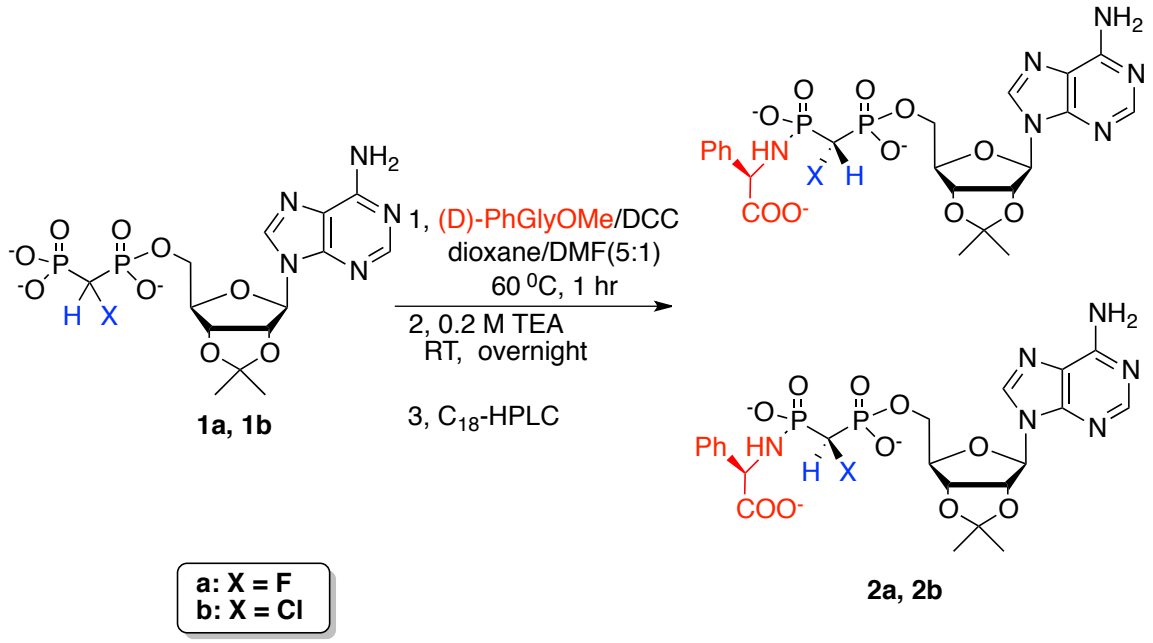
***R/S*-3c**

20 μmole of 2',3'-*O*-isopropylideneadenosine-5'-(methylenebromo)bisphosphonates **1c**, is added to 1 ml 1 N HCl and stirred to move the protecting group isopropylidene. After 1 h, the mixture is evaporated to remove excess of HCl followed by adding 1N Na_2CO_3 solution to adjust pD to neutral and lyophilized to dryness **4c**, which is directly dissolved in 0.25 ml fresh reaction buffer. Reaction mixture is diluted by 0.75 ml water and stays at RT. After 72 h, reaction is quenched by 0.4 ml 0.1 N EDTA before HPLC purification to give **3c**. (5.8 mg, 50%). >96% purity. ^{1}H NMR (500 MHz, Deuterium Oxide, pH = 10) δ

8.51 (s, 1H), 8.50 (s, 0.6H), 8.13 (s, 0.4H), 6.02 (d,*J* = 5.8 Hz, 1H), 4.58 – 4.44 (m, 1H), 4.27 (s, 1H), 4.23 – 3.95 (m, 3H). ^{31}P NMR (202 MHz, Deuterium Oxide, pH = 10) δ 12.62 (d, *J* = 4.5 Hz), 12.44 (d, *J* = 5.1 Hz), 0.72 (dd, *J* = 25.5, 4.7 Hz), -5.87 (dd, *J* = 25.4, 2.9 Hz). ESI-MS [M-H]-: calcd. $C_{11}H_{16}BrN_5O_{12}P_3^-$, Exact Mass: 581.9; found, 582.1 m/z.

Synthesis of α,β-CHF(Cl)-ATP individual isomers (Chemical method using auxiliary ligand)

Synthesis of (D)-phenylglycine derivatives of α,β-CHF(Cl)-ADP individual isomers (2a and 2b)

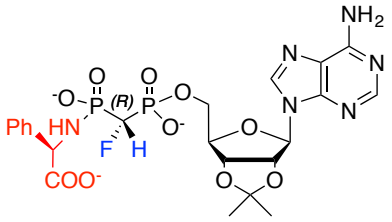


A general procedure for preparation of (D)-phenylglycine-α,β-CHF(Cl)-ADP individual isomers (2a and 2b)

1 as di(tetra-n-butylammonium salt (prepared by mixing 2 equiv. of tetra-n-butylammonium bicarbonate with **1** followed by lyophilization) and 7 equiv. DCC are dissolved by dioxane/DMF (5:1) solution containing 20 equiv. NEt_3 in a dried capped glass vial (6 ml), 7 equiv. of *(D)*-phenylglycine methyl ester (HCl salt) is added and the mixture is stirred at 65 °C for 1 h. After removing the precipitate (DCU), reaction mixture is evaporated under vacuum to dry solid. 0.2 M NEt_3 is added to the dry solid and stirred overnight at RT for cleavage of methyl group in the *(D)*-phenylglycine ester. The mixture is filtered and the filtrate extracted by $CHCl_3$ (3x). The aqueous phase is loaded to RP-HPLC for purification (**Condition C**). The fractions containing product are pooled, and triethylammonium bicarbonate is removed by rotary evaporation. The resulting solid

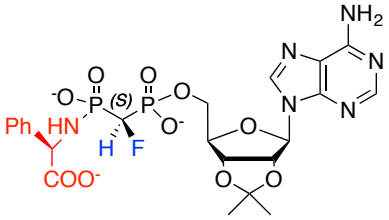
residue is dissolved in 1 ml water and then lyophilized to yield white solids.

R-(D)-PhGlyOH-CHF-ADP [(R)-2a] and S-(D)-PhGlyOH-CHF-ADP [(S)-2a]



***R*-2a**

or 2a-2



***S*-2a**

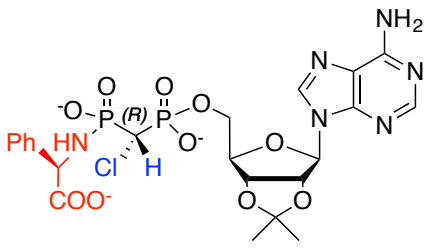
or 2a-1

1a (di(tetra-n-butylammonium) salt, 78 mg, 0.065 mmol) and DCC (90 mg, 0.455 mmol) are dissolved by 3 ml dioxane/DMF (5:1) solution containing 0.175ml NEt_3 (1.3 mmol) in a dried capped glass vial (6 ml), *(D)*-phenylglycine methyl ester (HCl salt, 90 mg, 0.455 mmol) is added and the mixture is stirred at 65 °C for 1 h (> 95% conversion as identified by ^{31}P NMR) before workup to yield (*S*)-**2a** (12 mg, 26%) and (*R*)-**2a** (13.9 mg, 30%,. HPLC retention time: 15.7 minutes [(*S*)-**2a**] and 17.3minutes [(*R*)-**2a**]; >97% purity.

(*S*)-**2a**: ^{1}H NMR **(**500 MHz, D_2O, pH = 10.9**)** δ 8.36 (s, 1H), 8.28 (s, 1H), 7.42 (d, *J* = 7.4 Hz, 2H), 7.25 (t, *J* = 7.6 Hz, 2H), 7.16 (t, *J* = 7.4 Hz, 1H), 6.26 (d, *J* = 3.3 Hz, 1H), 5.30 (dd, *J* = 6.1, 3.2 Hz, 1H), 5.12 (dd, *J* = 6.1, 1.9 Hz, 1H), 4.83 (s, 1H), 4.62 (s, 1H), 4.30 (dt, *J* = 46.1, 11.5 Hz, 1H), 4.02 (dt, *J* = 11.6, 4.0 Hz, 1H), 3.92 (dd, *J* = 10.9, 6.3 Hz, 1H), 1.70 (s, 1H), 1.49 (s, 1H). ^{31}P NMR (202 MHz, D_2O, pH = 10.9) δ 11.26 (dd, *J* = 62.1, 12.3 Hz), 9.82 (dd, *J* = 59.8, 12.4 Hz). ^{19}F NMR (470 MHz, D_2O, pH = 10.9) δ -218.07 (td, *J* = 61.0, 46.1 Hz). ESI-MS $[M-H]^-$: calcd. $C_{22}H_{26}FN_6O_{10}P_2^-$, Exact Mass: 615.1; found, 615.5 m/z.

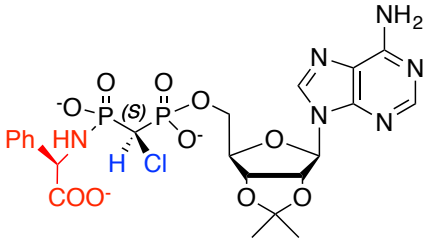
(*R*)-**2a**: ^{1}H NMR **(**500 MHz, D_2O, pH = 10.0**)** δ 8.37 (s, 1H), 8.29 (s, 1H), 7.38 (d, *J* = 7.5 Hz, 2H), 7.21 (t, *J* = 7.7 Hz, 2H), 7.10 (q, *J* = 7.9, 7.4 Hz, 1H), 6.25 (d, *J* = 3.4 Hz, 1H), 5.20 (dt, *J* = 5.4, 2.7 Hz, 1H), 5.10 (dd, *J* = 6.1, 2.1 Hz, 1H), 4.83 (s, 1H), 4.57 (s, 1H), 4.21 (dt, *J* = 45.4, 11.7 Hz, 1H), 4.07 – 3.99 (m, 1H), 3.96 – 3.85 (m, 1H), 1.69 (s, 3H), 1.47 (s, 3H). ^{31}P NMR (202 MHz, D_2O, pH = 10.0) δ 11.41 (dd, *J* = 61.7, 11.2 Hz), 9.96 (dd, *J* = 60.7, 11.2 Hz). ^{19}F NMR (470 MHz, D_2O, pH = 10.0) δ -217.38 (td, *J* = 61.2, 45.4 Hz). ESI-MS $[M-H]^-$: calcd. $C_{22}H_{26}FN_6O_{10}P_2^-$, Exact Mass: 615.1; found, 615.5 m/z.

R-(D)-PhGlyOH-CHCl-ADP [(R)-2b] and S-(D)-PhGlyOH-CHCl-ADP [(S)-2b]



***R*-2b**

or 2b-2



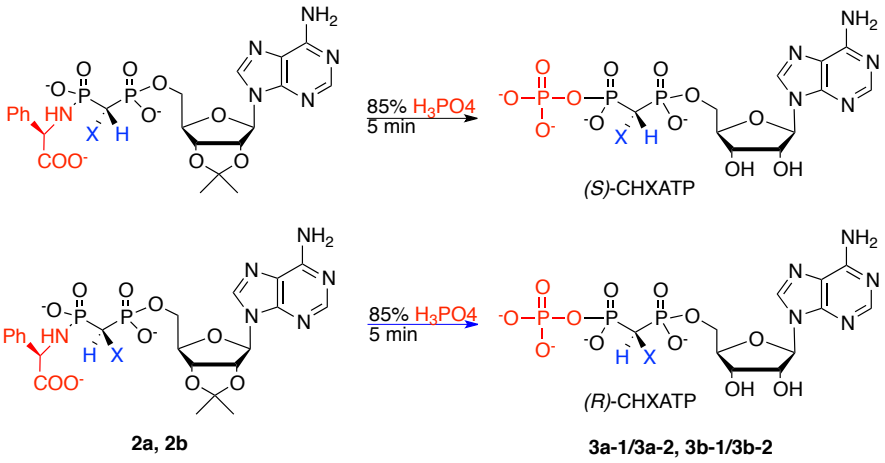
***S*-2b**

or 2b-2

1b (di(tetra-n-butylammonium) salt, 78 mg, 0.065 mmol) and DCC (90 mg, 0.455 mmol) are dissolved by 3 ml dioxane/DMF (5:1) solution containing 0.175 ml NEt_3 (1.3 mmol) in a dried capped glass vial (6 ml), *(D)*-phenylglycine methyl ester (HCl salt, 90 mg, 0.455 mmol) is added and the mixture is stirred at 65 °C for 1 h (> 90% conversion as identified by ^{31}P NMR) before workup to yield (*S*)-**2b** (10.9 mg, 23%) and (*R*)-**2b** (14.7 mg, 31%). HPLC retention time: 18.5 minutes [(*S*)-**2b**] and 20.3 minutes [(*R*)-**2b**]; >97% purity.

(*S*)-**2b**: ^{1}H NMR (500 MHz, D_2O, pH = 10.2) δ 8.39 (s, 1H), 8.27 (s, 1H), 7.44 (d, *J* = 7.6 Hz, 2H), 7.28 (t, *J* = 7.6 Hz, 2H), 7.17 (t, *J* = 7.4 Hz, 1H), 6.26 (d, *J* = 3.4 Hz, 1H), 5.33 (dd, *J* = 6.0, 3.2 Hz, 1H), 5.15 – 5.05 (m, 1H), 4.7 (s, 1H), 4.62 (d, *J* = 4.3 Hz, 1H), 4.00 (d, *J* = 11.1 Hz, 1H), 3.85 (s, 1H), 3.34 – 3.27 (m, 1H), 1.70 (s, 3H), 1.49 (s, 3H). ^{31}P NMR (202 MHz, D_2O, pH = 10.2) δ 12.52 (s), 11.20 (s). ESI-MS $[M-H]^-$: calcd. $C_{22}H_{26}ClN_6O_{10}P_2^-$, Exact Mass: 631.1; found, 631.5 m/z.
(*R*)-**2b**: ^{1}H NMR (500 MHz, D_2O, pH = 10.2) δ 8.43 (s, 1H), 8.27 (s, 1H), 7.41 (d, *J* = 7.6 Hz, 2H), 7.25 (t, *J* = 7.6 Hz, 2H), 7.15 (t, *J* = 7.4 Hz, 1H), 6.26 (d, *J* = 3.4 Hz, 1H), 5.28 (dd, *J* = 6.0, 3.2 Hz, 1H), 5.18 (m, 1H), 4.7 (s, 1H), 4.60 (d, *J* = 4.3 Hz, 1H), 4.06 (d, *J* = 11.1 Hz, 1H), 3.95 (s, 1H), 3.28 – 3.23 (m, 1H), 1.69 (s, 3H), 1.47 (s, 3H). ^{31}P NMR (202 MHz, D_2O, pH = 10.2) δ 12.75 (s), 11.30 (s). ESI-MS $[M-H]^-$: calcd. $C_{22}H_{26}ClN_6O_{10}P_2^-$, Exact Mass: 631.1; found, 631.5 m/z.

Synthesis of α,β-CHF(Cl)-ATP individual isomers (chemical phosphorylation)

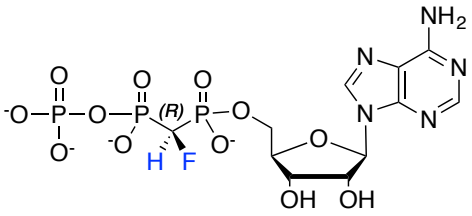


A general procedure for preparation of α,β-CHF(Cl)-ATP and α,β-CHF(Cl)-ADP isomers (3a, 3b and 4a, 4b)

1 equiv. of **2** is mixed with 20 equiv. 85% H_3PO_4 solution followed by stirring or vortexing for 5 min. The reaction mixture is then quenched by adding 30 equiv. 2 N Na_2CO_3 solution dropwise before Ion-exchange HPLC separation (using **Condition B**). The fractions containing products are pooled, and triethylammonium bicarbonate in mobile phase is removed by rotary evaporation, yielding **3a**, **3b**, **4a** and **4b**. ADP

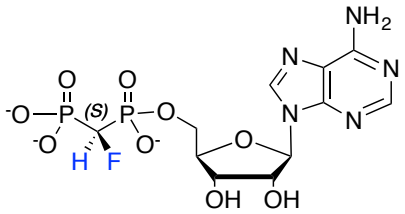
analogues **4a** and **4b** are further purified by RP-HPLC (using **Condition D**). The resulting solid residue is dissolved in 1 ml of water and then lyophilized to yield white solids.

(R)-CHF-ATP (R-3a) and (S)-CHF-ADP (S-4a)



***R*-3a**

or 3a-1



***S*-4a**

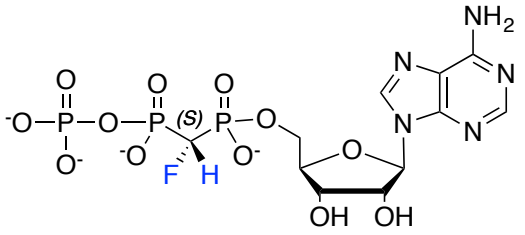
or 4a-1

(*S*)-**2a** (26 mg, 0.03 mmol) is mixed with 85% H_3PO_4 (0.045 ml, 0.6 mmol) followed by stirring or vortexing for 5 min (~85% and ~15% conversions for **3** and **4**, respectively, identify by ^{31}P NMR). The reaction is then quenched by adding 0.9 ml 2 N Na_2CO_3 dropwise before Ion-exchange HPLC separation (**Condition B**) to yield **3a-1** (monotriethylammonium salt, 13.0 mg, 70%) and Ion-exchange (**Condition B**) /RP-HPLC separations to yield **4a-1** (2.0 mg, 10.5%).

HPLC retention time: 12.2 minutes (**3a-1**); >97% purity. ^1H NMR (400 MHz, D_2O, pH = 9.7) δ 8.60 (s, 1H), 8.28 (s, 1H), 6.16 (d, *J* = 5.9 Hz, 1H), 5.16 (td, *J* = 48.9, 24.4 Hz, 1H), 4.79 (br, 1H), 4.62 (dd, *J* = 5.1, 3.5 Hz, 1H), 4.39 (s, 1H), 4.35 – 4.20 (m, 2H). ^{31}P NMR (162 MHz, D_2O, pH = 9.7) δ 11.33 (dd, *J* = 61.1, 14.8 Hz), -0.66 (ddd, *J* = 61.4, 25.8, 14.7 Hz), -5.62 (d, *J* = 24.2 Hz). ^{19}F NMR (376 MHz, D_2O, pH = 9.7) δ -219.11 (td, *J* = 61.2, 45.5 Hz). ESI-MS [M-H]-: calcd $C_{11}H_{16}FN_5O_{12}P_3^-$, 522.0; found, 522.2 m/z.

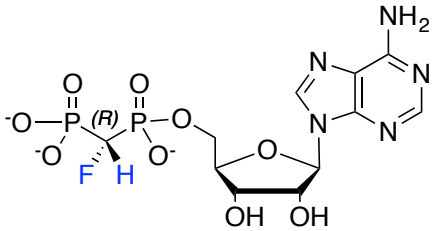
Ion-exchange (**Condition B**) and RP-HPLC retention times (**4a-1**): 14.4 minutes and 13 minutes, respectively; >96% purity. ^1H NMR (500 MHz, D_2O, pH = 10.4) δ 8.45 (s, 1H), 8.14 (s, 1H), 6.02 (d, *J* = 5.2 Hz, 1H), 4.58 (t, *J* = 12.0 Hz, 1H), 4.48 (t, *J* = 4.6 Hz, 1H), 4.24 (s, 1H), 4.22-4.09 (m, 2H). ^{31}P NMR (202 MHz, D_2O, pH = 10.4) δ 13.94 (dd, *J* = 63.5, 11.3 Hz), 6.86 (dd, *J* = 56.1, 11.3 Hz). ^{19}F NMR (470 MHz, D_2O, pH = 10.4) δ -216.68 (ddd, *J* = 63.6, 56.2, 45.3 Hz). ESI-MS [M-H]-: calcd. $C_{11}H_{15}FN_5O_9P_2^-$, 442.0; found, 442.3 m/z.

(S)-CHF-ATP (S-3a) and (R)-CHF-ADP (R-4a)



***S*-3a**

or 3a-2



***R*-4a**

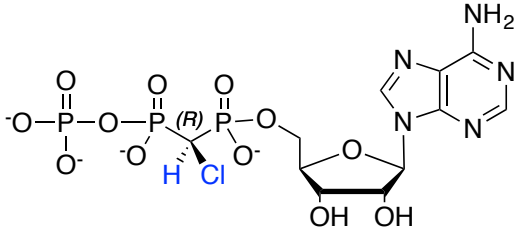
or 4a-2

(*R*)-**2a** (26 mg, 0.03 mmol) is mixed with 85% H_3PO_4 (0.045 ml, 0.6 mmol) followed by stirring or vortexing for 5 min (~85% and ~15% conversions for **3** and **4**, respectively, identify by ^{31}P NMR). The reaction is then quenched by adding 0.9 ml 2 N Na_2CO_3 dropwise before Ion-exchange HPLC separation (**Condition B**) to yield **3a-2** (monotriethylammonium salt, 11.9 mg, 64%) and Ion-exchange (**Condition B**) /RP-HPLC separations to yield **4a-2** (1.8 mg, 9.6%).

HPLC retention time: 12.2 minutes (**3a-2**); >97% purity. ^1H NMR **(**400 MHz, D_2O, pH = 9.9**)** δ 8.59 (s, 1H), 8.28 (s, 1H), 6.15 (d, *J* = 6.0 Hz, 1H), 5.16 (ddd, 45.3, 14.1, 12.1 Hz, 1H), 4.79 (br, 1H), 4.61 (dd, *J* = 5.1, 3.5 Hz, 1H), 4.42 – 4.38 (m, 1H), 4.33 – 4.21 (m, 2H). ^{31}P NMR (162 MHz, D_2O, pH = 9.9) δ 11.48 (dd, *J* = 60.9, 14.8 Hz), -0.60 (ddd, *J* = 61.4, 25.8, 14.7 Hz), -5.61 (d, *J* = 25.6 Hz). ^{19}F NMR (376 MHz, D_2O, pH = 9.9) δ -219.63 (td, *J* = 61.1, 45.3 Hz). ESI-MS [M-H]-: calcd. $C_{11}H_{16}FN_5O_{12}P_3^-$, 522.0; found, 522.2 m/z.

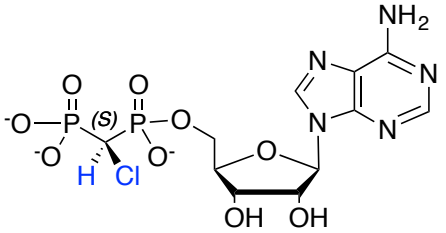
Ion-exchange (**Condition B**) and RP-HPLC retention times (**4a-2**): 14.4 minutes and 13 minutes, respectively; >96% purity. ^1H NMR **(**500 MHz, D_2O, pH = 10.4**)** δ 8.45 (s, 1H), 8.14 (s, 1H), 6.02 (d, *J* = 5.4 Hz, 2H), 4.57 (t, *J* = 12.0 Hz, 1H), 4.47 (t, *J* = 4.6 Hz, 1H), 4.24 (s, 1H), 4.22-4.05 (m, 1H). ^{31}P NMR (202 MHz, D_2O, pH = 10.4) δ 13.95 (dd, *J* = 63.3, 11.5 Hz), 6.83 (dd, *J* = 56.7, 11.0 Hz). ^{19}F NMR (470 MHz, D_2O, pH = 10.4) δ -217.21 (ddd, *J* = 63.1, 56.0, 45.3 Hz). ESI-MS [M-H]-: calcd. $C_{11}H_{15}FN_5O_9P_2^-$, 442.0; found, 442.3 m/z.

(R)-CHCl-ATP (R-3b) and (S)-CHCl-ADP (S-4b)



***R*-3b**

or 3b-1



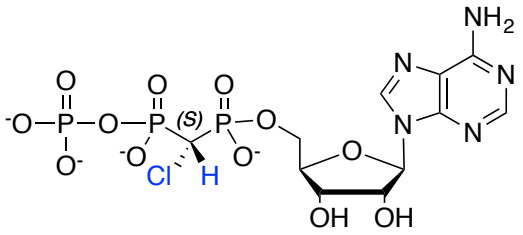
***S*-4b**

or 4b-1

(*S*)-**2b** (10.8 mg, 0.015 mmol) is mixed with 85% H_3PO_4 (0.023 ml, 0.3 mmol) followed by stirring or vortexing for 5 min (~85% and ~15% conversions for **3** and **4**, respectively, identify by ^{31}P NMR). The reaction is then quenched by adding 0.45 ml 2 N Na_2CO_3 dropwise before Ion-exchange HPLC separation (**Condition B**) to yield **3b-1** (monotriethylammonium salt, 6.2 mg, 65%) and Ion-exchange (**Condition B**) /RP-HPLC separations to yield **4b-1** (0.9 mg, 9.5%).

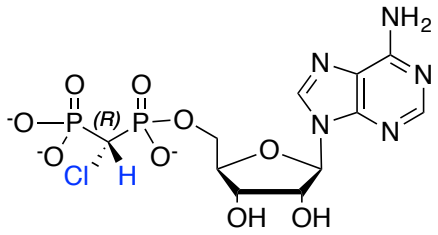
HPLC retention time: 13.7 minutes (**3b-1**); >97% purity. ^1H NMR (400 MHz, D_2O, pH = 10.7) δ 8.63 (s, 1H), 8.28 (s, 1H), 6.15 (d, *J* = 5.4 Hz, 1H), 4.62 (s, 1H), 4.40 (s, 1H), 4.36 – 4.17 (m, 3H). ^{31}P NMR (162 MHz, D_2O, pH = 10.7) δ 12.69 (d, *J* = 6.3 Hz), 0.84 (dd, *J* = 25.6, 6.3 Hz), -6.04 (d, *J* = 25.5 Hz). ESI-MS [M-H]-: calcd. $C_{11}H_{16}ClN_5O_{12}P_3^-$, 538.0; found, 538.2 m/z.
Ion-exchange- and RP-HPLC retention times (**4b-1**): 15.8 minutes and 13.5 minutes, respectively; >96% purity. ^1H NMR (500 MHz, Deuterium Oxide, pH = 10.2) δ 8.48 (s, 1H), 8.14 (s, 1H), 6.02 (d, *J* = 5.7 Hz, 1H), 4.48 (s, 1H), 4.25 (s, 1H), 4.22 – 4.04 (m, 2H). ^{31}P NMR (162 MHz, Deuterium Oxide, pH = 10.2) δ 15.40 , 8.23. ESI-MS [M-H]-: calcd. $C_{11}H_{13}ClN_5O_9P_2^-$, 458.02; found, 558.2 m/z. ESI-MS [M-H]-: calcd. $C_{11}H_{13}ClN_5O_9P_2^-$, 458.0; found, 458.2 m/z.

(S)-CHCl-ATP (S-3b) and (R)-CHCl-ADP (R-4b)



***S*-3b**

or 3b-2



***R*-4b**

or 4b-2

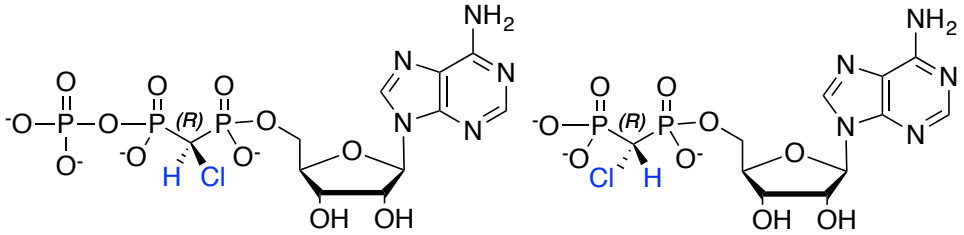
(*R*)-**2b** (14.0 mg, 0.02 mmol) is mixed with 85% H_3PO_4 (0.03 ml, 0.4 mmol) followed by stirring or vortexing for 5 min (~85% and ~15% conversions for **3** and **4**, respectively, identify by ^{31}P NMR). The reaction is then quenched by adding 0.6 ml 2 N Na_2CO_3 dropwise before Ion-exchange HPLC separation (**Condition B**) to yield **3b-2** (monotriethylammonium salt, 7.7 mg, 60%) and Ion-exchange (**Condition B**) /RP-HPLC separations to yield **4b-2** (1.2 mg, 9.1%).

HPLC retention time: 13.7 minutes (**3b-2**); >97% purity. ^1H NMR (400 MHz, D_2O, pH = 10.7) δ 8.60 (s, 1H), 8.24 (s, 1H), 6.12 (d, *J* = 5.8 Hz, 1H), 4.79 (br, 1H), 4.63 (s, 1H), 4.35 (s, 1H), 4.32 – 4.13 (m, 3H). ^{31}P NMR (162 MHz, D_2O, pH = 10.7) δ 12.87 (d, *J* = 5.8 Hz), 0.91 (dd, *J* = 25.5, 6.0 Hz), -5.98 (d, *J* = 25.7 Hz). ESI-MS [M-H]-: ESI-MS [M-H]-: calcd. $C_{11}H_{16}ClN_5O_{12}P_3^-$, 538.0; found, 538.2 m/z.

Ion-exchange- and RP-HPLC retention times (**4b-2**): 15.8 minutes and 13.5 minutes, respectively; >96% purity. ^{1}H NMR (500 MHz, Deuterium Oxide, pH = 10.2) δ 8.49 (s, 1H), 8.14 (s, 1H), 6.02 (d, *J* = 5.6 Hz, 1H), 4.52 (s, 1H), 4.25 (s, 1H), 4.22 – 4.03 (m, 2H). ^{31}P NMR (162 MHz, Deuterium Oxide, pH = 10.2) δ 15.44 , 8.17. ESI-MS [M-H]-: calcd. $C_{11}H_{13}ClN_5O_9P_2^-$, 458.0; found, 458.2 m/z.

Resolution of α,β-CHCl-ATP and α,β-CHBr-ATP individual isomers by Src kinase/YopH phosphatase

(R)-CHCl-ATP (R-3b) and (R)-CHCl-ADP (R-4b)



***R*-3b** ***R*-4b**

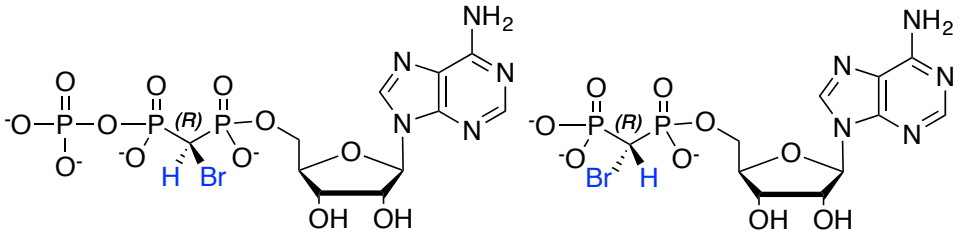
or 3b-1 ***or 4b-1***

1 mL reaction mixture of (*R/S*)-α,β-CHClATP (8 mM), Src-peptide (IYGEFKKK, 0.4 mM), YopH (50 nM) and Src (200 nM) in reaction buffer (100 mM Tris , 150 mM KCl, 10 mM $MgCl_2$ and 5 mM DTT) was incubated at RT for 24 h. The reaction was then subjected to Ion-exchange HPLC separation (**Condition B**) to yield **3b-1** (monotriethylammonium salt, 2.4 mg, 38%) and **4b-1** (2.2 mg, 41%).

Ion-exchange-HPLC retention time: 13.7 minutes (**3b-1**); >97% purity. ^{1}H NMR (500 MHz, Deuterium Oxide, pH = 10.5) δ 8.49 (s, 1H), 8.14 (s, 1H), 6.02 (d, *J* = 5.9 Hz, 1H), 4.51 – 4.44 (m, 1H), 4.27 (s, 1H), 4.21 – 4.11 (m, 2H). ^{31}P NMR (202 MHz, Deuterium Oxide, pH = 10.5) δ 12.76 (d, *J* = 6.3 Hz), 1.09 (dd, *J* = 25.5, 6.4 Hz), -5.76 (d, *J* = 25.3 Hz). ESI-MS [M-H]-: calcd. $C_{11}H_{16}ClN_5O_{12}P_3^-$, 538.0; found, 538.2 m/z.

Ion-exchange-HPLC retention time (**4b-1**): 15.8 minutes >96% purity. ^{1}H NMR (500 MHz, Deuterium Oxide, pH = 10.5) δ 8.47 (s, 1H), 8.14 (s, 1H), 6.02 (d, *J* = 5.4 Hz, 1H), 4.51 (t, *J* = 4.7 Hz, 1H), 4.25 (d, *J* = 2.5 Hz, 1H), 4.23 – 4.03 (m, 2H). ^{31}P NMR (202 MHz, Deuterium Oxide, pH = 10.5) δ 15.55, 8.31. ESI-MS [M-H]-: calcd. $C_{11}H_{13}ClN_5O_9P_2^-$, 458.0; found, 458.2 m/z.

(R)-CHBrATP (R-3c] and (R)-CHBrADP (R-4c]



***R*-3c** ***R*-4c**

1 mL reaction mixture of (R/S)-α,β-CHBrATP (8 mM), Src-peptide (IYGEFKKK, 0.4 mM), YopH (250 nM) and Src (500 nM) in reaction buffer (100 mM Tris , 150 mM KCl, 10 mM $MgCl_2$ and 5 mM DTT) is incubated at RT for 72 h. The reaction is then subjected to Ion-exchange HPLC separation (**Condition B**) to yield (*R*)-**3c** (monotriethylammonium salt, 2.8 mg, 40%) and (*S*)-**4c** (monotriethylammonium salt, 2.4 mg, 41%).

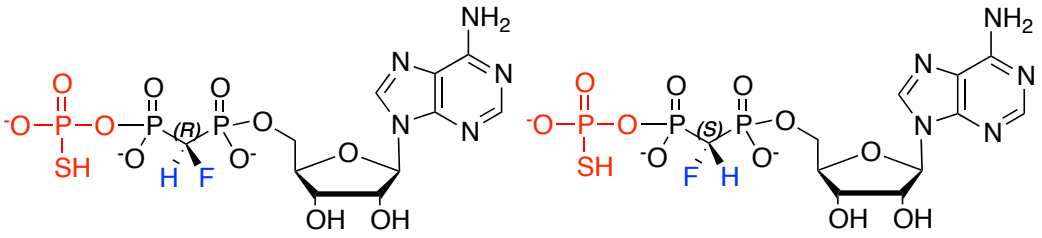
Ion-exchange-HPLC retention time: 13.5 minutes (*R*-**3c**); >97% purity. ^{1}H NMR (500 MHz, Deuterium Oxide, pH = 10.5) δ 8.51 (s, 1H), 8.14 (s, 1H), 6.02 (d, *J* = 5.8 Hz, 1H), 4.49 (s, 1H), 4.27 (s, 1H), 4.19 – 4.03 (m, 2H). ^{31}P NMR (202 MHz, Deuterium Oxide, pH = 10.5) δ 12.46 (d, *J* = 5.1 Hz), 0.70 (dd, *J* = 25.4, 5.0 Hz), -5.86 (d, *J* = 25.4 Hz). ESI-MS [M-H]-: calcd. $C_{11}H_{16}BrN_5O_{12}P_3^-$ 581.9; found, 582.2 m/z.

Ion-exchange-HPLC retention time (*R*-**4c**): 15.5 minutes >96% purity. ^{1}H NMR (500 MHz, Deuterium Oxide, pH = 10.2) δ 8.49 (s, 1H), 8.13 (s, 0H), 6.02 (d, *J* = 4.9 Hz, 1H), 4.53 (s, 1H), 4.25 (s, 1H), 4.22 – 3.99 (m, 2H). ^{31}P NMR (202 MHz, Deuterium Oxide, pH = 10.2) δ 15.22 , 7.68. ESI-MS [M-H]-: calcd. $C_{11}H_{15}BrN_5O_9P_2^-$, 502.0; found, 502.2 m/z.

Preparation of α,β-CHF-γS-ATP isomers (5a-1 and 5a-2)

The synthetic route of α,β-γS-CHF-ATP isomer was inspired by a previously reported different synthetic route toward γS-ATP.[6] We discovered that (*D*)-phenylglycine ADP conjugates (**2a**) was compatible with Zn^{2+} promoted reaction with thiophosphate. *R*-**2a** or *S*-**2a** (0.03 mmol) was dissolved in 0.15 ml of DMF, and mixed with 0.25 ml DMF solution of mixture of thiolphosphate triethylammonium salt (0.3 M) and anhydrous zinc chloride (0.8 M). After 10 h, the reaction was quenched by adding 0.6 ml of EDTA solution (0.5 M) and 4 ml of triethylammonium bicarbonate (0.5 M) sequentially at 0 ^{o}C. Chromatography (**Condition E**) performed on a C_{18} column yielded a mixture of 2',3'-isopropylidene-α,β-CHF-γS-ATP and 2',3'-isopropylidene-α,β-CHF-ADP (both have retention time at 9.0 min), which was freeze-dried. The solid was added with 1 ml of 0.2 N HCl at 0 ^{o}C and frozen in -20 ^{o}C overnight to remove the isopropylidene group. The reaction was thawed and quenched by slowly adding 0.15 ml of 2 N Na_2CO_3 solution at 0 ^{o}C. The mixture was finally subjected to Ion-exchange HPLC separation (**Condition F**) to yield **5a-1** or **5a-2**.

(R)-α,β-γS-CHF-ATP (5a-1) and (S)-α,β-γS-CHF-ATP (5a-2)



5a-1 **5a-2**

5a-1 HPLC (**Condition F**) retention time: 28 minutes; yield：10 micromoles (determined by UV), 33% over 2 steps. Purity: > 97%. ^{1}H NMR **(**500 MHz, D_2O, pH = 10**)** δ 8.46 (s, 1H), 8.14 (s, 1H), 6.02 (d, *J* = 5.8 Hz, 2H), 5.26 (ddd, *J* = 45.1, 14.3, 12.0 Hz, 1H), 4.49 (t, *J* = 4.4 Hz, 1H), 4.29 - 4.21 (m, 1H), 4.16 (qdd, *J* = 11.9, 5.3, 3.1 Hz, 2H). ^{19}F NMR (470 MHz, D_2O, pH = 10) δ -218.95 (td, *J* = 61.3, 45.2 Hz). ^{31}P NMR (202 MHz, D_2O, pH = 10) δ 32.87 (d, *J* = 33.5 Hz), 11.25 (dd, *J* = 61.2, 15.8 Hz), -1.28 (ddd, *J* = 61.2, 33.5, 15.6 Hz). ESI-MS [M-H]-: calcd $C_{11}H_{16}FN_5O_{11}P_3S^-$ Exact Mass: 538.0; found, 538.1 m/z.

5a-2 HPLC retention (**Condition F**) time: 28 minutes; yield：8 micromoles (determined by UV), 25% over 2 steps. Purity: > 96%. ^{1}H NMR **(**400 MHz, D_2O, pH = 10**)** δ 8.45 (s, 1H), 8.14 (s, 1H), 6.02 (d, *J* = 5.9 Hz, 1H), 5.25 (ddd, *J* = 45.1, 14.4, 11.9 Hz, 1H), 4.48 (t, *J* = 4.4 Hz, 1H), 4.29 - 4.24 (m, 1H), 4.24 - 4.06 (m, 2H). ^{19}F NMR (470 MHz, D_2O, pH = 10) δ -219.41 (td, *J* = 61.1, 45.1 Hz). ^{31}P NMR (202 MHz, D_2O, pH = 10) δ 32.89 (dd, *J* = 33.5, 6.3 Hz), 11.34 (dd, *J* = 60.9, 15.8 Hz), (-0.89) – (-1.53) (m). ESI-MS [M-H]-: calcd $C_{11}H_{16}FN_5O_{11}P_3S^-$ Exact Mass: 538.0; found, 538.0 m/z.

Figures

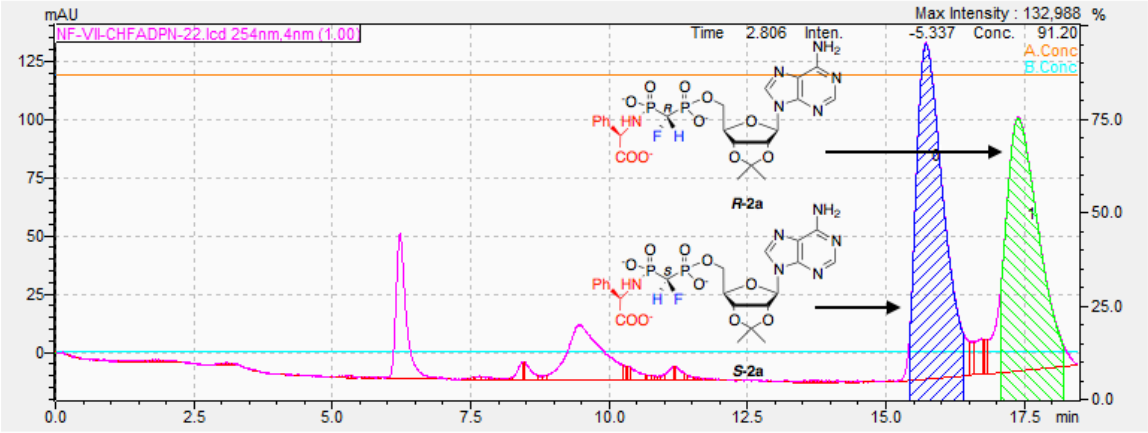


Figure S1.Preparative HPLC separation of ***S*-2a** (**2a-1**) and ***R*-2a**.(**2a-2**)

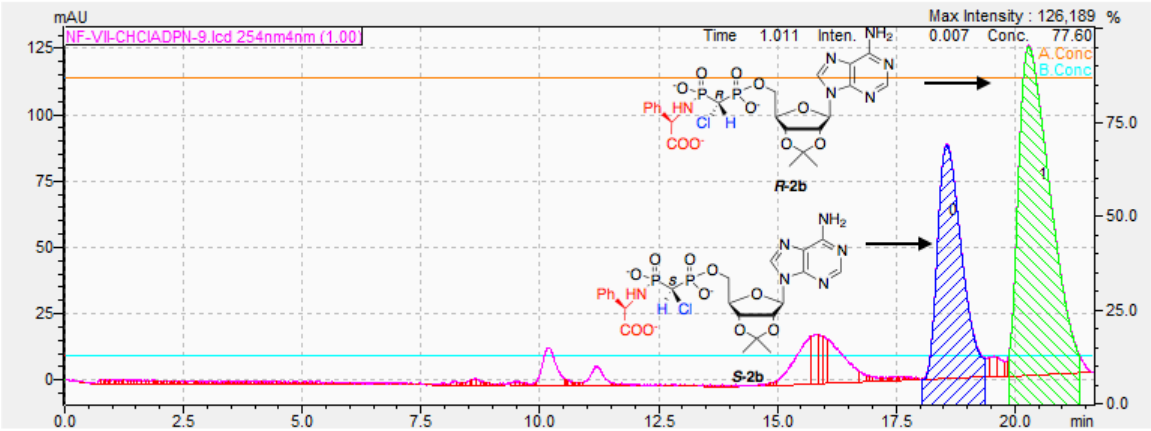


Figure S2. Preparative HPLC separation of ***S*-2b** (**2b-1**) and ***R*-2b**.(**2b-2**)

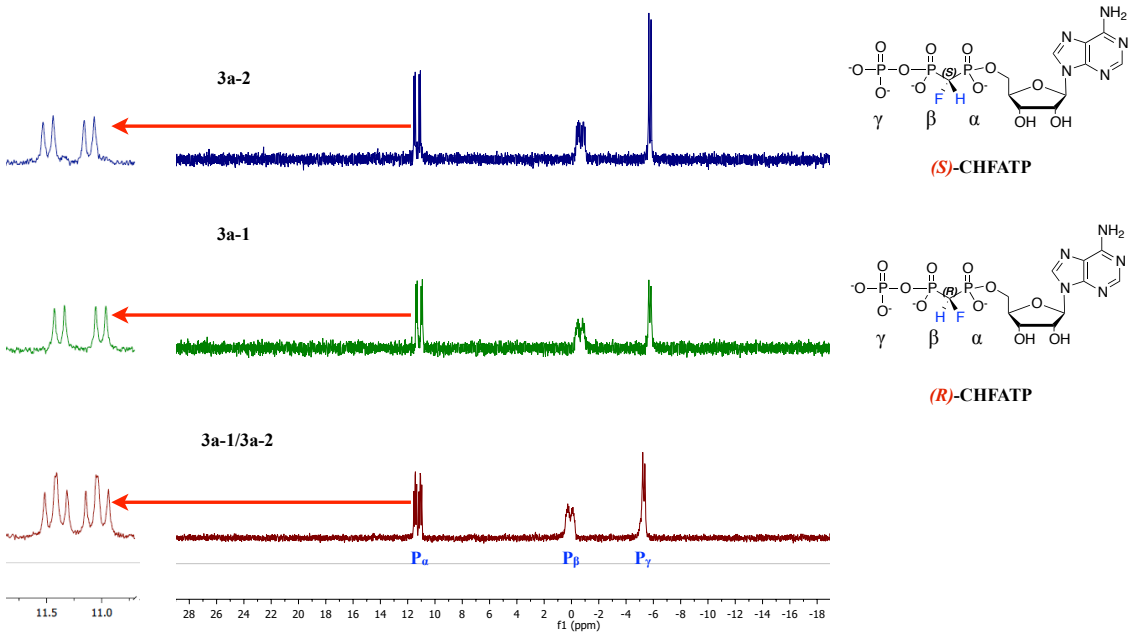


Figure S3. ^{31}P NMR (162 MHz, D_2O, pH = 10.0) spectra of individual diastereomers (top panel and middle panel) and diastereomeric mixture (1:1 ratio of two individual isomers, bottom panel) of α,β-CHF-ATP.

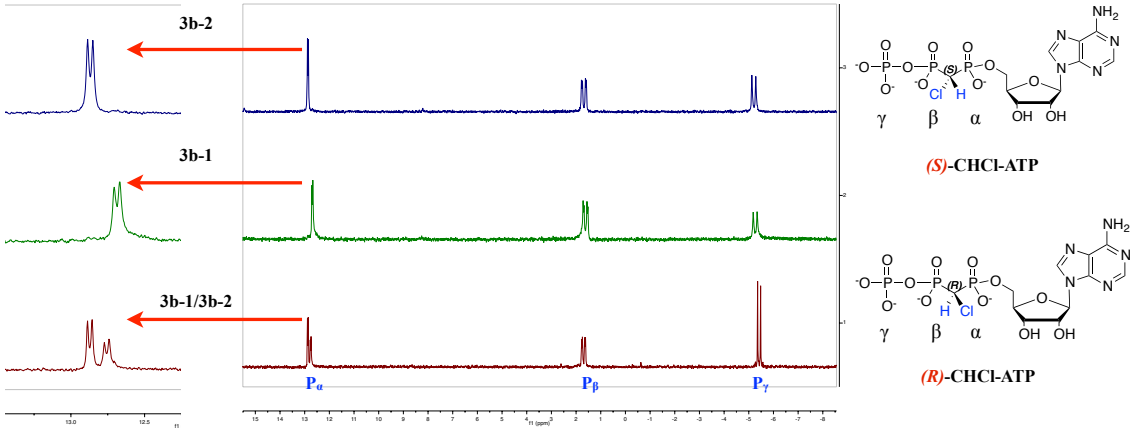


Figure S4. ^{31}P NMR (162 MHz, D_2O, pH = 10.0) spectra of individual diastereomers of α,β-CHCl-ATP.

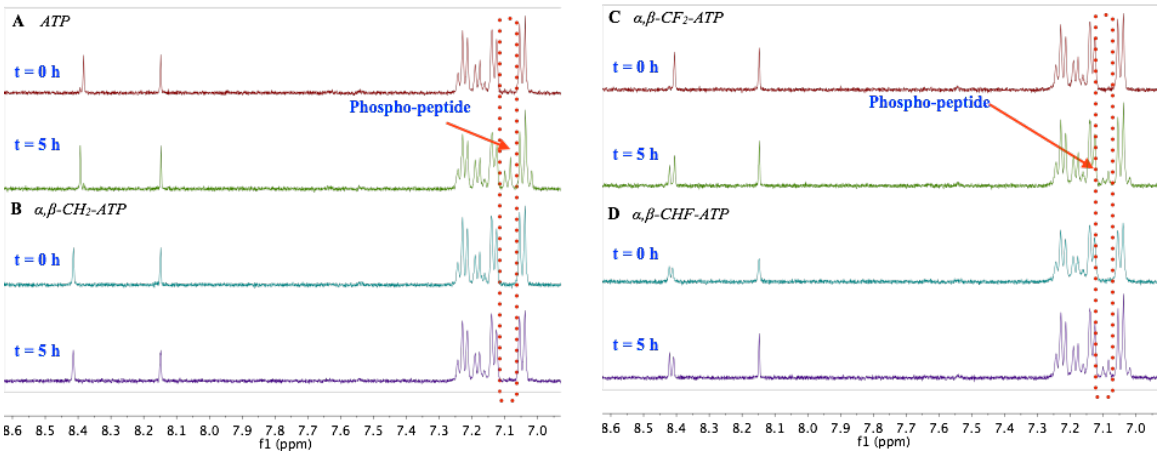


Figure S5. Use of ^{1}H NMR to monitor the phosphoryl transfer reaction catalyzed by the Src kinase. Proton peaks in the aromatic region were measured to monitor the accumulation of the phosphorylated peptide for (A) ATP, (B) α,β-CH_2-ATP, (C) α,β-CF_2-ATP, and (D) α,β-CHF-ATP. Assay conditions: 0.5 mM ATP analog, 0.89 mM peptide and 100 nM Src kinase were incubated in 100 mM Tris/10 mM $MgCl_2$/150 mM KCl buffer contains 10% D_2O. Spectrums were acquired at 0 h and 5 h with water peak suppression method PRESAT to improve the quality.

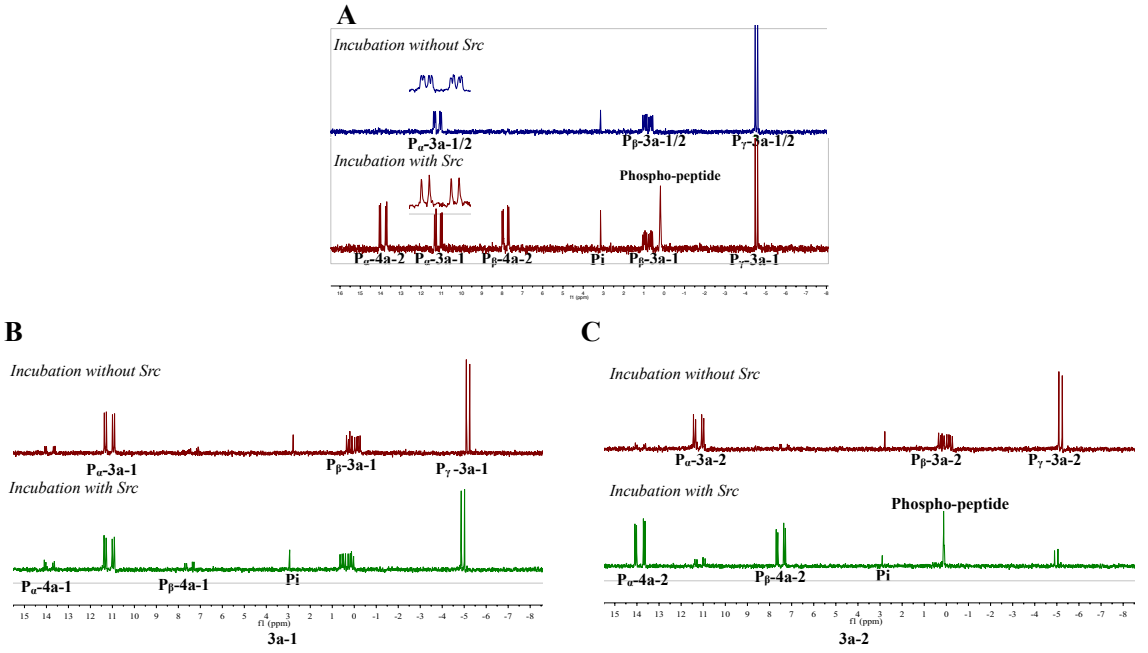


Figure S6. ^{31}P NMR (202 MHz, 10% D_2O, pH = 9.4) spectra reveal that Src kinase selectively utilizes one diastereomer of α,β-(R/S)-CHF-ATP. Spectra of a diastereomeric mixture (A) or pure diastereomers (B & C) of α,β-CHF-ATP were acquired after incubation with or without Src kinase.

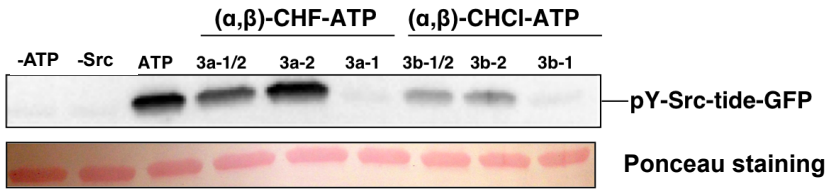


Figure S7. Kinase assay based on western blot reveal that **3a-2** and **3b-2** were utilized by Src to phosphorylate GFP-fused Src-tide with high efficiency while their diastereomers could not. The Src kinase, GFP-Src-tide and various nucleotides were incubated for 30 min. The samples were resolved by SDS-PAGE gel, transferred to nitrocellulose membrane, and finally probed with phospho-tyrosine antibody or stained with ponceau.

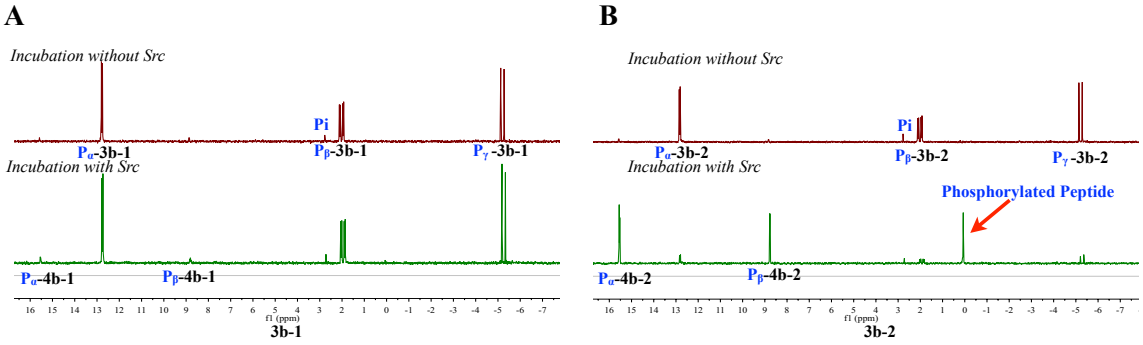
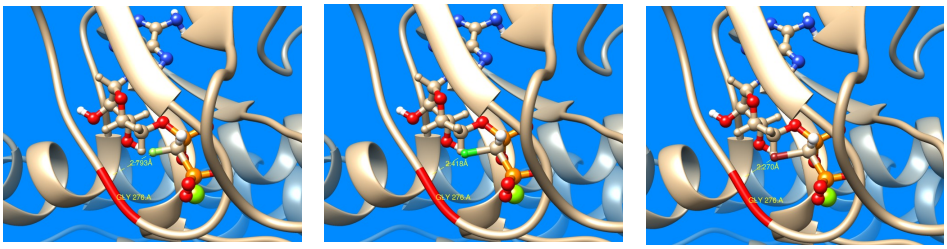


Figure S8. NMR spectroscopy confirmed the selective utilization of one diastereomer of α,β-CHCl-ATP by Src. ^{31}P NMR (202 MHz, 10% D_2O, pH = 9.4) spectrums were acquired for **3b-1** (A) and **3b-2** (B) before and after incubation with the kinase. 1): The ATP analogue alone. 2): The ATP analog, Src and peptide were incubated in kinase assay buffer for 5 h.



(S)-CHFADP/Src (S)-CHClADP/Src (S)-CHBrADP/Src

Inactive CHX-ATP isomers	van der waals radius of halogen X (Å)	Sum of VDW radius N and X d1 (Å)	Predicted Distance between Gly 276-N and X d2 (Å)	d2<d1 (crash)
CHF	1.47	3.02	2.79	yes
CHCl	1.75	3.30	2.42	yes
CHBr	1.85	3.4	2.27	yes

van der waals radius of nitrogen: 1.55Å

Figure S9. Proposed models of Src/(S)-CHX-ADP imply that the inactive diastereomer suffer from not only loss of H-bond but also steric clash with the main chain amide bond of Q275/G276 within the glycine-rich loop. Models were constructed by replacing the CHF linker in the crystal structure of Src/(R)-CHF-ADP complex with (S)-CHX group.

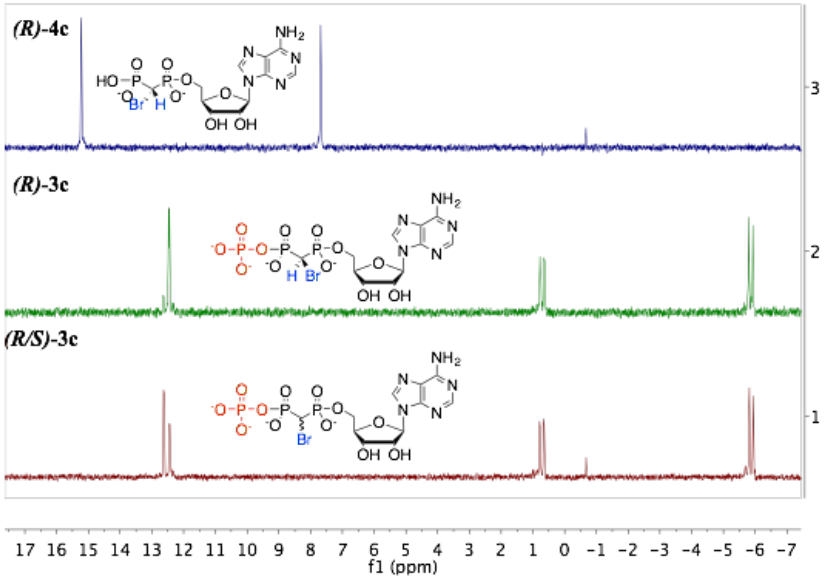


Figure S10. ^{31}P NMR spectra of enzymatically resolved α,β-CHBr-ADP and α,β-CHBr-ATP diastereomeric isomers (top and middle panels) and the spectrum before enzymatic resolution (bottom panel).

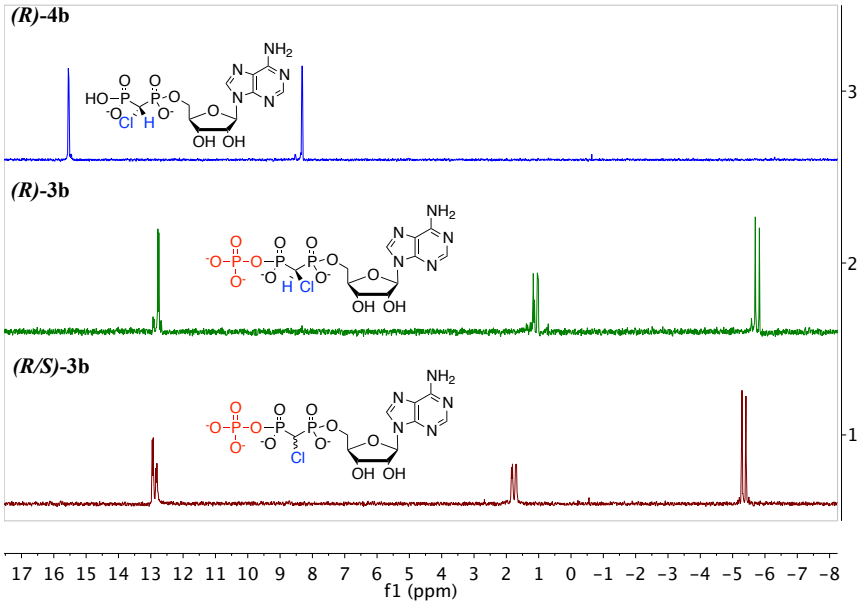


Figure S11. ^{31}P NMR spectra of enzymatically resolved α,β-CHCl-ADP and α,β-CHCl-ATP diastereomeric isomers (top and middle panels) and the spectrum before enzymatic resolution (bottom panel).

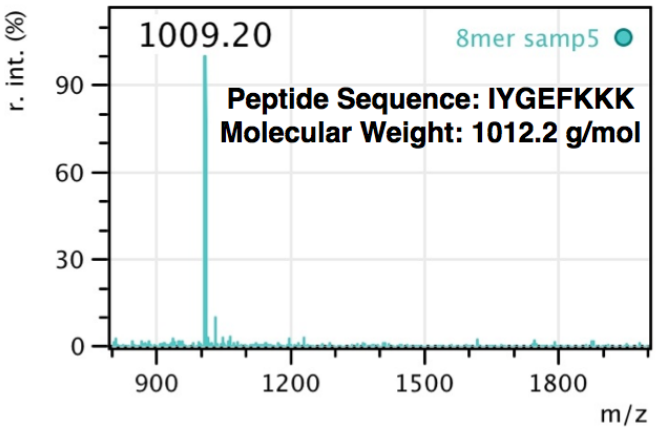


Figure S12. MALDI MS spectrum of the substrate peptide used in the Src kinase assay.

Tables

Table S1. K_i values of ADP and ADP analogues against Src kinase *in vitro*

Compound	K_i (mM)
ADP	0.037
4a-1	0.23
4a-2	0.17
4b-1	0.32
4b-2	0.038

Table S2. Crystallographic data collection and refinement statistics

	Src-**4a-2**	Src-**3a-2**	Src-**4b-2**	Src-**3b-2**
Resolution range (Å)	43.49 - 2.30 (2.38 - 2.30)	43.45 - 2.10 (2.18 - 2.10)	43.35 - 2.44 (2.53 - 2.44)	36.55 - 2.01 (2.08 - 2.01)
Space group	P 1	P 1	P 1	P 1
Unit cell	42.03, 63.60, 74.00, 79.14, 89.53, 88.48	42.29, 63.69, 73.82, 79.07 89.23 89.24	42.12, 63.68, 74.16, 78.69 89.92 90.36	42.28, 63.98, 74.00, 101.03, 89.83, 90.60
Unique reflections	31522 (2277)	41453 (2947)	27299 (2543)	48688 (4455)
Completeness (%)	94.43 (68.81)	94.08 (67.05)	97.43 (91.64)	96.46 (88.69)
Mean I/sigma	10.00 (2.29)	15.18 (1.46)	9.05 (2.49)	13.9(3.1)
Wilson B-factor	19.88	40.14	36.75	31.93
R-work	0.1828 (0.2277)	0.1996 (0.3720)	0.1920 (0.2458)	0.1679 (0.2277)
R-free	0.2205 (0.2879)	0.2456 (0.3602)	0.2390 (0.3013)	0.1982 (0.2807)
Number of non-hydrogen atoms	4653	4533	4487	4747
Macromolecules	4290	4290	4262	4284
Ligands	58	66	57	64
Water	305	177	168	399
Protein residues	534	534	529	532
RMS(bonds)	0.009	0.009	0.011	0.010
RMS(angles)	1.40	1.18	1.54	1.20
Ramachandran favored (%)	96	94	97	98
Ramachandran outliers (%)	0.95	0.38	0.58	0
Clashscore	5.44	6.71	8.87	4.17
Average B-factor	26.00	55.70	48.90	48.20
Macromolecules	25.60	55.40	48.80	47.90
Ligands	36.60	97.30	69.90	67.30
Water	29.50	47.20	43.90	48.40
Note: Statistics for the highest-resolution shell are shown in parentheses.				

Table S3. Kinetic parameters for ATP and α,β-CHF-ATP as substrates for a panel of kinases

	ATP		**3a-1** or *(R)*-CHF		**3a-2** or *(S)*-CHF		Stereo-specificity
	K_M (mM)	k_{cat} (min^{-1})	K_M (mM)	k_{cat} (min^{-1})	K_M (mM)	k_{cat} (min^{-1})	$(k_{cat}/K_M)_S/(k_{cat}/K_M)_R$
ERK2	0.057	30.6	0.047	4.3	0.024	18.5	8
CDK2	0.067	39.4	0.146	1.5	0.036	5.3	14
EGFR	0.0024	17.7	0.0082	10.3	0.0011	23.1	16
PKAα	0.008	35.2	0.010	2.3	0.0010	4.4	20
Src	0.070	41.8	0.114	1.3	0.049	13.0	23
CRAF	0.019	23.2	0.214	10.2	0.018	20.6	24
CK1α	0.0034	16.4	0.048	3.2	0.0027	6.3	35
ZAP70	0.0022	7.5	0.079	1.7	0.0083	31.2	174
MEK1	0.140	3.0	n.a.	n.a.	0.027	0.40	n.a
CaMK1α	0.76	66.5	n.a.	n.a.	0.129	0.38	n.a.
MARK1	0.0093	50.5	n.a.	n.a.	n.a.	n.a.	n.a.
DYRK1A	0.049	13.3	n.a.	n.a.	0.0082	3.3	n.a.

Table S4. Reaction conditions used for the Caliper kinase assays

Kinase	[E], nM	Metal	Kinase substrate
CDK2/CycA	0.1	Mg^{2+}	PKTPKKAKKL
CK1α	5	Mg^{2+}	KRRRALSVASLPGL
DYRK1A	0.04	Mg^{2+}	RRFRPASPLRGPPK
MAPK1/ERK2	1	Mg^{2+}	IPTSPITTTYFFFKKK
MARK1	0.02	Mg^{2+}	KKKVSRSGLYRSPSMPENLNRPR
PKAα	0.015	Mg^{2+}	AKRRRLSSRA
SRC	0.55	Mg^{2+}	GEEPLYWSFPAKKK
ZAP70	0.33	Mn^{2+}	EEEEYFFIIAKKKK
CAMK1	2	Mg^{2+}	KKLRRTLSVA
CRAF	45	Mn^{2+}	N/A (proprietary)
EGFR	1.5	Mn^{2+}	KKKKEEIYFFF
MEK1	70	Mg^{2+}	N/A (proprietary)

References:

(1) McKenna, C. E.; Khawli, L. A.; Ahmad, W.-Y.; Pham, P.; Bongartz, J.-P. *Phosphorus and Sulfur and the Related Elements* **1988**, *37*, 1.

(2) Clark, V. M.; Todd, A. R.; Zussman, J. *Journal of the Chemical Society (Resumed)* **1951**, 2952.

(3) Seeliger, M. A.; Young, M.; Henderson, M. N.; Pellicena, P.; King, D. S.; Falick, A. M.; Kuriyan, J. *Protein Science : A Publication of the Protein Society* **2005**, *14*, 3135.

(4) Witt, J. J.; Roskoski Jr, R. *Analytical Biochemistry* **1975**, *66*, 253.

(5) Davisson, V. J.; Davis, D. R.; Dixit, V. M.; Poulter, C. D. *J Org Chem* **1987**, *52*, 1794.

(6) Kowalska, J.; Lewdorowicz, M.; Darzynkiewicz, E.; Jemielity, J. *Tetrahedron Lett* **2007**, *48*, 5475.

HPLC traces

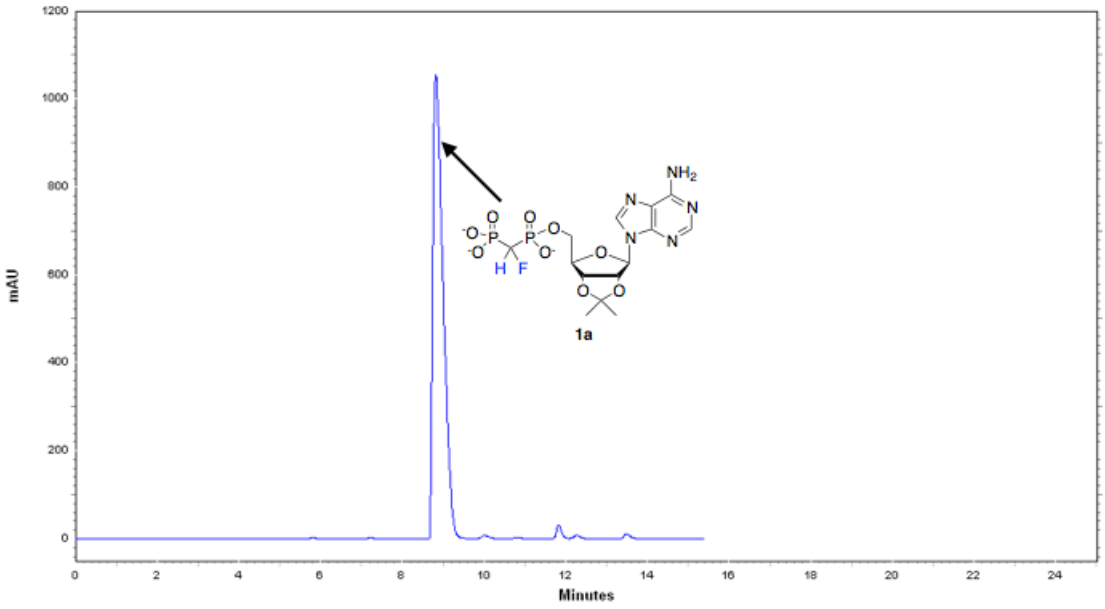


Figure S13. Preparative HPLC purification of 1a.

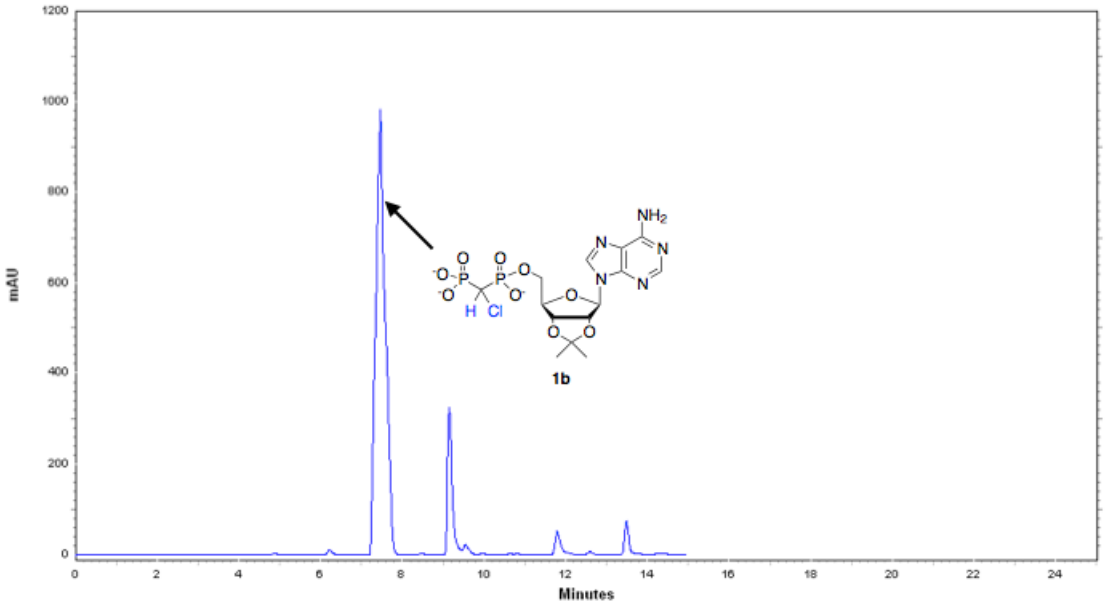


Figure S14. Preparative HPLC purification of **1b**.

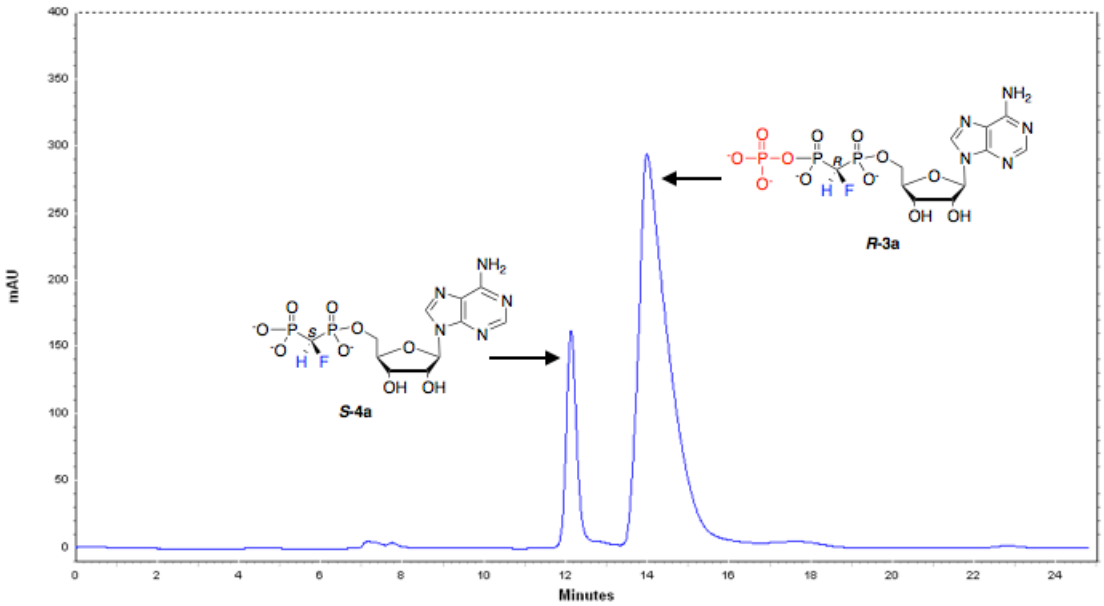


Figure S15. Preparative HPLC separation of **4a-1** (*S*) and **3a-1** (*R*).

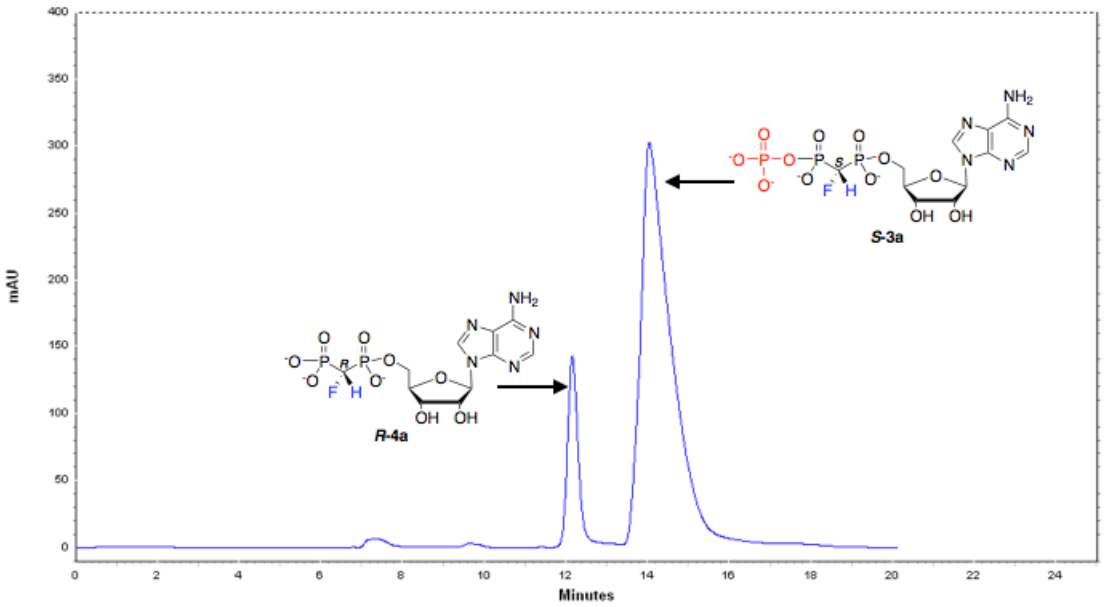


Figure S16. Preparative HPLC separation of **4a-2** (*R*) and **3a-2** (*S*).

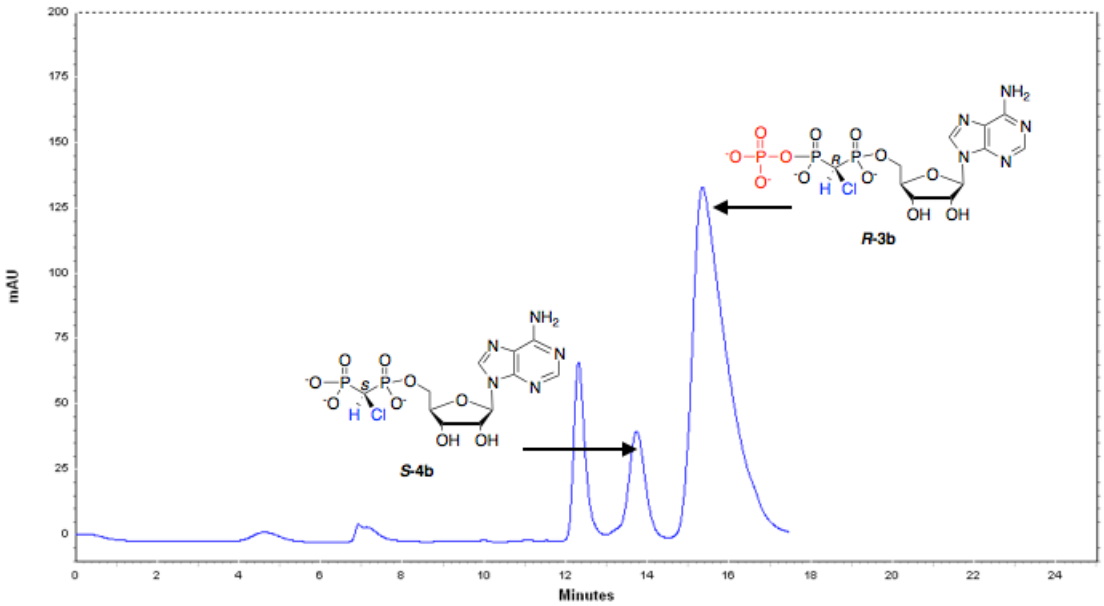


Figure S17. Preparative HPLC separation of **4b-1** (*S*) and **3b-1** (*R*).

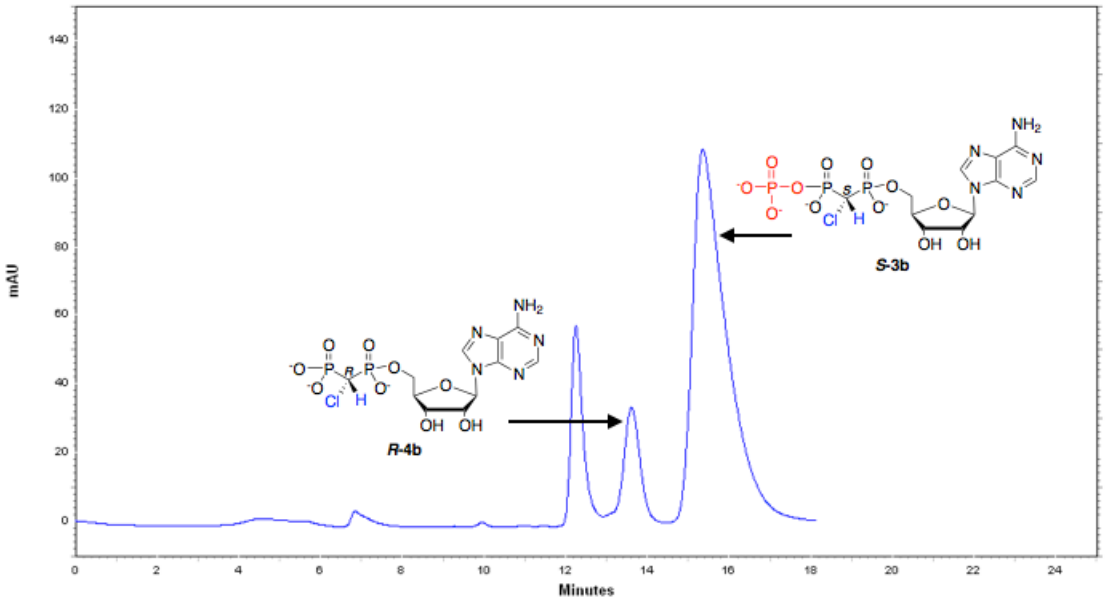


Figure S18. Preparative HPLC separation of **4b-2** (*R*) and **3b-2** (*S*).

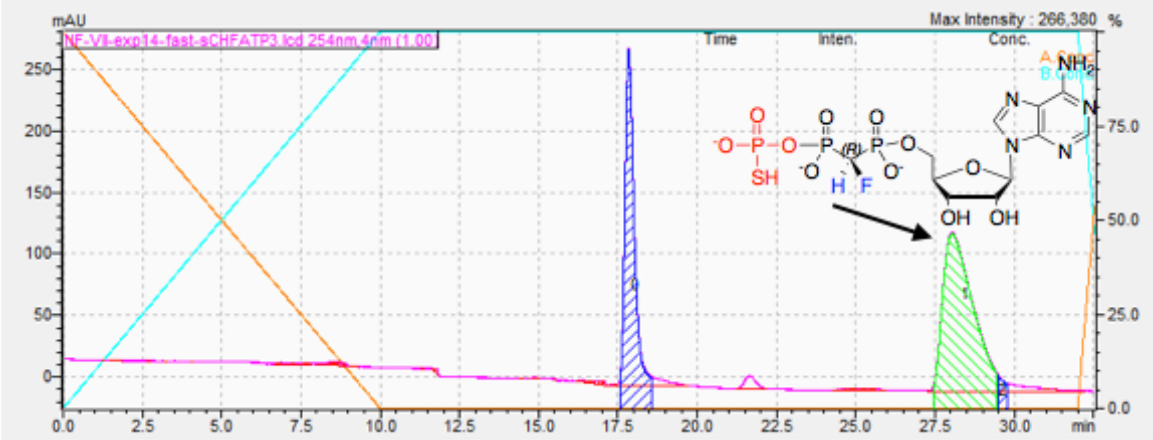


Figure S19. Preparative HPLC purification of **5a-1** (*R*).

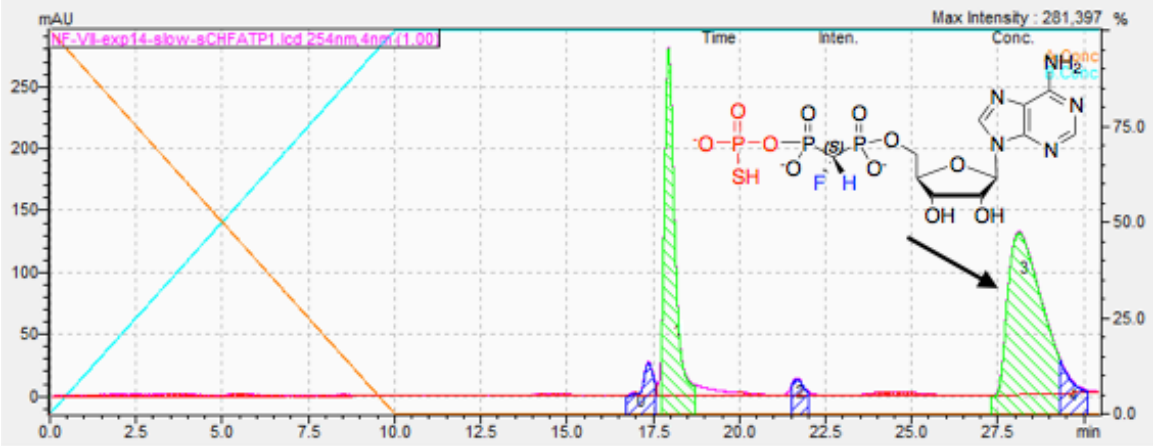


Figure S20. Preparative HPLC purification of **5a-2** (*S*).

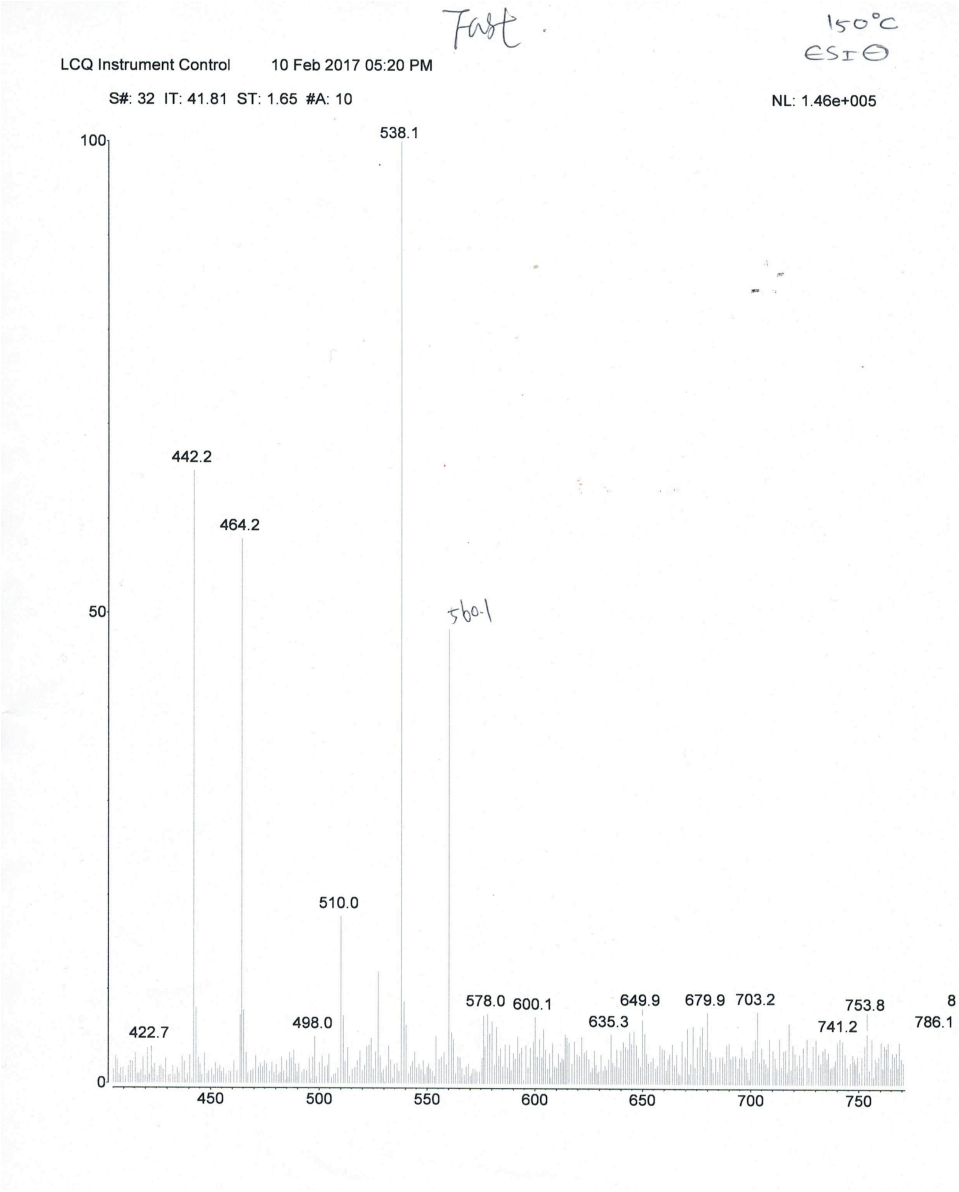


Figure S21. ESI-MS(-) spectrum of **5a-1** (*R*).

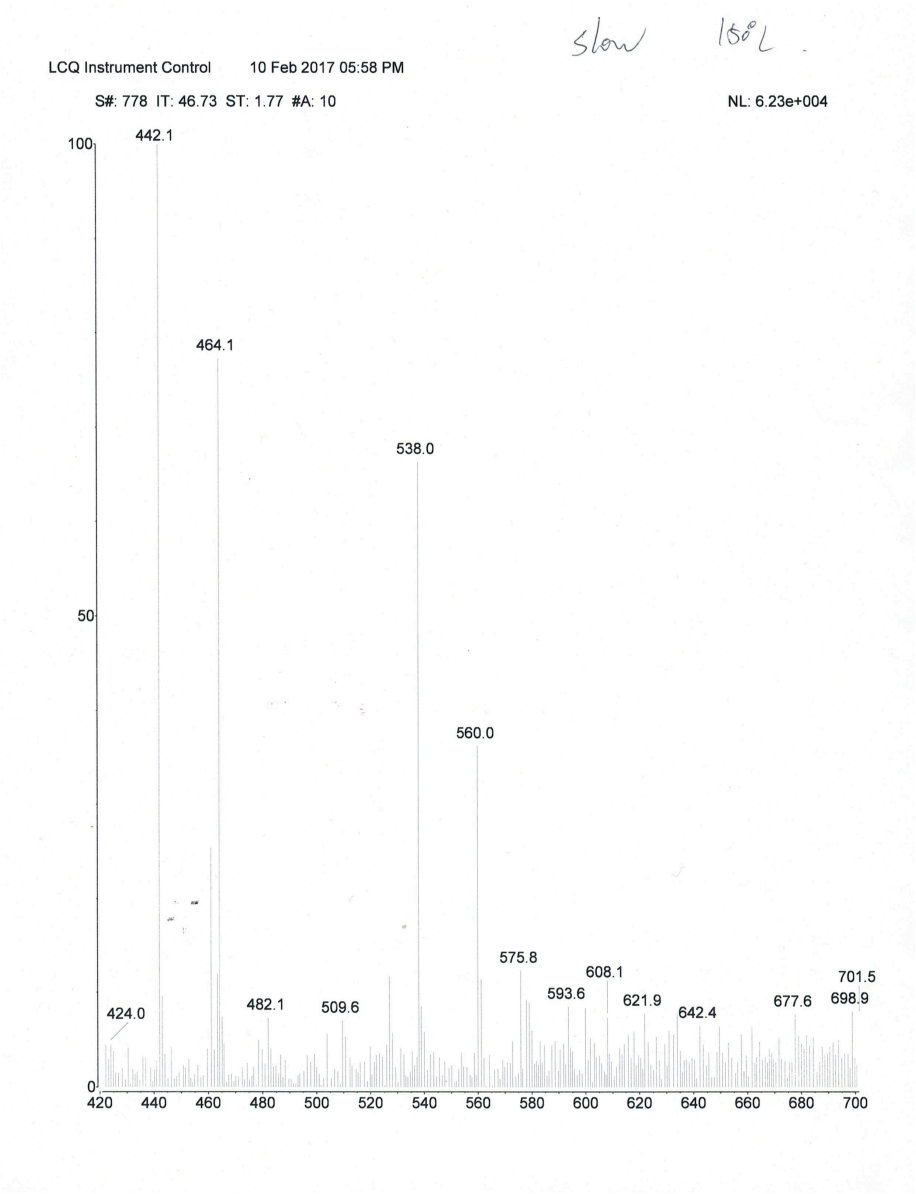
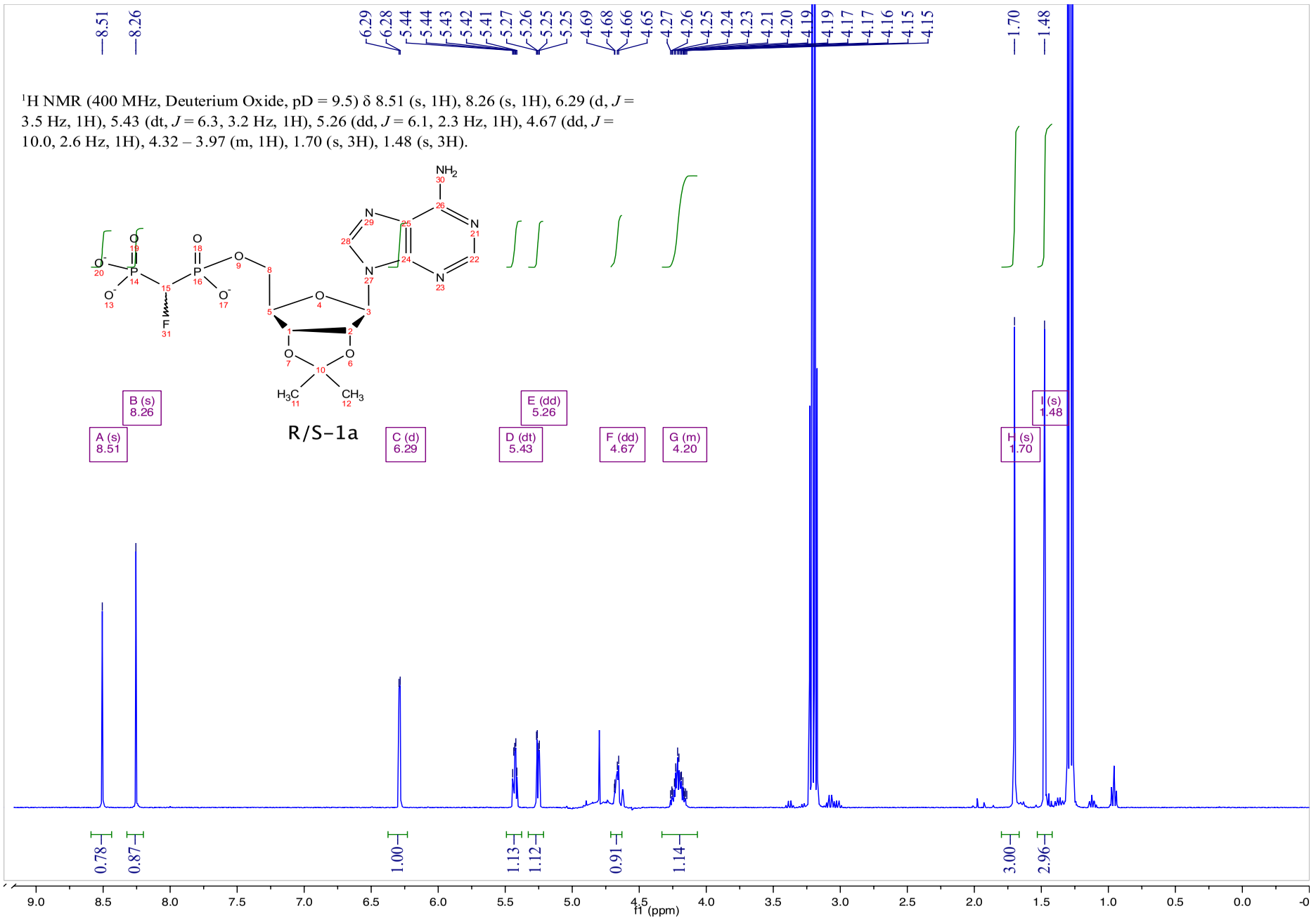
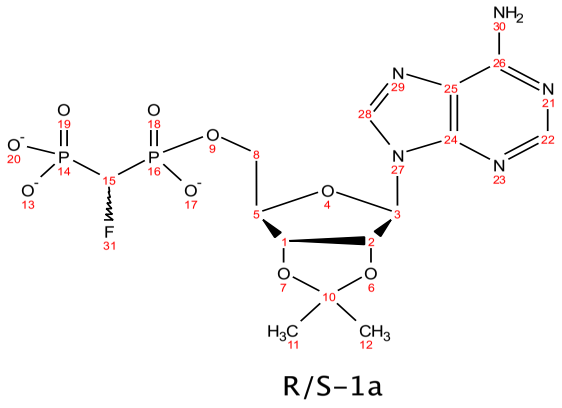


Figure S22. ESI-MS(-) spectrum of **5a-2** (*S*).

NMR spectra


8.51
8.26
6.29
6.28
5.44
5.44
5.43
5.42
5.41
5.27
5.26
5.25
5.25
4.69
4.68
4.66
4.65
4.27
4.26
4.25
4.24
4.23
4.21
4.20
4.19
4.19
4.17
4.17
4.16
4.15
4.15
1.70
1.48
[1]H NMR (400 MHz, Deuterium Oxide, pD = 9.5) δ 8.51 (s, 1H), 8.26 (s, 1H), 6.29 (d, J = 3.5 Hz, 1H), 5.43 (dt, J = 6.3, 3.2 Hz, 1H), 5.26 (dd, J = 6.1, 2.3 Hz, 1H), 4.67 (dd, J = 10.0, 2.6 Hz, 1H), 4.32 – 3.97 (m, 1H), 1.70 (s, 3H), 1.48 (s, 3H).
R/S-1a
A (s) 8.51
B (s) 8.26
C (d) 6.29
D (dt) 5.43
E (dd) 5.26
F (dd) 4.67
G (m) 4.20
H (s) 1.70
I (s) 1.48
0.78
0.87
1.00
1.13
1.12
0.91
1.14
3.00
2.96
f1 (ppm)

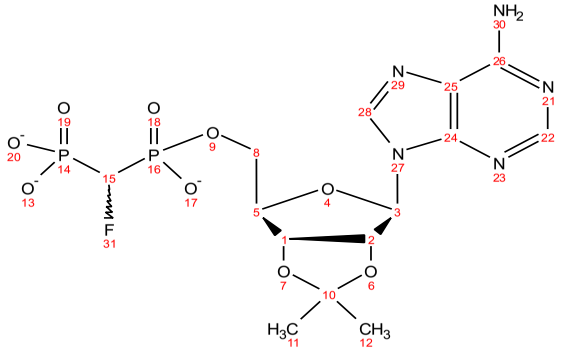
^{31}P NMR (162 MHz, Deuterium Oxide, pD = 9.5) δ 13.69 (dt, *J* = 63.4, 13.2 Hz), 6.96 (ddd, *J* = 55.8, 12.1, 1.5 Hz).



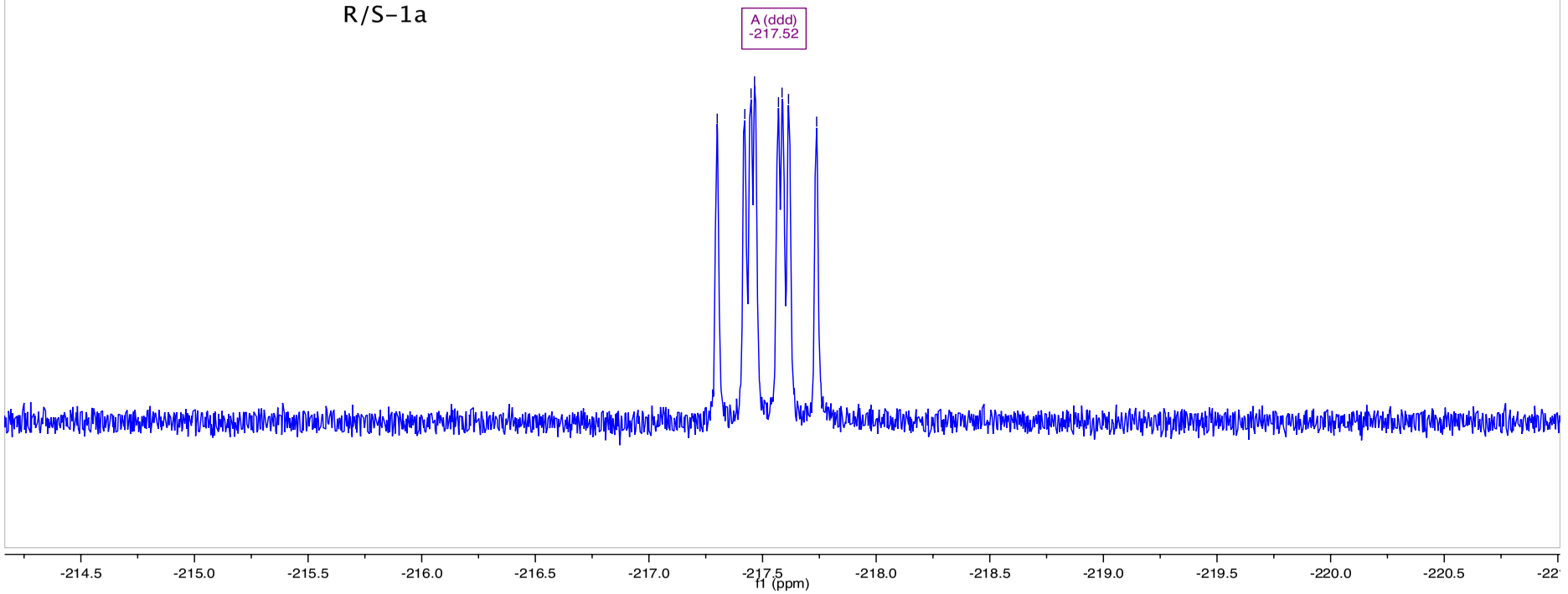
R/S-1a

-217.30
-217.42
-217.45
-217.46
-217.57
-217.59
-217.61
-217.74

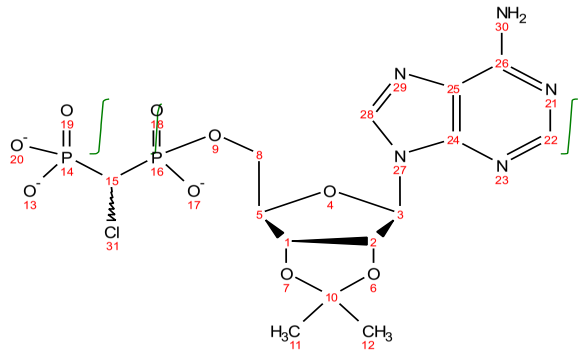
^{19}F NMR (376 MHz, Deuterium Oxide, pD = 9.5) δ -217.52 (ddd, *J* = 63.2, 56.6, 46.1 Hz).



R/S-1a

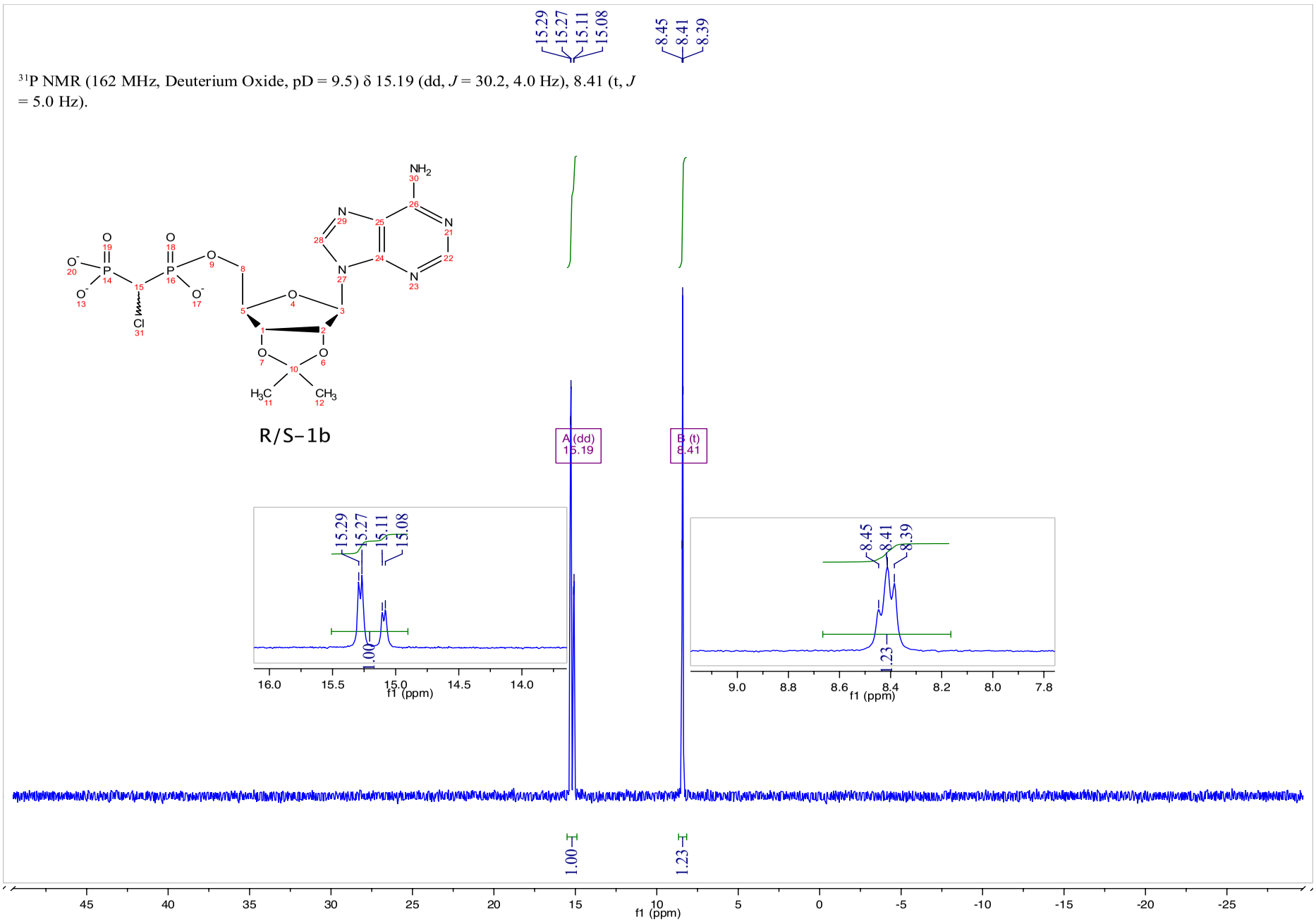


^{1}H NMR (400 MHz, Deuterium Oxide, pD = 9.5) δ 8.53 (s, 1H), 8.26 (s, 1H), 6.29 (d, *J* = 3.4 Hz, 2H), 5.52 – 5.35 (m, 3H), 5.27 (td, *J* = 5.9, 2.2 Hz, 3H), 4.70 – 4.63 (m, 1H), 4.32 – 4.05 (m, 2H).

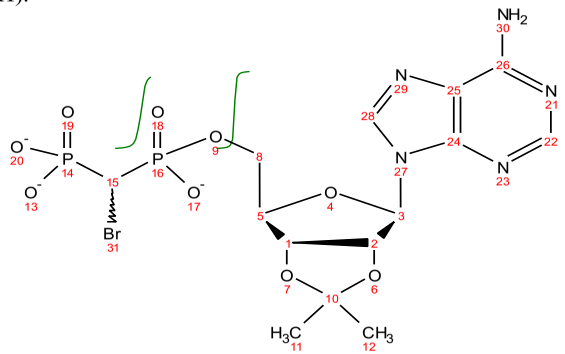


R/S-1b

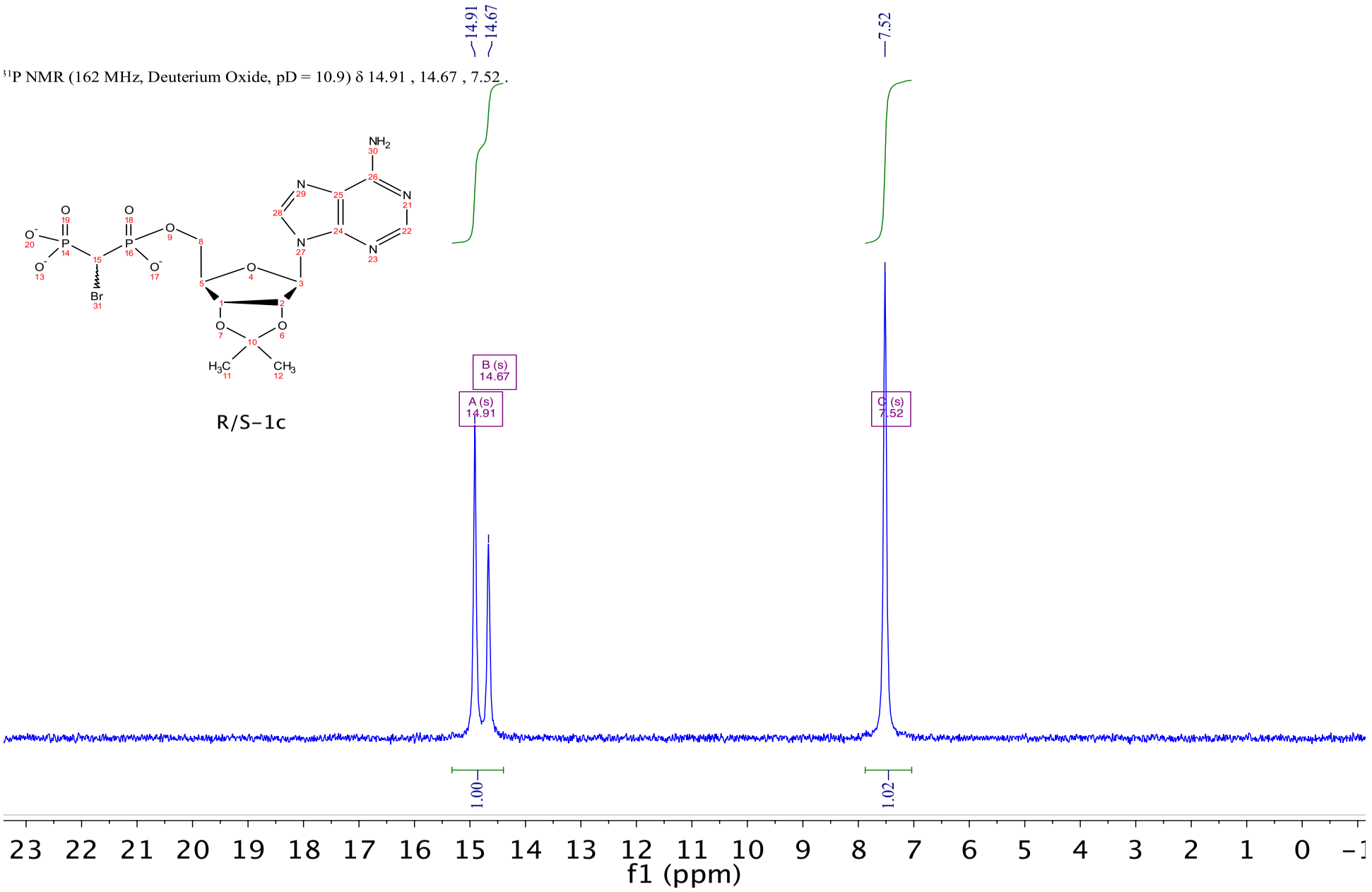
^{31}P NMR (162 MHz, Deuterium Oxide, pD = 9.5) δ 15.19 (dd, *J* = 30.2, 4.0 Hz), 8.41 (t, *J* = 5.0 Hz).



[1]H NMR (500 MHz, Deuterium Oxide, pD = 10.9) δ 8.38 (d, *J* = 6.3 Hz, 1H), 8.09 (s, 1H), 6.13 (t, *J* = 3.7 Hz, 1H), 5.91 (s, 0.2H), 5.30 (q, *J* = 5.1 Hz, 1H), 5.14 (d, *J* = 5.5 Hz, 1H), 4.89 (d, *J* = 5.8 Hz, 0.3H), 4.58 (s, 0.7H), 4.54 (t, *J* = 4.8 Hz, 1H), 4.17 – 3.94 (m, 2H), 1.56 (s, 3H), 1.33 (s, 3H).

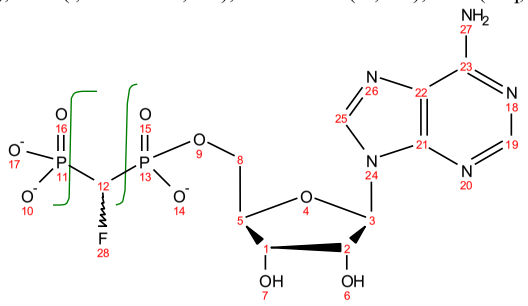


^{31}P NMR (162 MHz, Deuterium Oxide, pD = 10.9) δ 14.91 , 14.67 , 7.52 .



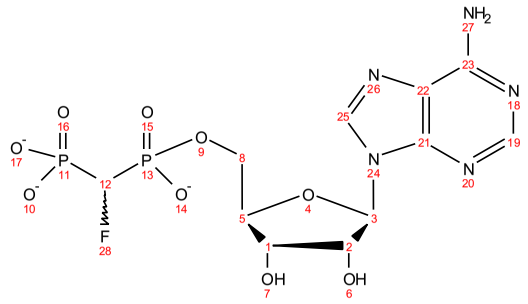
R/S-1c

^{1}H NMR (400 MHz, Deuterium Oxide, pD = 6.5) δ 8.51 (s, 1H), 8.33 (s, 1H), 6.04 (d, *J* = 5.1 Hz, 1H), 4.99 (td, *J* = 12.7, 5.7 Hz, 0.5H), 4.92 – 4.82 (m, 0.5H), 4.64 (q, *J* = 5.2 Hz, 1H), 4.42 (t, *J* = 4.7 Hz, 1H), 4.31 – 4.25 (m, 1H), 4.20 (ddq, *J* = 14.8, 8.7, 2.8 Hz, 1H).



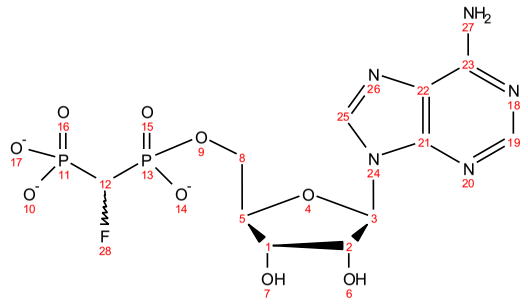
R/S-4a

^{31}P NMR (162 MHz, Deuterium Oxide, pD = 6.5) δ 10.53 (dd, J = 65.7, 12.5 Hz), 9.12 (dd, J = 60.2, 12.6 Hz).



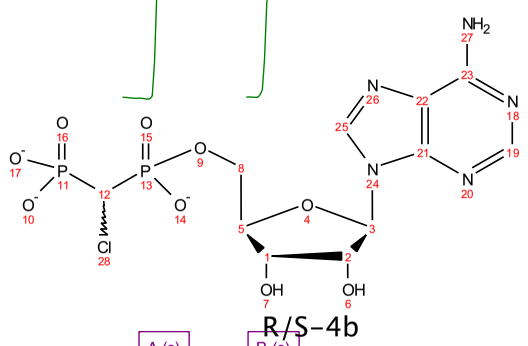
R/S-4a

^{19}F NMR (376 MHz, Deuterium Oxide, pD = 6.5) δ -224.22 (dddd, *J* = 65.5, 60.4, 45.2, 12.8 Hz).



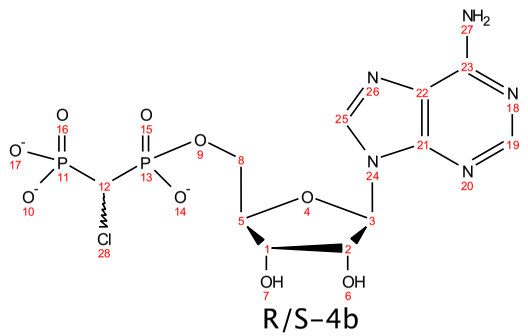
R/S-4a

^{1}H NMR (400 MHz, Deuterium Oxide, pD =10) δ 8.60 (s, 1H), 8.27 (s, 1H), 6.15 (d, *J* = 5.1 Hz, 1H), 4.67 – 4.60 (m, 1H), 4.39 (s, 1H), 4.35 – 4.06 (m, 2H).



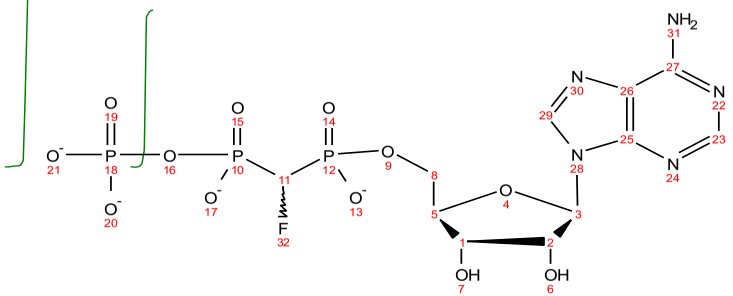
R/S-4b

^{31}P NMR (162 MHz, Deuterium Oxide, pD = 10) δ 15.53 (dd, *J* = 6.0, 3.6 Hz), 8.40 (dd, *J* = 8.4, 3.7 Hz).



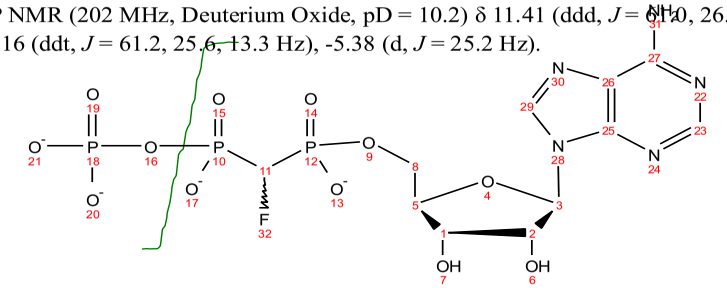
R/S-4b

^{1}H NMR (500 MHz, Deuterium Oxide, pD = 10.2) δ 8.58 (s, 1H), 8.26 (s, 2H), 6.15 (d, *J* = 5.9 Hz, 2H), 5.25 – 5.06 (m, 2H), 4.63 – 4.54 (m, 2H), 4.43 – 4.35 (m, 2H), 4.33 – 4.21 (m, 2H).



R/S-3a

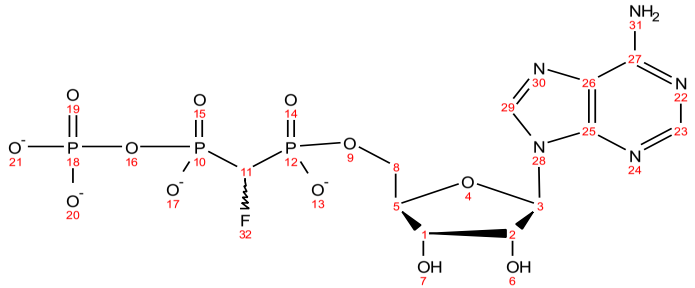
^{31}P NMR (202 MHz, Deuterium Oxide, pD = 10.2) δ 11.41 (ddd, J = 61.0, 26.5, 14.8 Hz), -0.16 (ddt, J = 61.2, 25.6, 13.3 Hz), -5.38 (d, J = 25.2 Hz).



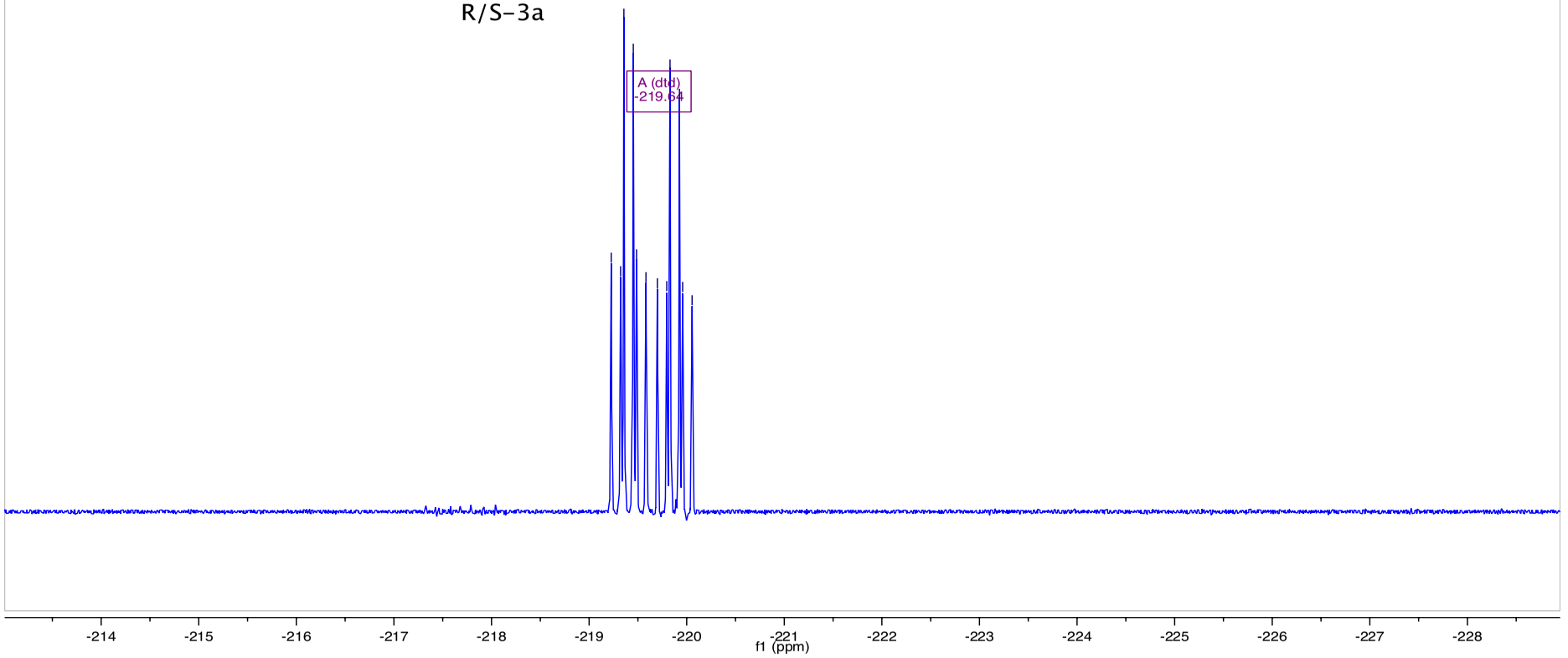
R/S-3a

-219.23 -219.32 -219.36 -219.45 -219.49 -219.58 -219.70 -219.80 -219.83 -219.93 -219.96 -220.06

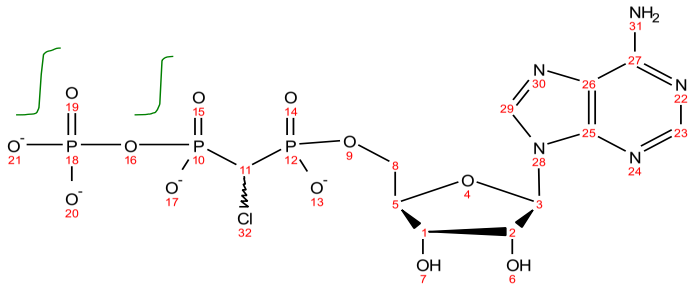
^{19}F NMR (470 MHz, Deuterium Oxide, pD = 10.2) δ -219.64 (dtd, *J* = 222.2, 61.0, 45.3 Hz).



R/S-3a

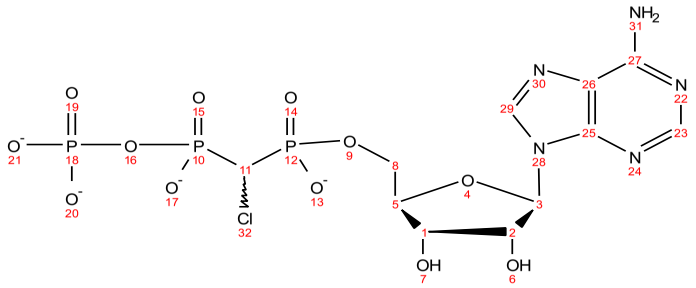


^{1}H NMR (500 MHz, Deuterium Oxide, pD = 10) δ 8.58 (s, 1H), 8.22 (s, 1H), 6.13 (d, *J* = 5.8 Hz, 2H), 4.64 – 4.54 (m, 1H), 4.42 – 4.34 (m, 1H), 4.34 – 4.13 (m, 3H).

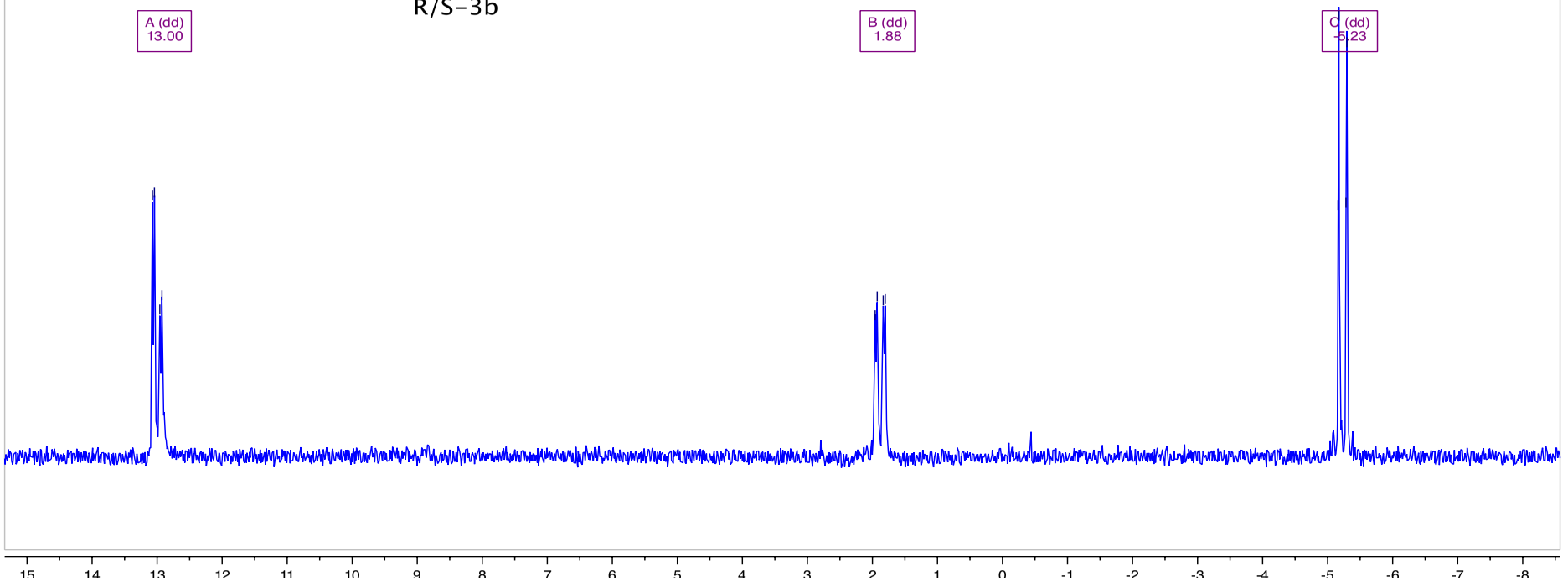


R/S-3b

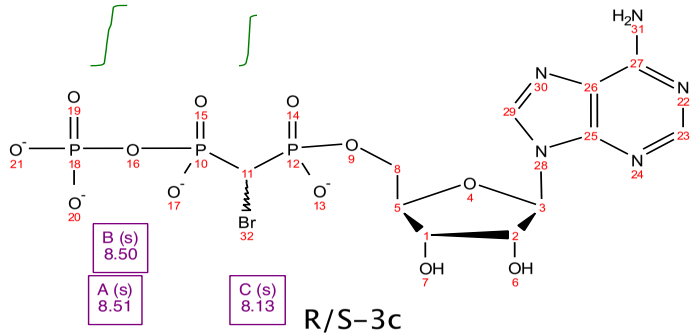
^{31}P NMR (202 MHz, Deuterium Oxide, pD = 10) δ 13.00 (dd, *J* = 23.3, 6.5 Hz), 1.88 (dd, *J* = 24.9, 6.3 Hz), -5.23 (dd, *J* = 24.7, 2.7 Hz).



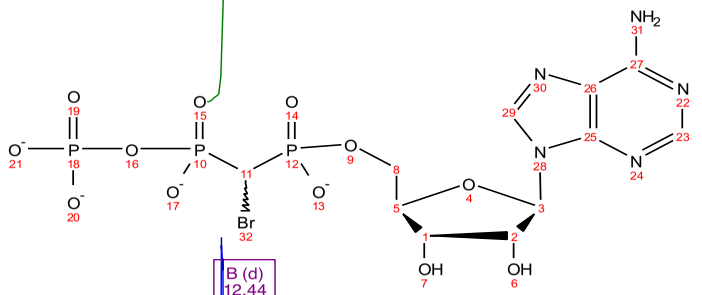
R/S-3b

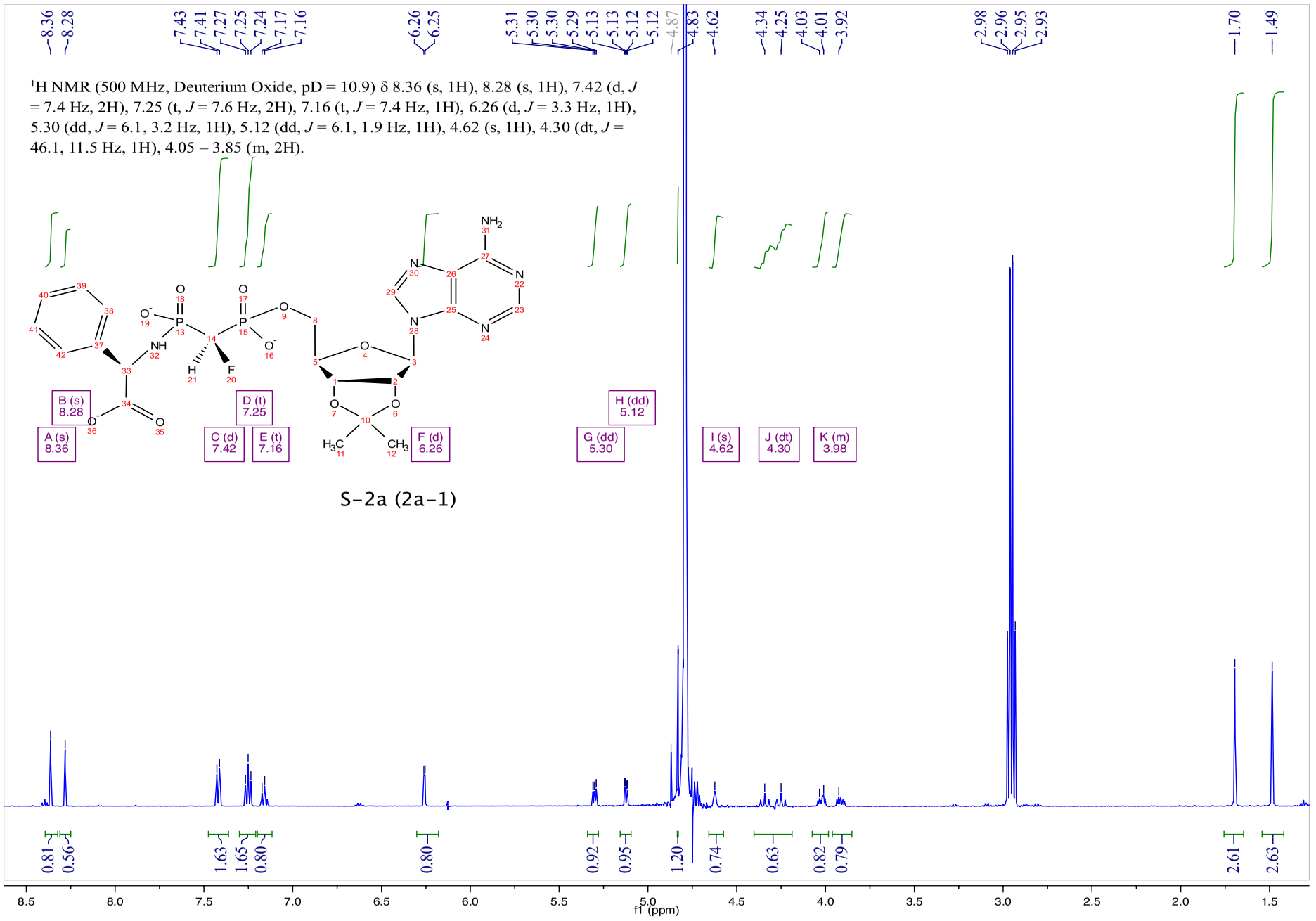


[1]H NMR (500 MHz, Deuterium Oxide, pD = 10) δ 8.51 (s, 1H), 8.50 (s, 0.6H), 8.13 (s, 0.4H), 6.02 (d, *J* = 5.8 Hz, 1H), 4.58 – 4.44 (m, 1H), 4.27 (s, 1H), 4.23 – 3.95 (m, 3H).

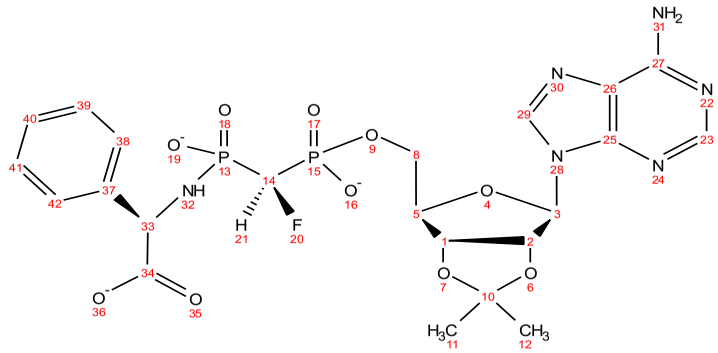


^{1}P NMR (202 MHz, Deuterium Oxide, pD = 10) δ 12.62 (d, *J* = 4.5 Hz), 12.44 (d, *J* = 5.1 Hz), .72 (dd, *J* = 25.5, 4.7 Hz), -5.87 (dd, *J* = 25.4, 2.9 Hz).




8.36
8.28
7.43
7.41
7.27
7.25
7.24
7.17
7.16
6.26
6.25
5.31
5.30
5.30
5.29
5.13
5.13
5.12
5.12
4.87
4.83
4.62
4.34
4.25
4.03
4.01
3.92
2.98
2.96
2.95
2.93
1.70
1.49
[1]H NMR (500 MHz, Deuterium Oxide, pD = 10.9) δ 8.36 (s, 1H), 8.28 (s, 1H), 7.42 (d, J = 7.4 Hz, 2H), 7.25 (t, J = 7.6 Hz, 2H), 7.16 (t, J = 7.4 Hz, 1H), 6.26 (d, J = 3.3 Hz, 1H), 5.30 (dd, J = 6.1, 3.2 Hz, 1H), 5.12 (dd, J = 6.1, 1.9 Hz, 1H), 4.62 (s, 1H), 4.30 (dt, J = 46.1, 11.5 Hz, 1H), 4.05 – 3.85 (m, 2H).
B (s) 8.28
D (t) 7.25
H (dd) 5.12
A (s) 8.36
C (d) 7.42
E (t) 7.16
G (dd) 5.30
I (s) 4.62
J (dt) 4.30
K (m) 3.98
F (d) 6.26
S-2a (2a-1)
0.81
0.56
1.63
1.65
0.80
0.80
0.92
0.95
1.20
0.74
0.63
0.82
0.79
2.61
2.63
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
f1 (ppm)

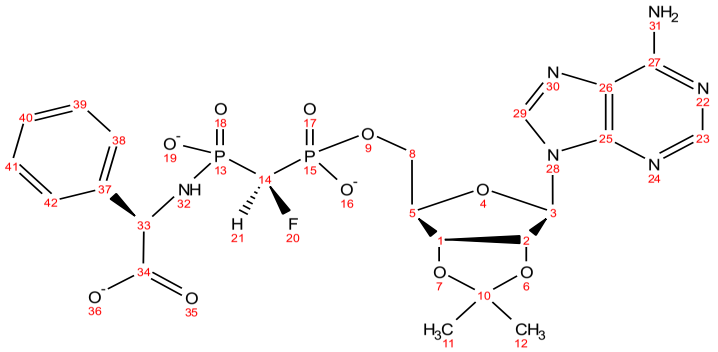
^{31}P NMR (202 MHz, Deuterium Oxide, pD = 10.9) δ 11.26 (dd, *J* = 62.1, 12.3 Hz), 9.82 (dd, *J* = 59.8, 12.4 Hz).

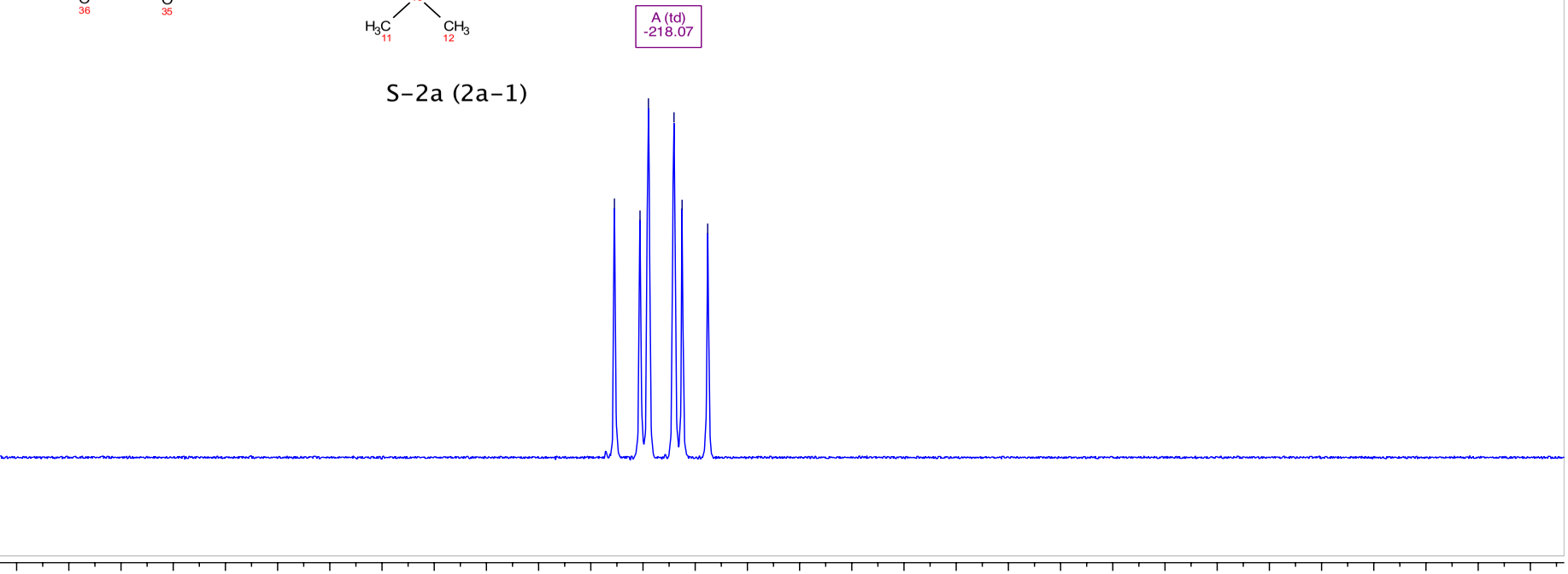


S-2a (2a-1)

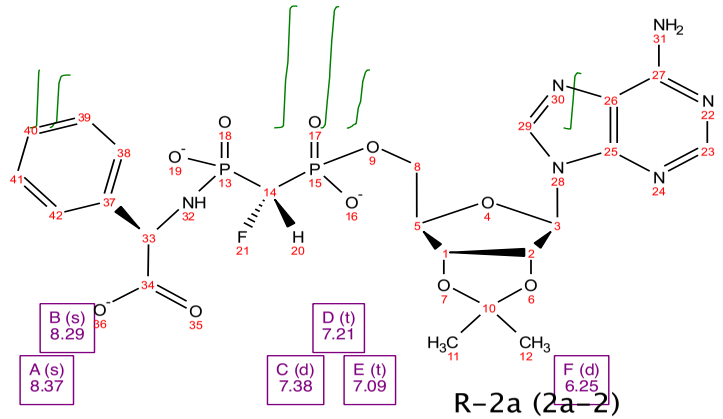
-217.89
-217.99
-218.02
-218.12
-218.15
-218.25

^{19}F NMR (470 MHz, Deuterium Oxide, pD = 10.9) δ -218.07 (td, *J* = 61.0, 46.1 Hz).



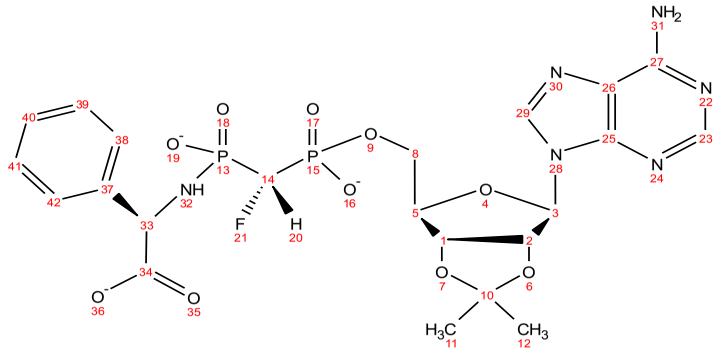


^{1}H NMR (500 MHz, Deuterium Oxide, pD = 10) δ 8.37 (s, 1H), 8.29 (s, 1H), 7.38 (d, J = 7.5 Hz, 2H), 7.21 (t, J = 7.7 Hz, 2H), 7.09 (t, J = 7.4 Hz, 1H), 6.25 (d, J = 3.4 Hz, 1H), 5.20 (dd, J = 6.1, 3.5 Hz, 1H), 5.11 (d, J = 2.0 Hz, 1H), 4.21 (d, J = 45.4 Hz, 0H), 4.08 – 3.88 (m, 2H), 1.69 (s, 3H), 1.47 (s, 3H).



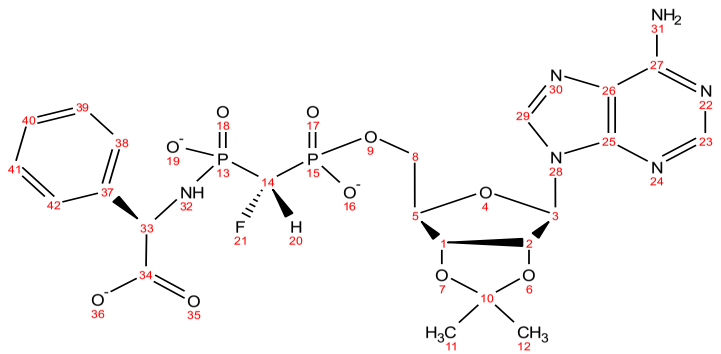
R-2a (2a-2)

^{31}P NMR (202 MHz, Deuterium Oxide, pD = 10) δ 11.41 (dd, *J* = 61.7, 11.2 Hz), 9.96 (dd, *J* = 60.7, 11.2 Hz).

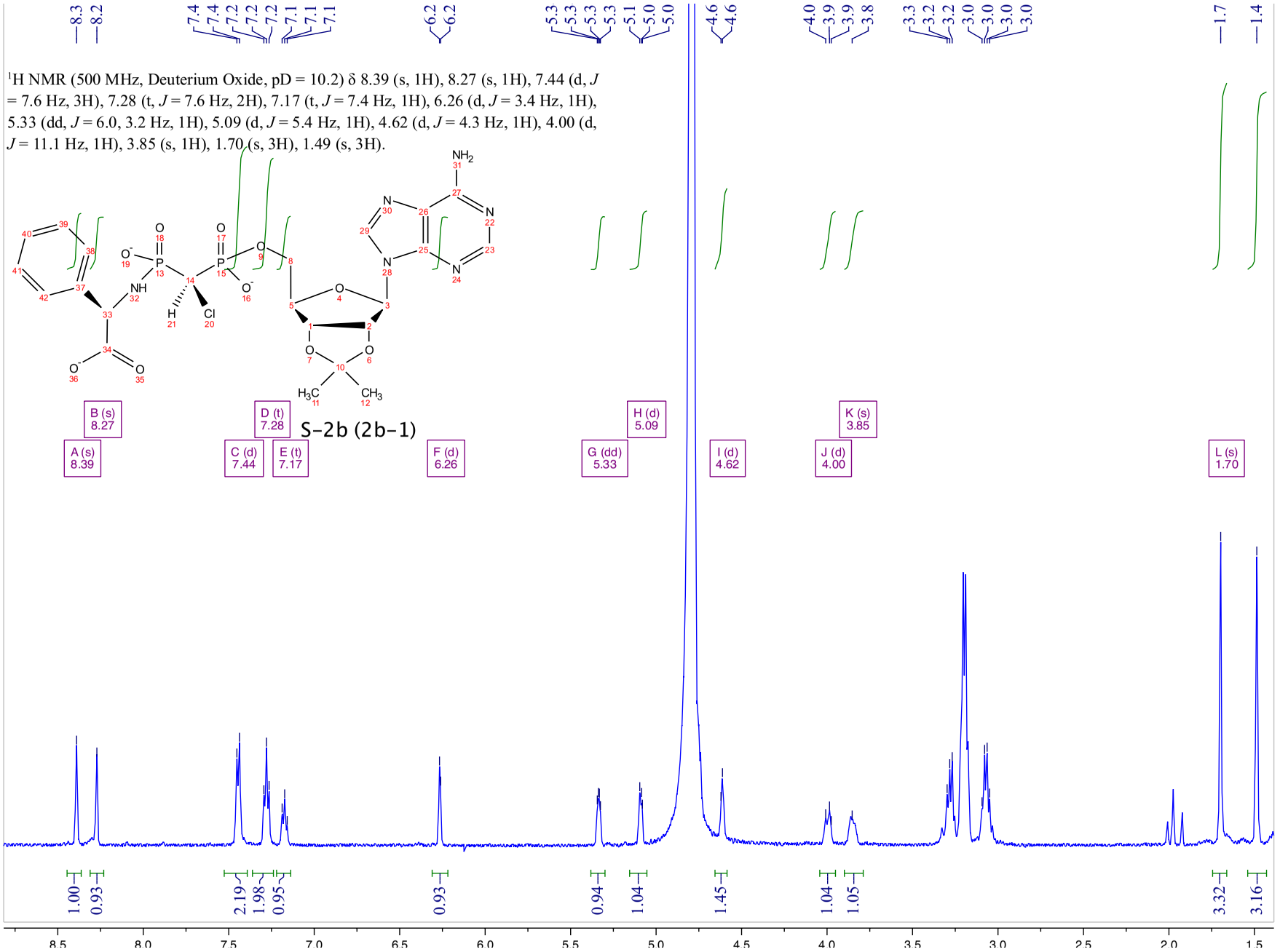


R-2a (2a-2)

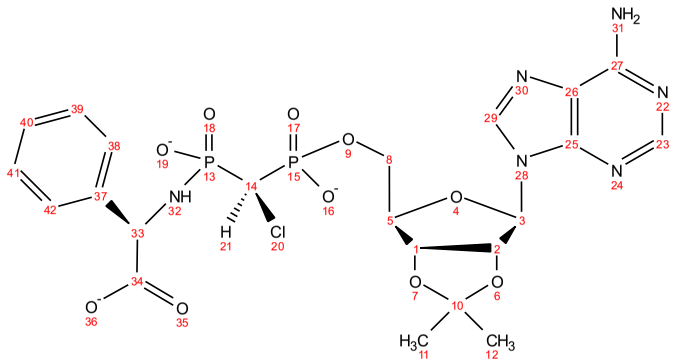
^{19}F NMR (470 MHz, Deuterium Oxide, pD =10) δ -217.38 (td, J = 61.2, 45.4 Hz).



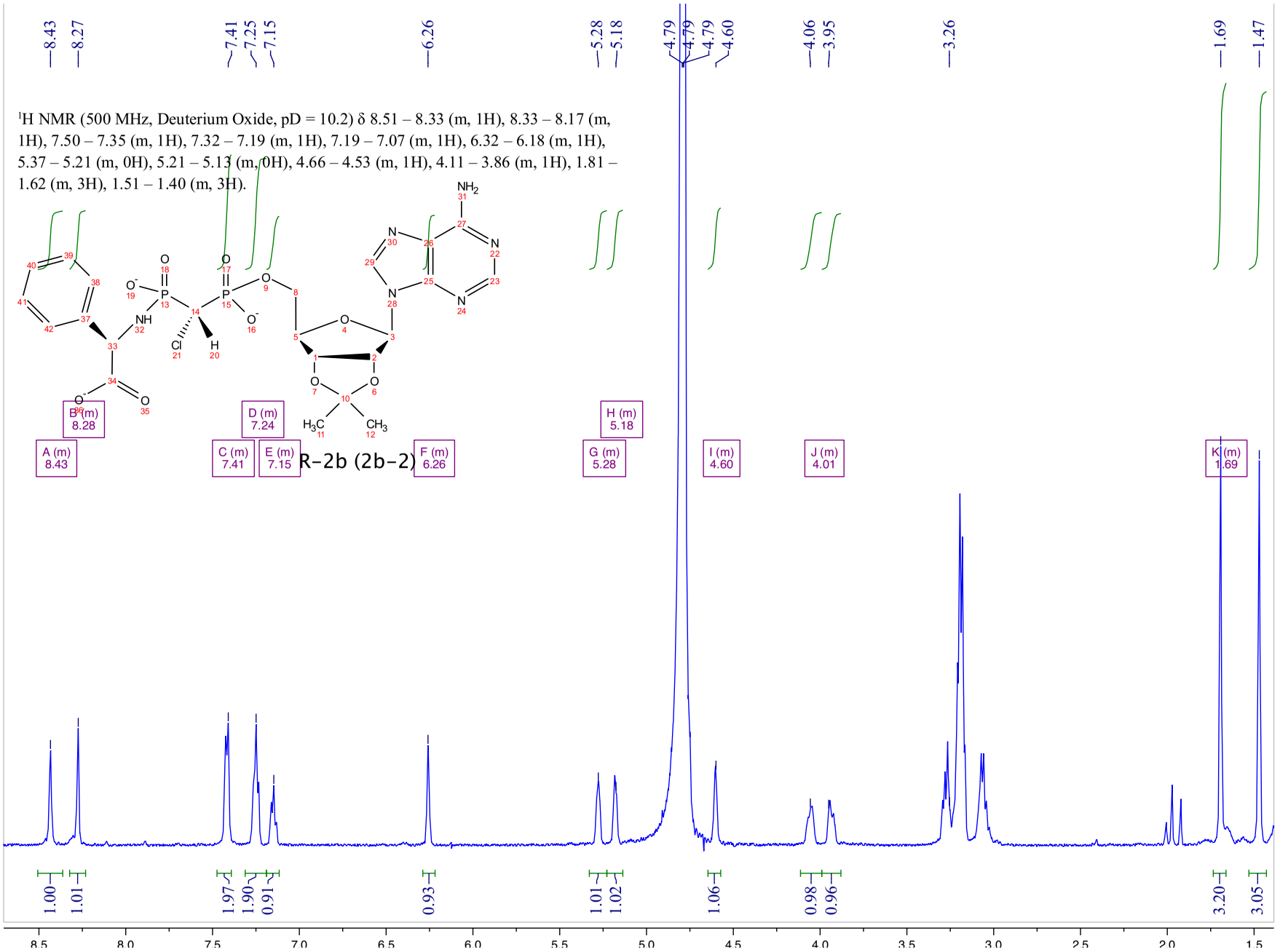
R-2a (2a-2)


8.3
8.2
7.4
7.4
7.2
7.2
7.2
7.1
7.1
7.1
6.2
6.2
5.3
5.3
5.3
5.3
5.1
5.0
5.0
4.6
4.6
4.0
3.9
3.9
3.8
3.3
3.2
3.2
3.0
3.0
3.0
3.0
1.7
1.4
[1]H NMR (500 MHz, Deuterium Oxide, pD = 10.2) δ 8.39 (s, 1H), 8.27 (s, 1H), 7.44 (d, J = 7.6 Hz, 3H), 7.28 (t, J = 7.6 Hz, 2H), 7.17 (t, J = 7.4 Hz, 1H), 6.26 (d, J = 3.4 Hz, 1H), 5.33 (dd, J = 6.0, 3.2 Hz, 1H), 5.09 (d, J = 5.4 Hz, 1H), 4.62 (d, J = 4.3 Hz, 1H), 4.00 (d, J = 11.1 Hz, 1H), 3.85 (s, 1H), 1.70 (s, 3H), 1.49 (s, 3H).
B (s) 8.27
D (t) 7.28
S-2b (2b-1)
H (d) 5.09
K (s) 3.85
A (s) 8.39
C (d) 7.44
E (t) 7.17
F (d) 6.26
G (dd) 5.33
I (d) 4.62
J (d) 4.00
L (s) 1.70
1.00
0.93
2.19
1.98
0.95
0.93
0.94
1.04
1.45
1.04
1.05
3.32
3.16
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5

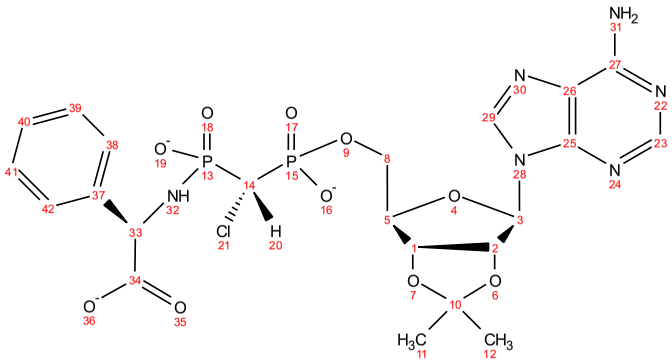
^{31}P NMR (202 MHz, Deuterium Oxide, pD = 10.2) δ 12.52 , 11.20 .



S-2b (2b-1)

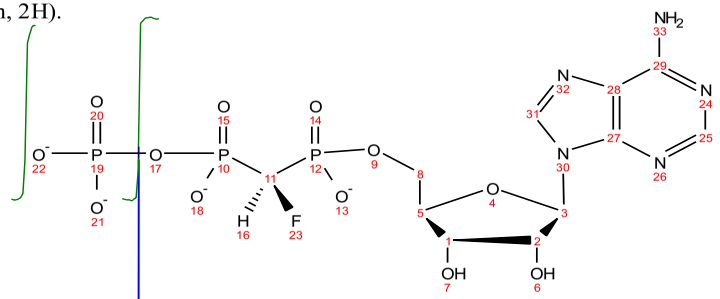

8.43
8.27
7.41
7.25
7.15
6.26
5.28
5.18
4.79
4.79
4.79
4.60
4.06
3.95
3.26
1.69
1.47
[1]H NMR (500 MHz, Deuterium Oxide, pD = 10.2) δ 8.51 – 8.33 (m, 1H), 8.33 – 8.17 (m, 1H), 7.50 – 7.35 (m, 1H), 7.32 – 7.19 (m, 1H), 7.19 – 7.07 (m, 1H), 6.32 – 6.18 (m, 1H), 5.37 – 5.21 (m, 0H), 5.21 – 5.13 (m, 0H), 4.66 – 4.53 (m, 1H), 4.11 – 3.86 (m, 1H), 1.81 – 1.62 (m, 3H), 1.51 – 1.40 (m, 3H).
B (m) 8.28
D (m) 7.24
H (m) 5.18
A (m) 8.43
C (m) 7.41
E (m) 7.15
F (m) 6.26
G (m) 5.28
I (m) 4.60
J (m) 4.01
K (m) 1.69
R-2b (2b-2)
1.00
1.01
1.97
1.90
0.91
0.93
1.01
1.02
1.06
0.98
0.96
3.20
3.05
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5

^{31}P NMR (202 MHz, Deuterium Oxide, pD = 10.2) δ 12.75 , 11.30 .



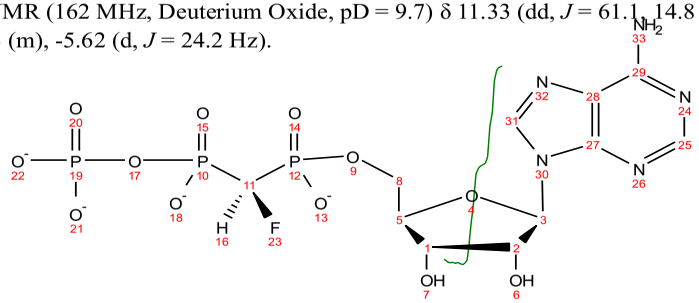
R-2b (2b-2)

^{1}H NMR (400 MHz, Deuterium Oxide, pD = 9.7) δ 8.60 (s, 1H), 8.28 (s, 1H), 6.16 (d, *J* = 5.9 Hz, 1H), 5.30 – 5.01 (m, 1H), 4.62 (dd, *J* = 5.1, 3.5 Hz, 1H), 4.39 (s, 1H), 4.35 – 4.21 (m, 2H).



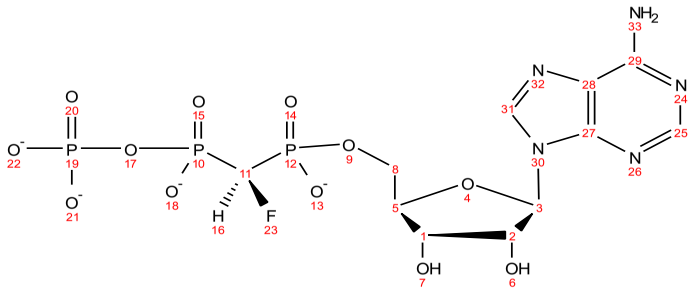
R-3a (3a-1)

^{31}P NMR (162 MHz, Deuterium Oxide, pD = 9.7) δ 11.33 (dd, *J* = 61.1, 14.8 Hz), 0.61 – -2.33 (m), -5.62 (d, *J* = 24.2 Hz).

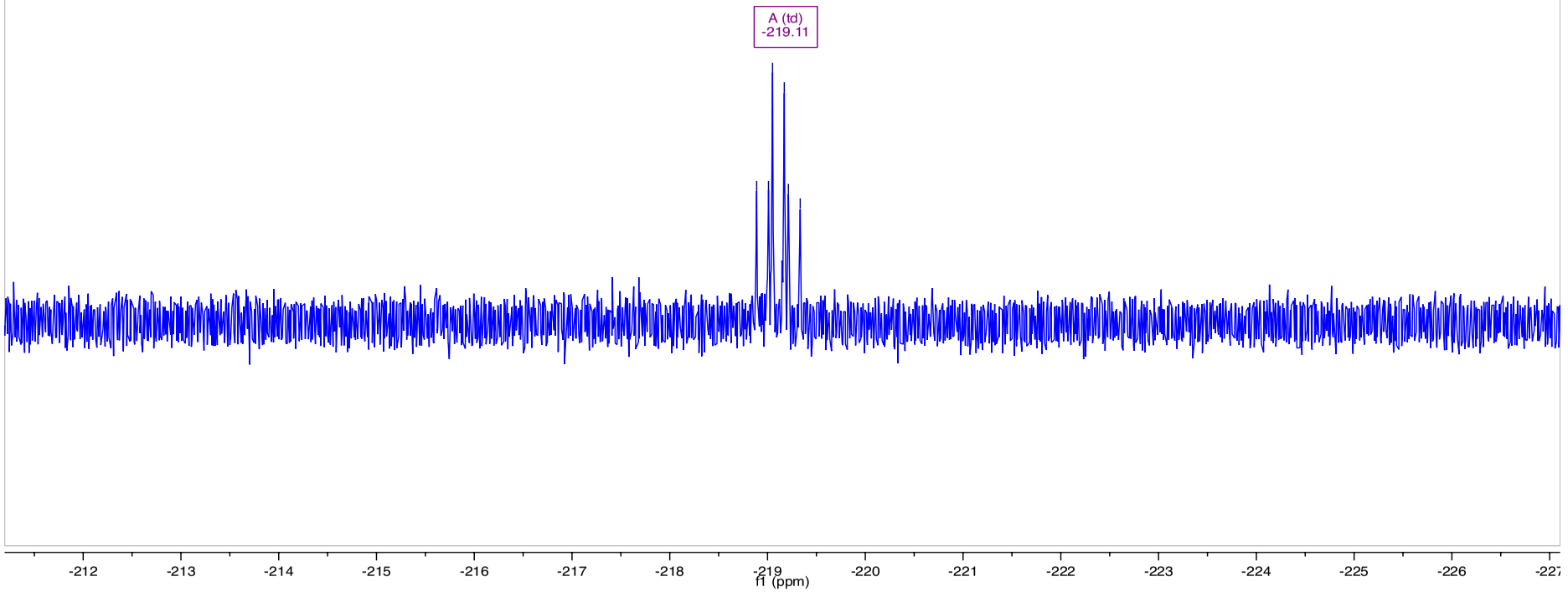


^{19}F NMR (376 MHz, Deuterium Oxide, pD = 9.7) δ -219.11 (td, J = 61.2, 45.5 Hz).

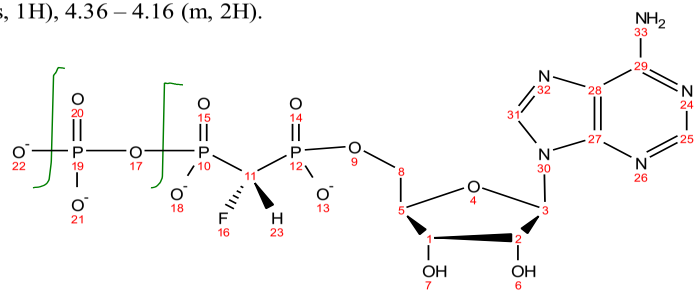
-218.89
-219.01
-219.05
-219.17
-219.21
-219.33



R-3a (3a-1)

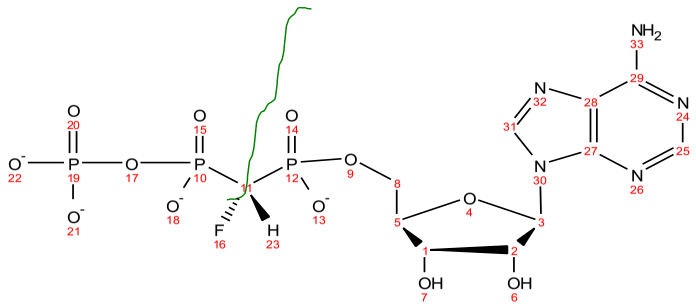


^{1}H NMR (400 MHz, Deuterium Oxide, pD = 9.9) δ 8.59 (s, 1H), 8.28 (s, 1H), 6.15 (d, *J* = 6.0 Hz, 1H), 5.16 (ddd, *J* = 45.3, 14.1, 12.1 Hz, 1H), 4.61 (dd, *J* = 5.1, 3.5 Hz, 1H), 4.40 (s, 1H), 4.36 – 4.16 (m, 2H).



S-3a (3a-2)

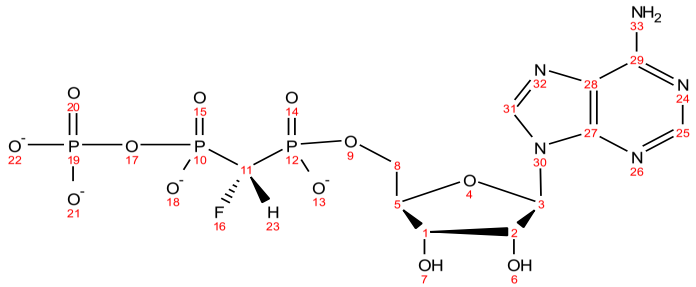
^{31}P NMR (162 MHz, Deuterium Oxide, pD = 9.9) δ 11.48 (dd, *J* = 60.9, 14.8 Hz), -0.60 (ddd, *J* = 61.4, 25.8, 14.7 Hz), -5.61 (d, *J* = 25.6 Hz).



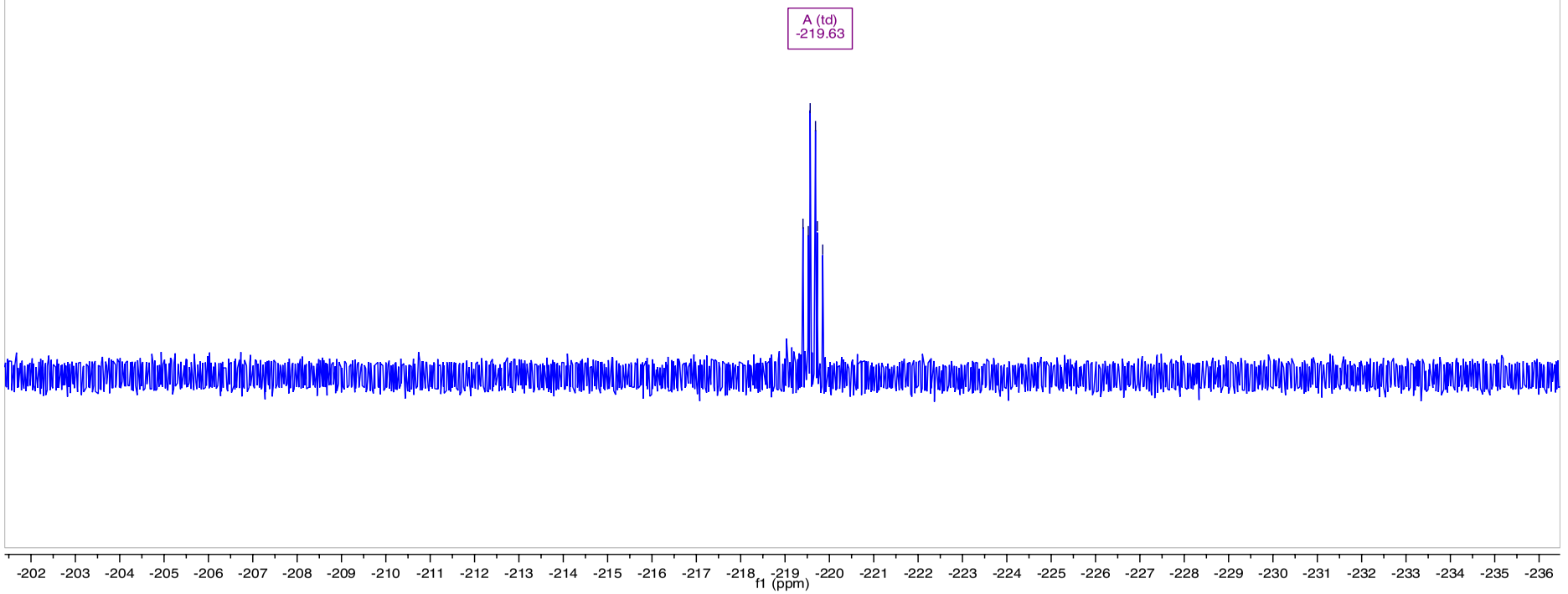
S-3a (3a-2)

^{19}F NMR (376 MHz, Deuterium Oxide, pD =9.9) δ -219.63 (td, J = 61.1, 45.3 Hz).

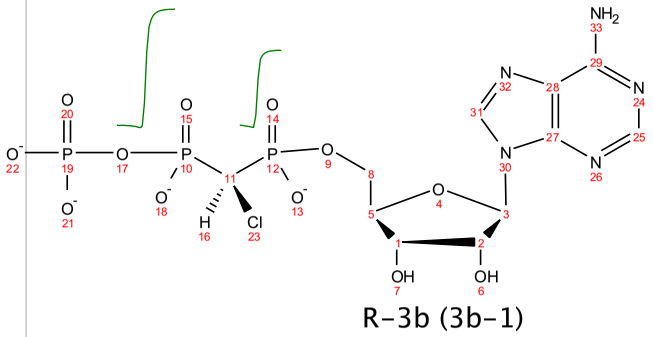
-219.40
-219.52
-219.57
-219.69
-219.73
-219.85



S-3a (3a-2)

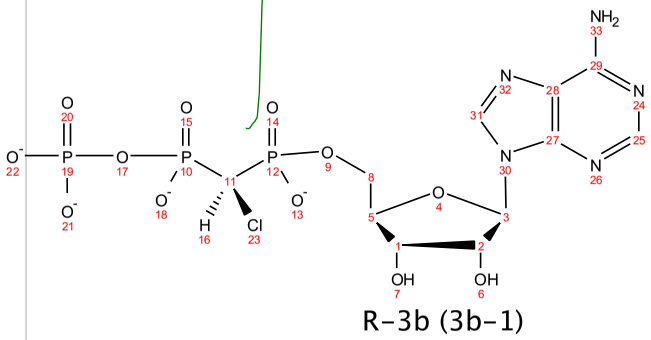


^{1}H NMR (400 MHz, Deuterium Oxide, pD = 10.7) δ 8.63 (s, 1H), 8.28 (s, 1H), 6.25 – 6.01 (m, 1H), 4.62 (s, 1H), 4.40 (s, 1H), 4.35 – 4.17 (m, 2H).



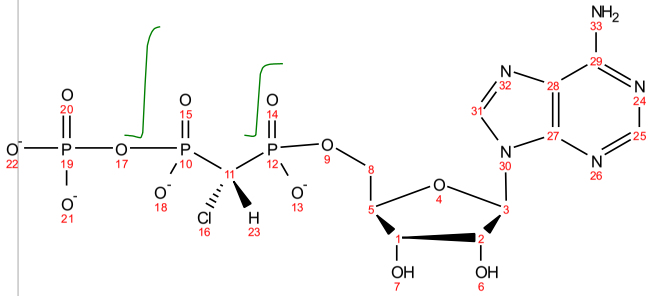
R-3b (3b-1)

^{31}P NMR (162 MHz, Deuterium Oxide, pD = 10.7) δ 12.69 (d, *J* = 6.3 Hz), 0.84 (dd, *J* = 25.6, 6.3 Hz), -6.04 (d, *J* = 25.5 Hz).



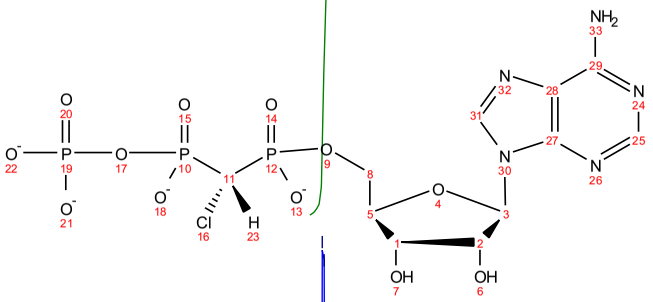
R-3b (3b-1)

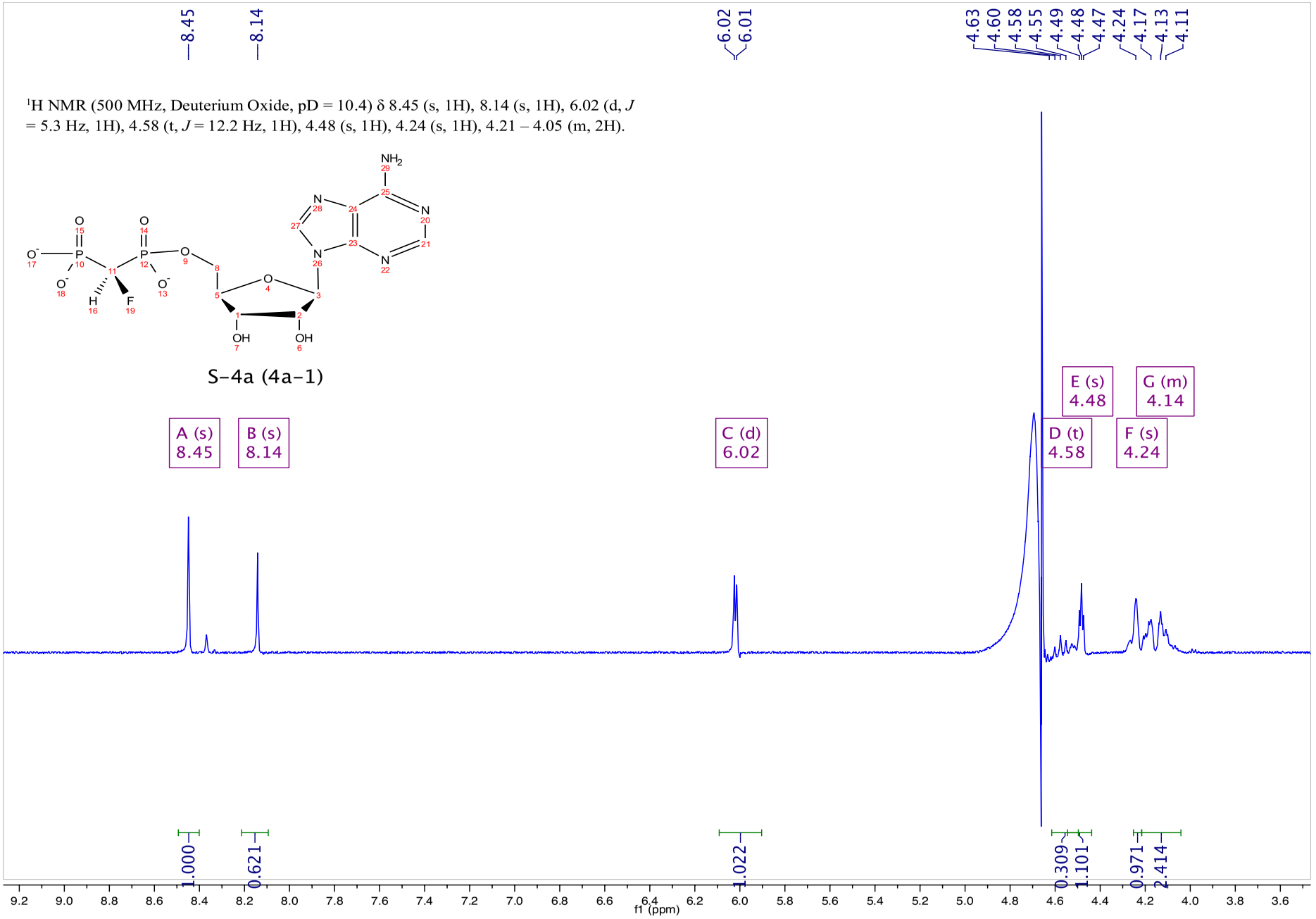
^{1}H NMR (400 MHz, Deuterium Oxide, pD =10.7) δ 8.60 (s, 1H), 8.24 (s, 1H), 6.12 (d, *J* = 5.8 Hz, 1H), 4.63 (s, 1H), 4.35 (s, 1H), 4.30 – 4.09 (m, 2H).



S-3b (3b-2)

^{31}P NMR (162 MHz, Deuterium Oxide, pD = 10.7) δ 12.87 (d, *J* = 5.8 Hz), 0.91 (dd, *J* = 25.5, 6.0 Hz), -5.98 (d, *J* = 25.7 Hz).

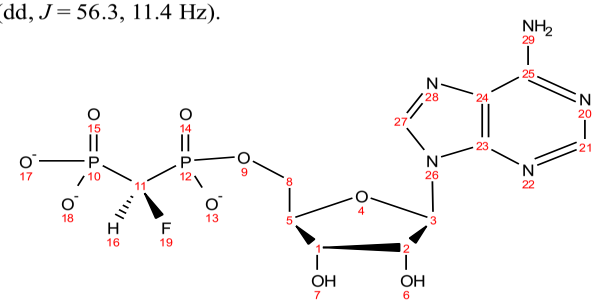




^{1}H NMR (500 MHz, Deuterium Oxide, pD = 10.4) δ 8.45 (s, 1H), 8.14 (s, 1H), 6.02 (d, *J* = 5.3 Hz, 1H), 4.58 (t, *J* = 12.2 Hz, 1H), 4.48 (s, 1H), 4.24 (s, 1H), 4.21 – 4.05 (m, 2H).

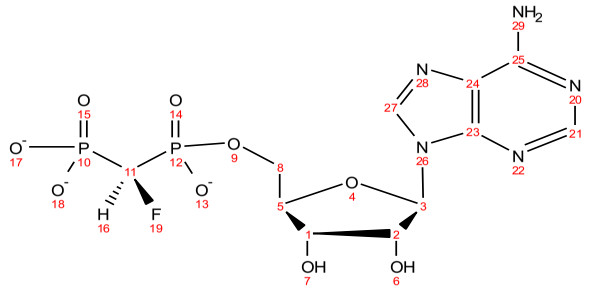
S-4a (4a-1)

^{31}P NMR (162 MHz, Deuterium Oxide, pD = 10.4) δ 13.92 (dd, *J* = 63.6, 11.4 Hz), 6.87 (dd, *J* = 56.3, 11.4 Hz).

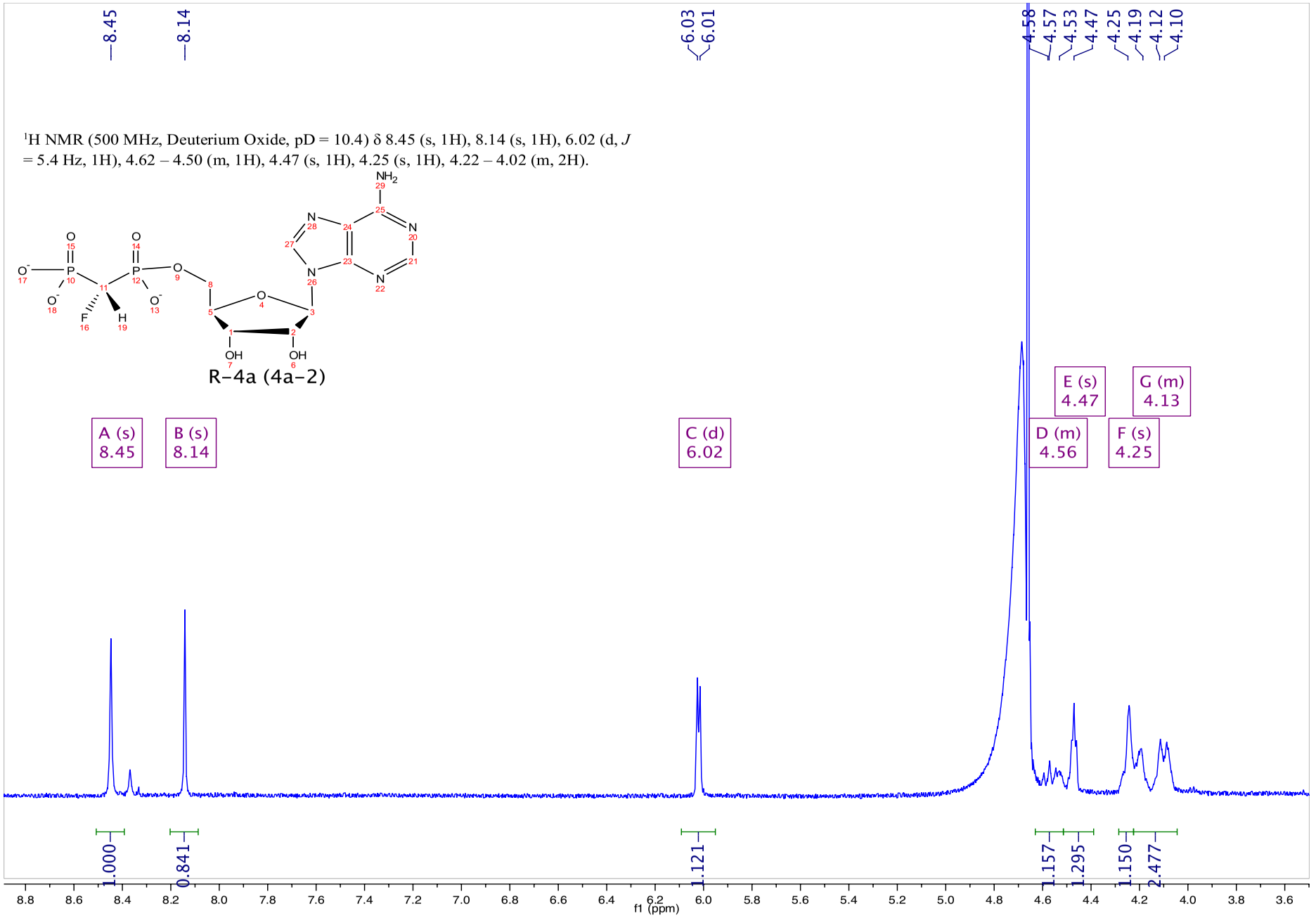


S-4a (4a-1)

^{19}F NMR (376 MHz, Deuterium Oxide, pD = 10.4) δ -216.70 (ddd, *J* = 63.6, 56.3, 45.4 Hz).



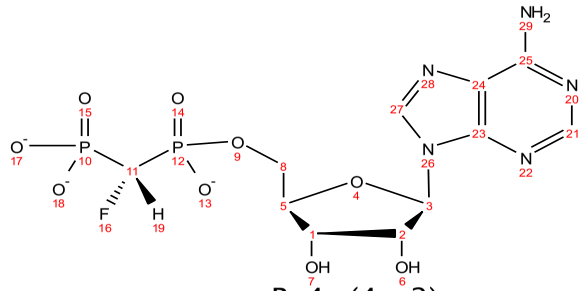
S-4a (4a-1)


8.45
8.14
6.03
6.01
4.58
4.57
4.53
4.47
4.25
4.19
4.12
4.10
[1]H NMR (500 MHz, Deuterium Oxide, pD = 10.4) δ 8.45 (s, 1H), 8.14 (s, 1H), 6.02 (d, J = 5.4 Hz, 1H), 4.62 – 4.50 (m, 1H), 4.47 (s, 1H), 4.25 (s, 1H), 4.22 – 4.02 (m, 2H).
R-4a (4a-2)
A (s) 8.45
B (s) 8.14
C (d) 6.02
D (m) 4.56
E (s) 4.47
F (s) 4.25
G (m) 4.13
1.000
0.841
1.121
1.157
1.295
1.150
2.477
f1 (ppm)

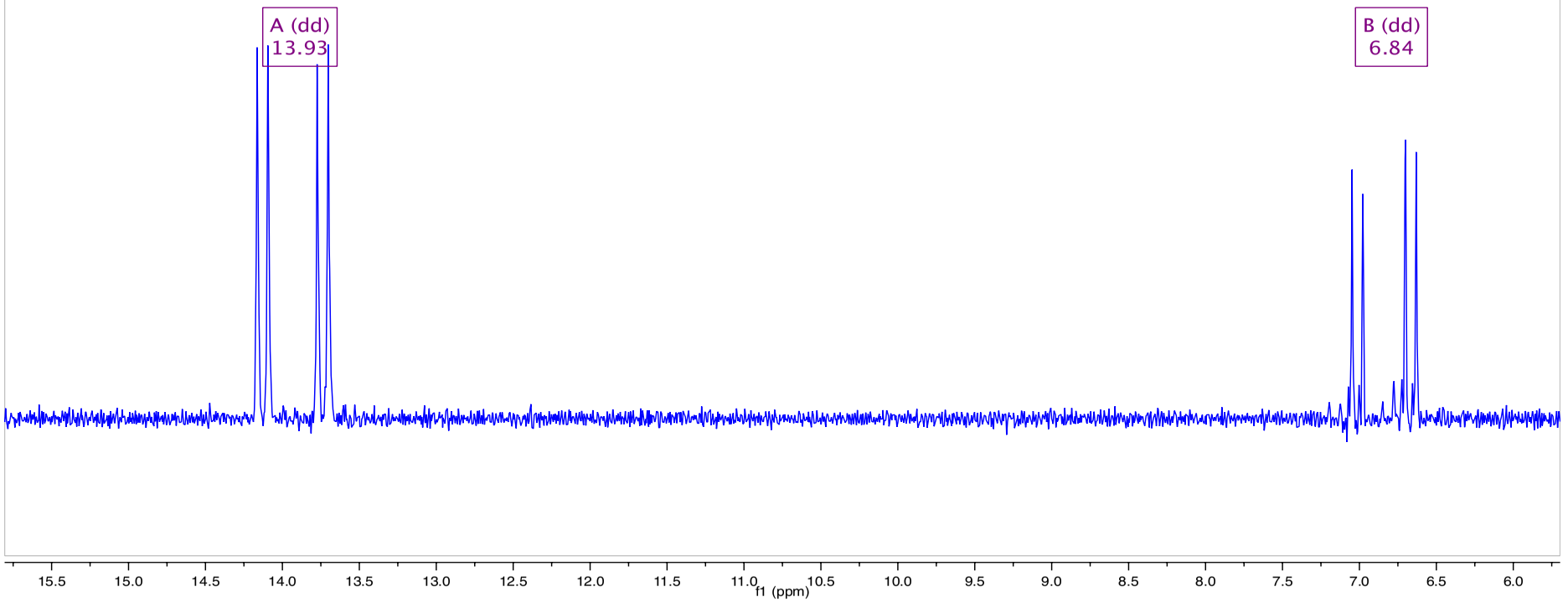
14.16
14.09
13.77
13.70

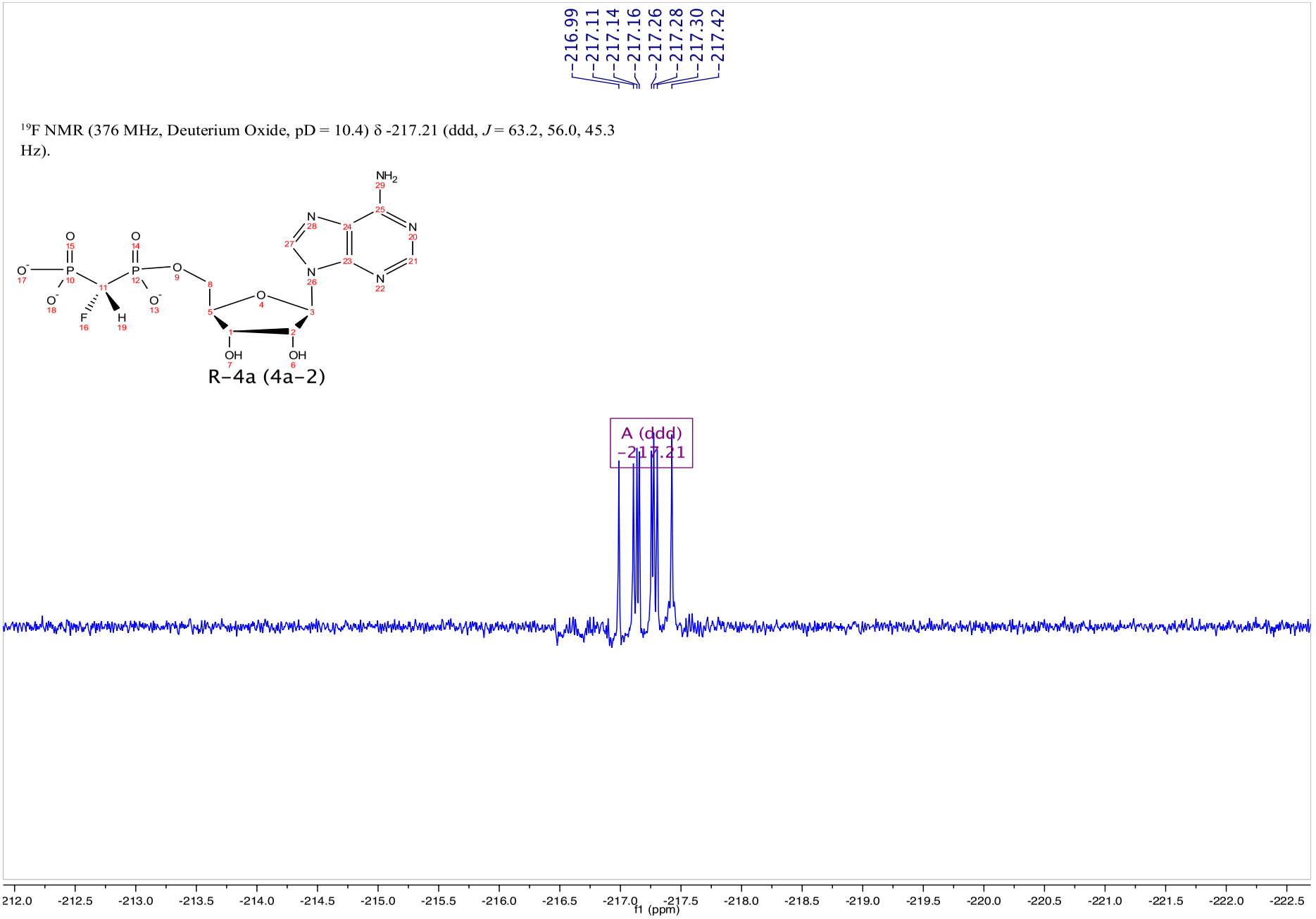
7.05
6.98
6.70
6.63

^{31}P NMR (162 MHz, Deuterium Oxide, pD = 10.4) δ 13.93 (dd, *J* = 63.3, 11.4 Hz), 6.84 (dd, *J* = 56.1, 11.4 Hz).



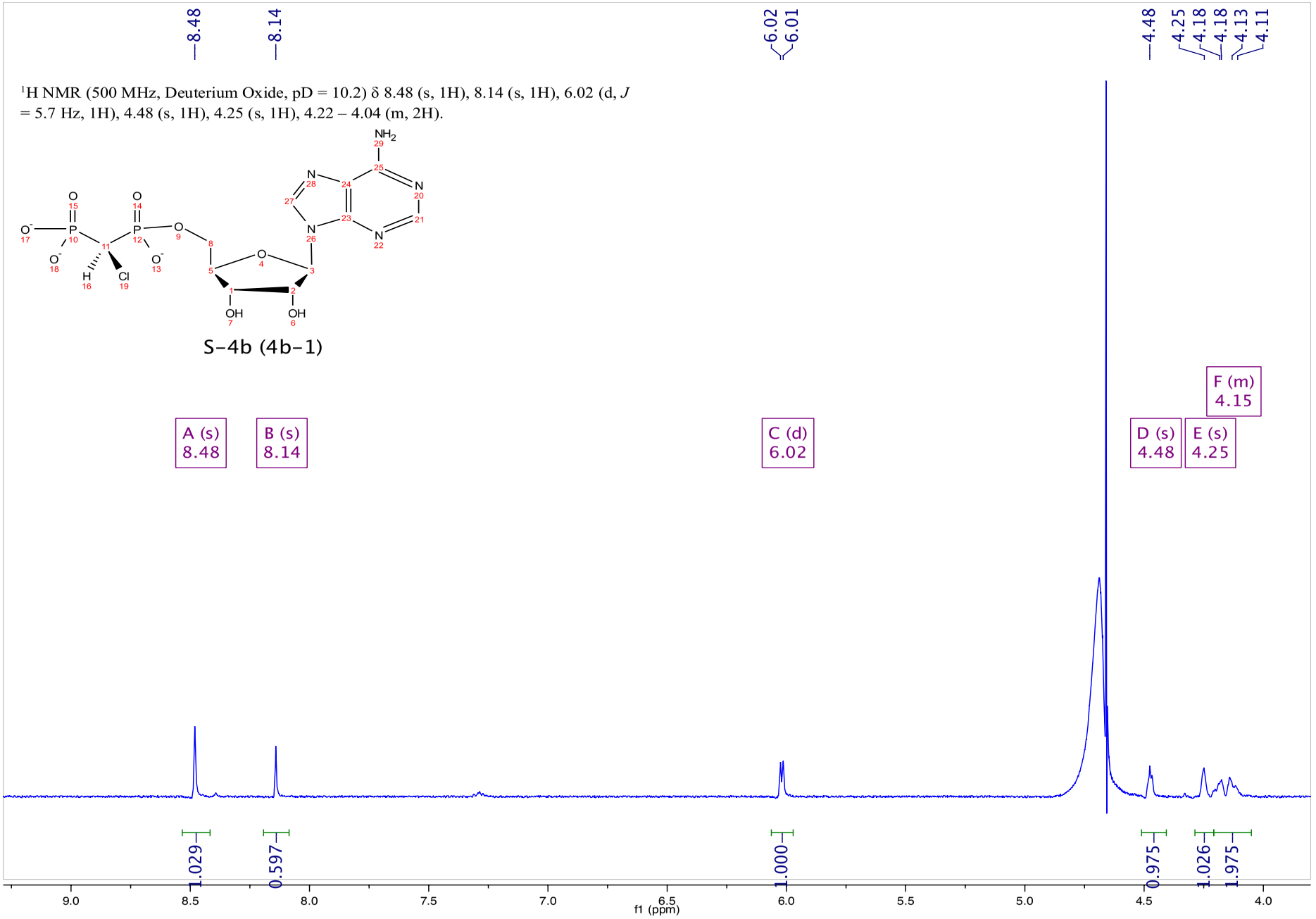
R–4a (4a–2)



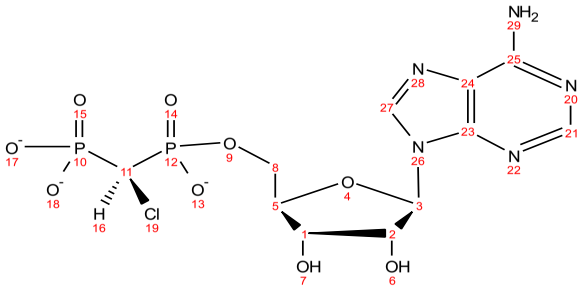


^{19}F NMR (376 MHz, Deuterium Oxide, pD = 10.4) δ -217.21 (ddd, J = 63.2, 56.0, 45.3 Hz).

R–4a (4a–2)

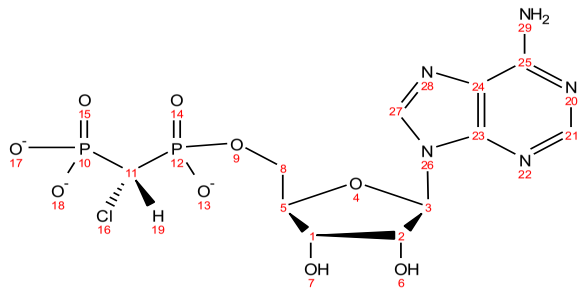

8.48
8.14
6.02
6.01
4.48
4.25
4.18
4.18
4.13
4.11
[1]H NMR (500 MHz, Deuterium Oxide, pD = 10.2) δ 8.48 (s, 1H), 8.14 (s, 1H), 6.02 (d, J = 5.7 Hz, 1H), 4.48 (s, 1H), 4.25 (s, 1H), 4.22 – 4.04 (m, 2H).
S-4b (4b-1)
A (s) 8.48
B (s) 8.14
C (d) 6.02
D (s) 4.48
E (s) 4.25
F (m) 4.15
1.029
0.597
1.000
0.975
1.026
1.975
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
f1 (ppm)

^{31}P NMR (162 MHz, Deuterium Oxide, pD = 10.2) δ 15.40 , 8.23 .



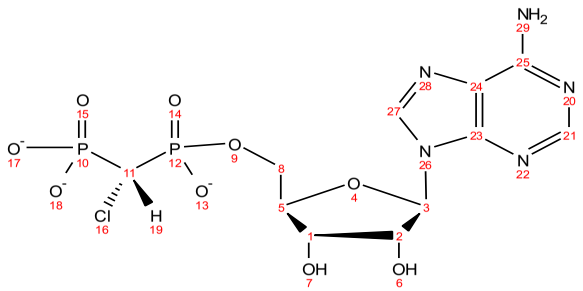
S-4b (4b-1)

^{1}H NMR (500 MHz, Deuterium Oxide, pD = 10.2) δ 8.49 (s, 1H), 8.14 (s, 1H), 6.02 (d, *J* = 5.6 Hz, 1H), 4.52 (s, 1H), 4.25 (s, 1H), 4.22 – 4.03 (m, 2H).



R-4b (4b-2)

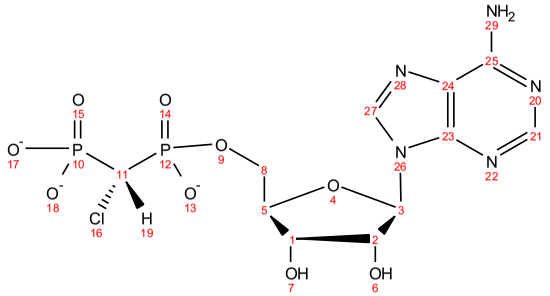
^{31}P NMR (162 MHz, Deuterium Oxide, pD = 10.2) δ 15.44 , 8.17 .



R-4b (4b-2)

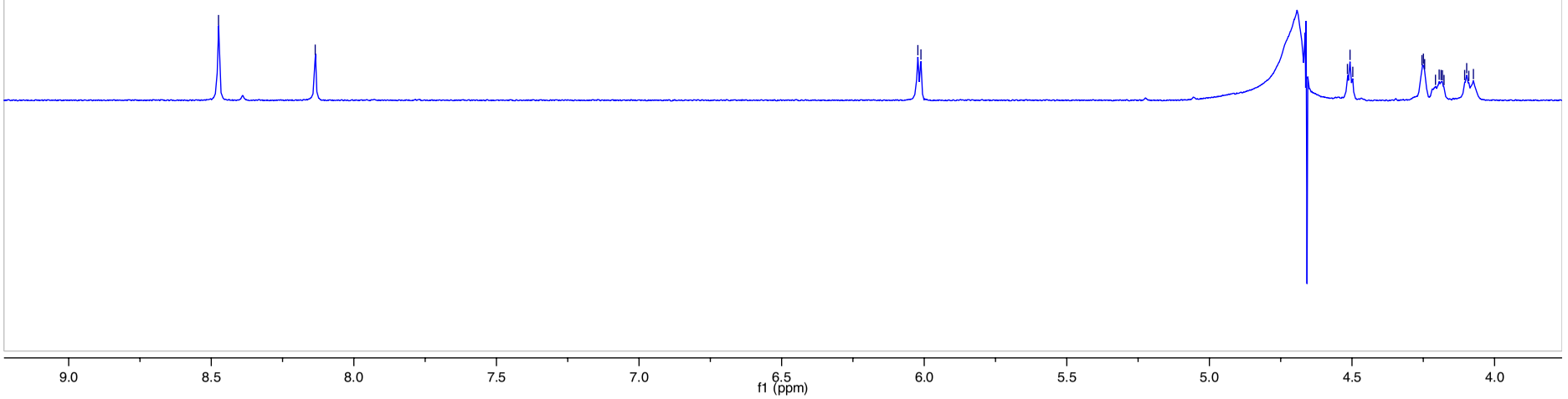
8.47 8.14 6.02 6.01 4.52 4.51 4.50 4.25 4.25 4.25 4.21 4.19 4.19 4.18 4.18 4.10 4.10 4.09 4.07

^{1}H NMR (500 MHz, Deuterium Oxide, pD = 10.5) δ 8.47 (s, 1H), 8.14 (s, 1H), 6.02 (d, *J* = 5.4 Hz, 1H), 4.51 (t, *J* = 4.7 Hz, 1H), 4.25 (d, *J* = 2.5 Hz, 1H), 4.23 – 4.03 (m, 2H).

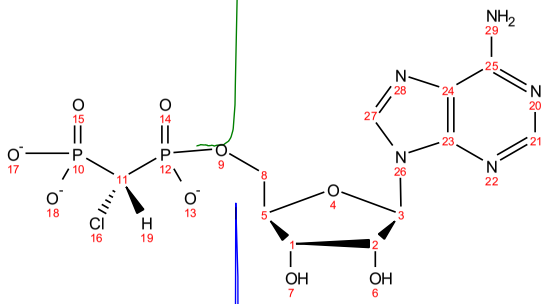


R-4b (4b-2) by Src/YopH

A (s) 8.47 | B (s) 8.14 | C (d) 6.02 | D (t) 4.51 | E (d) 4.25 | F (m) 4.14



^{31}P NMR (202 MHz, Deuterium Oxide, pD = 10.5) δ 15.55 , 8.31 .



R-4b (4b-2) by Src/YopH

15.55

8.31

A (s)
15.55

B (s)
8.31

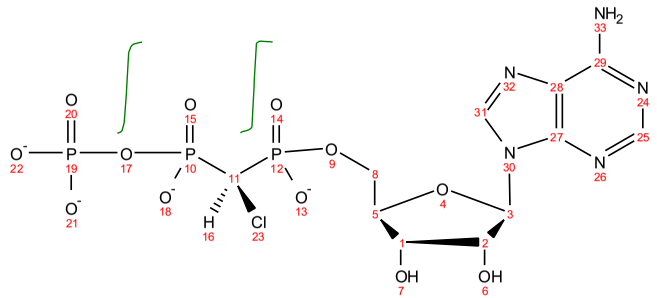
1.00

0.95

18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3

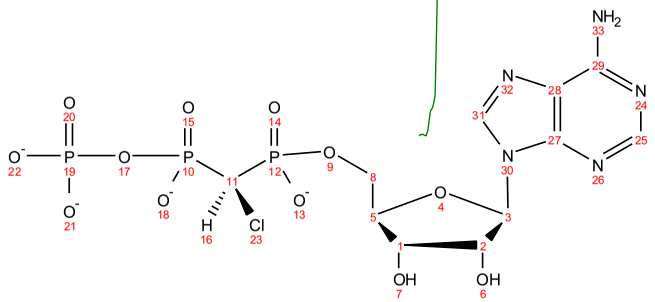
f1 (ppm)

^{1}H NMR (500 MHz, Deuterium Oxide, pD = 10.5) δ 8.49 (s, 1H), 8.14 (s, 1H), 6.02 (d, *J* = 5.9 Hz, 1H), 4.51 – 4.44 (m, 1H), 4.27 (s, 1H), 4.21 – 4.11 (m, 2H).

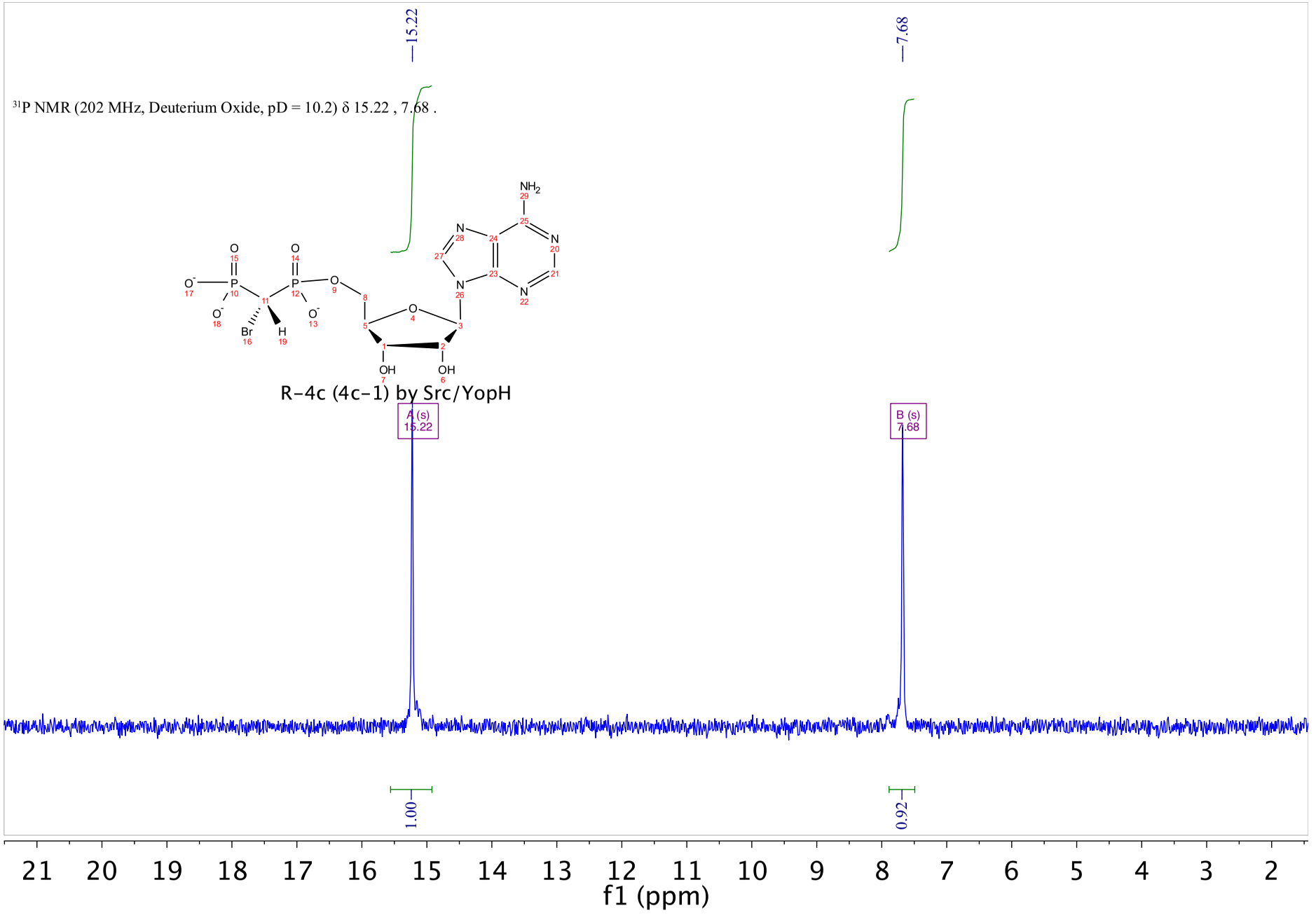


R-3b (3b-1) by Src/YopH

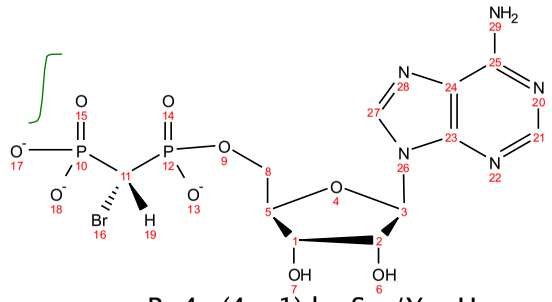
^{31}P NMR (202 MHz, Deuterium Oxide, pD = 10.5) δ 12.76 (d, *J* = 6.3 Hz), 1.09 (dd, *J* = 25.5, 6.4 Hz), -5.76 (d, *J* = 25.3 Hz).



^{31}P NMR (202 MHz, Deuterium Oxide, pD = 10.2) δ 15.22 , 7.68 .

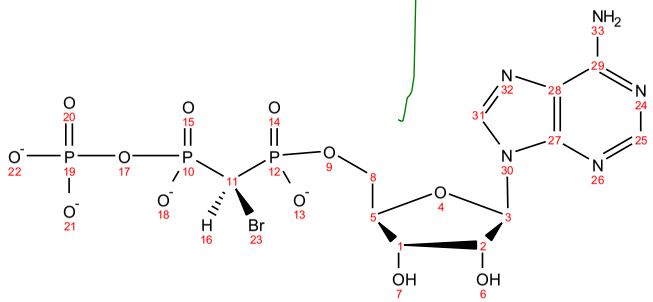


^{1}H NMR (500 MHz, Deuterium Oxide, pD = 10.2) δ 8.49 (s, 1H), 8.13 (s, 0H), 6.02 (d, J = 4.9 Hz, 1H), 4.53 (s, 1H), 4.25 (s, 1H), 4.22 – 3.99 (m, 2H).

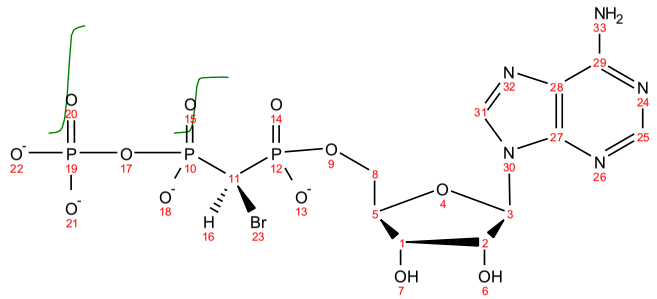


R-4c (4c-1) by Src/YopH

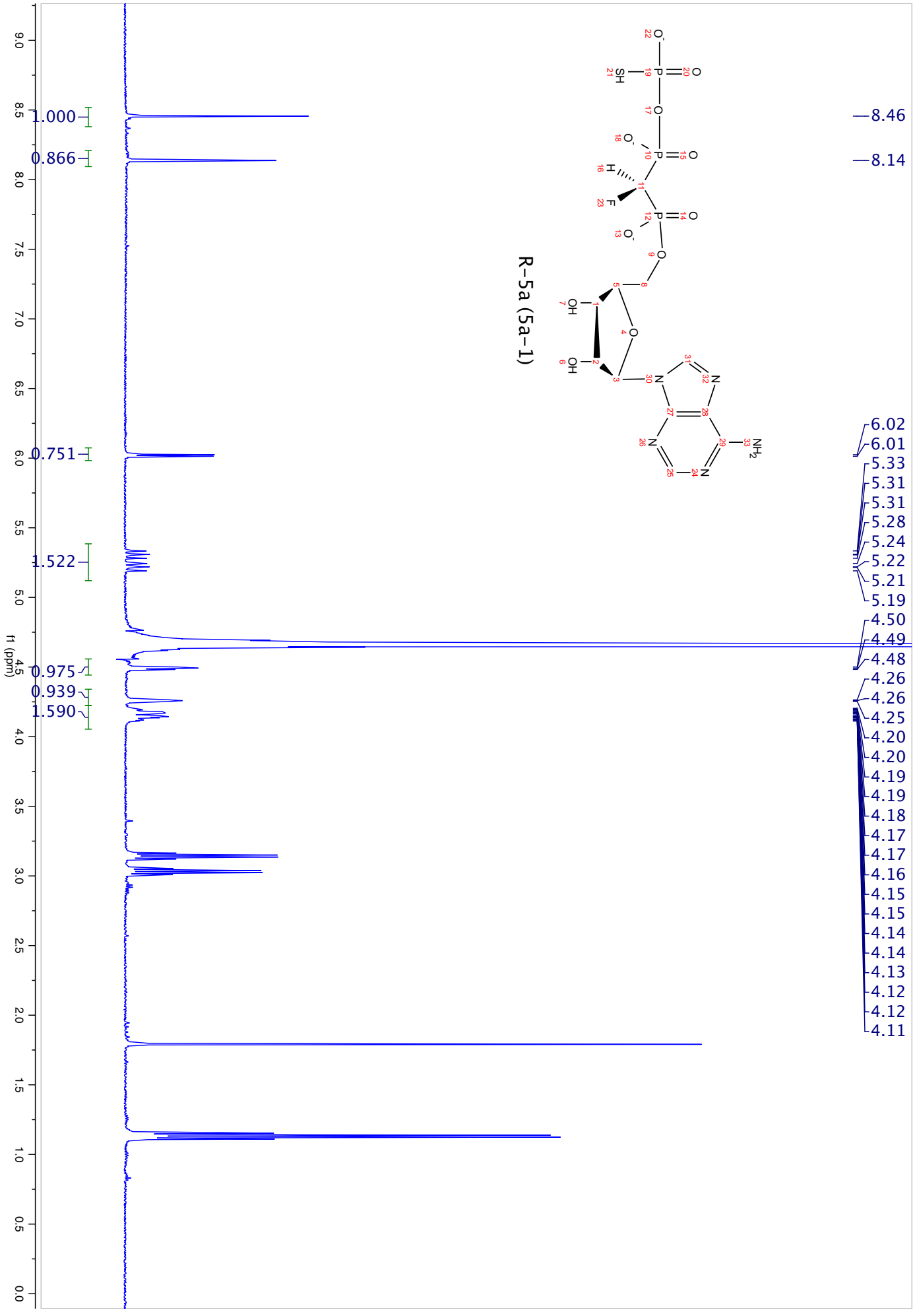
^{31}P NMR (202 MHz, Deuterium Oxide, pD = 10.5) δ 12.46 (d, *J* = 5.1 Hz), 0.70 (dd, *J* = 25.4, 5.0 Hz), -5.86 (d, *J* = 25.4 Hz).

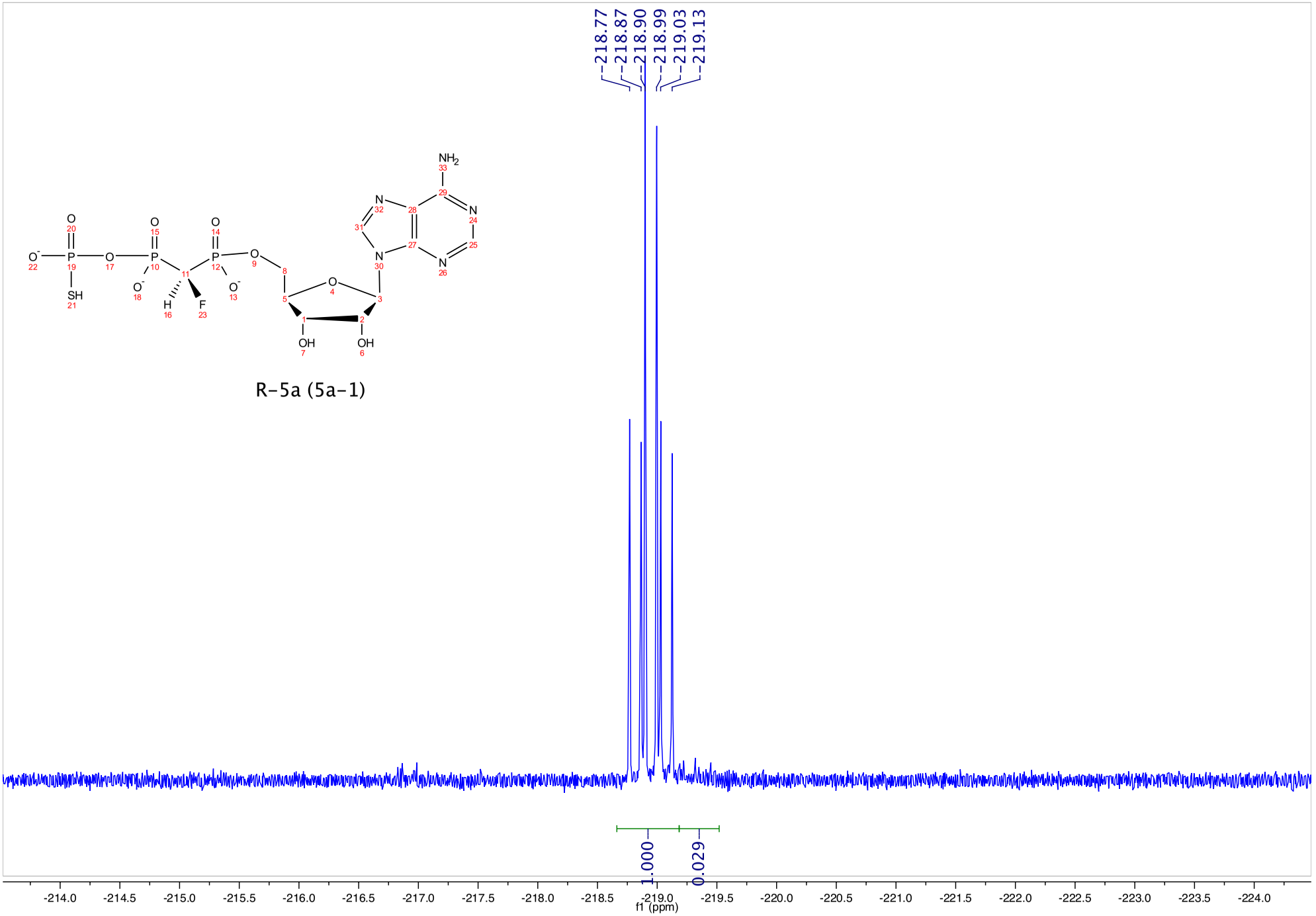


^{1}H NMR (500 MHz, Deuterium Oxide, pD = 10.5) δ 8.51 (s, 1H), 8.14 (s, 1H), 6.02 (d, *J* = 5.8 Hz, 1H), 4.49 (s, 1H), 4.27 (s, 1H), 4.19 – 4.03 (m, 2H).

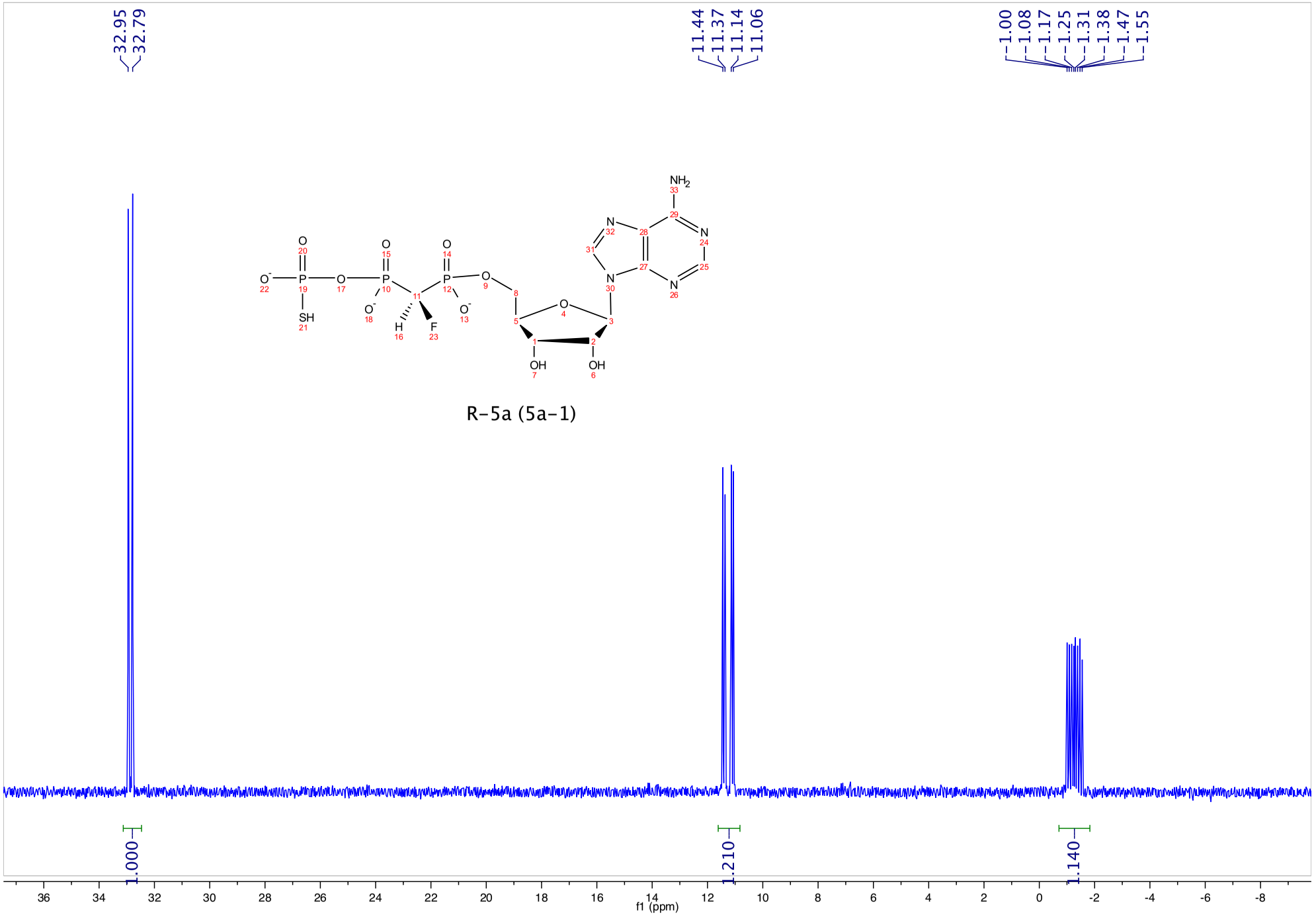


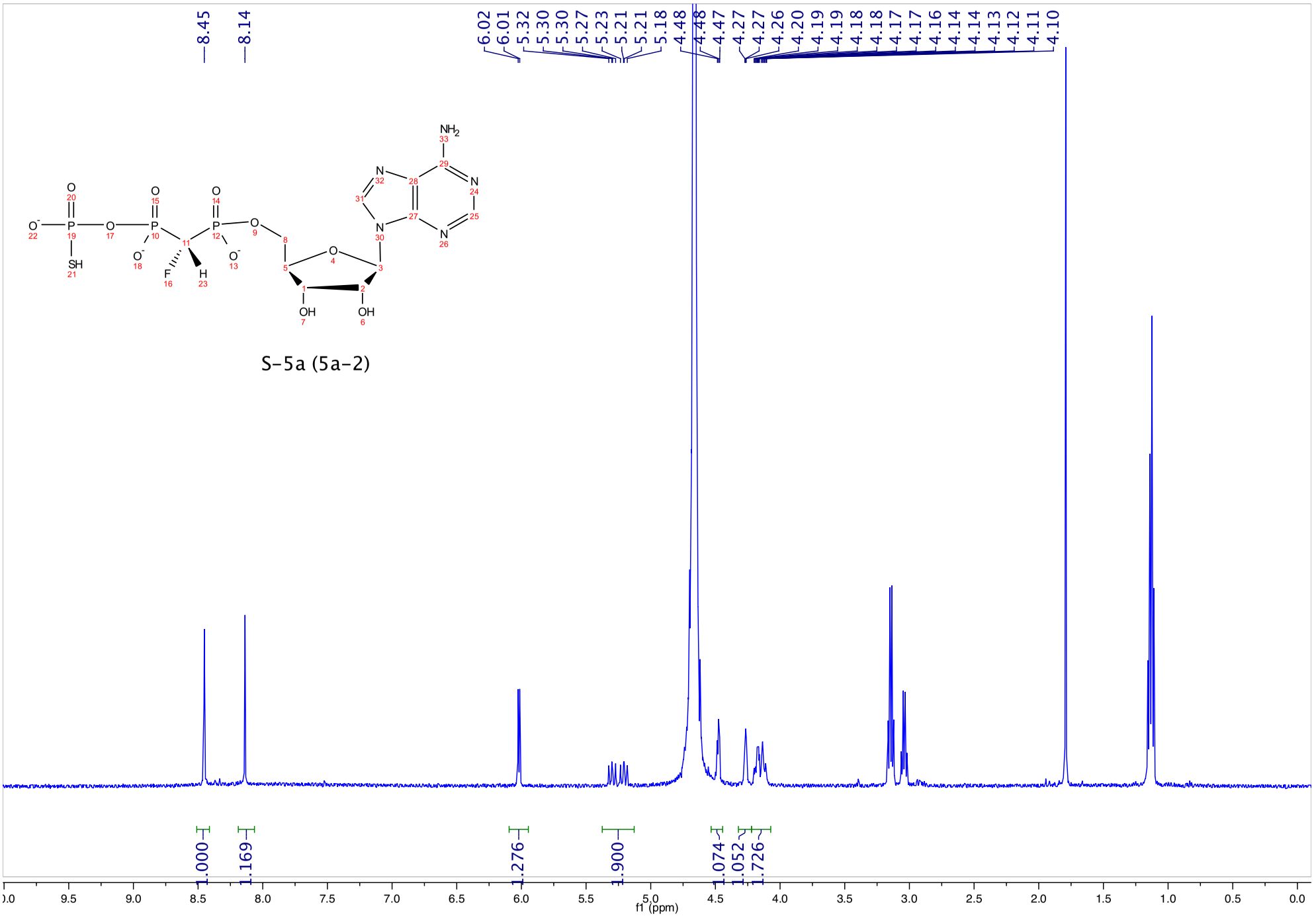
R-3c (3c-1) by Src/YopH

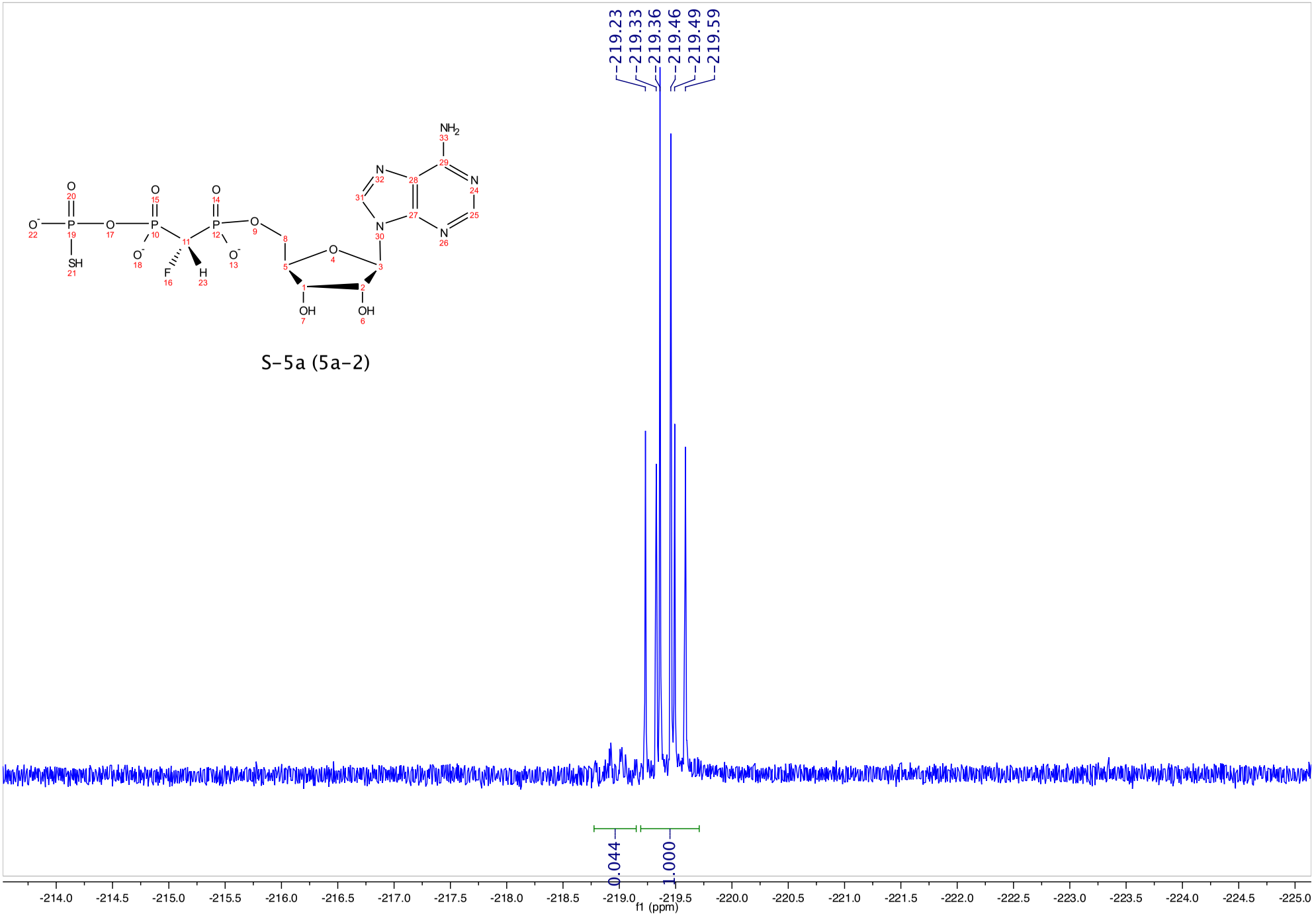

R-5a (5a-1)
1.000
0.866
0.751
1.522
0.975
0.939
1.590
8.46
8.14
6.02
6.01
5.33
5.31
5.31
5.28
5.24
5.22
5.21
5.19
4.50
4.49
4.48
4.26
4.26
4.25
4.20
4.20
4.19
4.19
4.18
4.17
4.17
4.16
4.15
4.15
4.14
4.14
4.13
4.12
4.12
4.11
f1 (ppm)


-218.77
-218.87
-218.90
-218.99
-219.03
-219.13
1.000
0.029
-214.0
-214.5
-215.0
-215.5
-216.0
-216.5
-217.0
-217.5
-218.0
-218.5
-219.0
-219.5
-220.0
-220.5
-221.0
-221.5
-222.0
-222.5
-223.0
-223.5
-224.0
f1 (ppm)

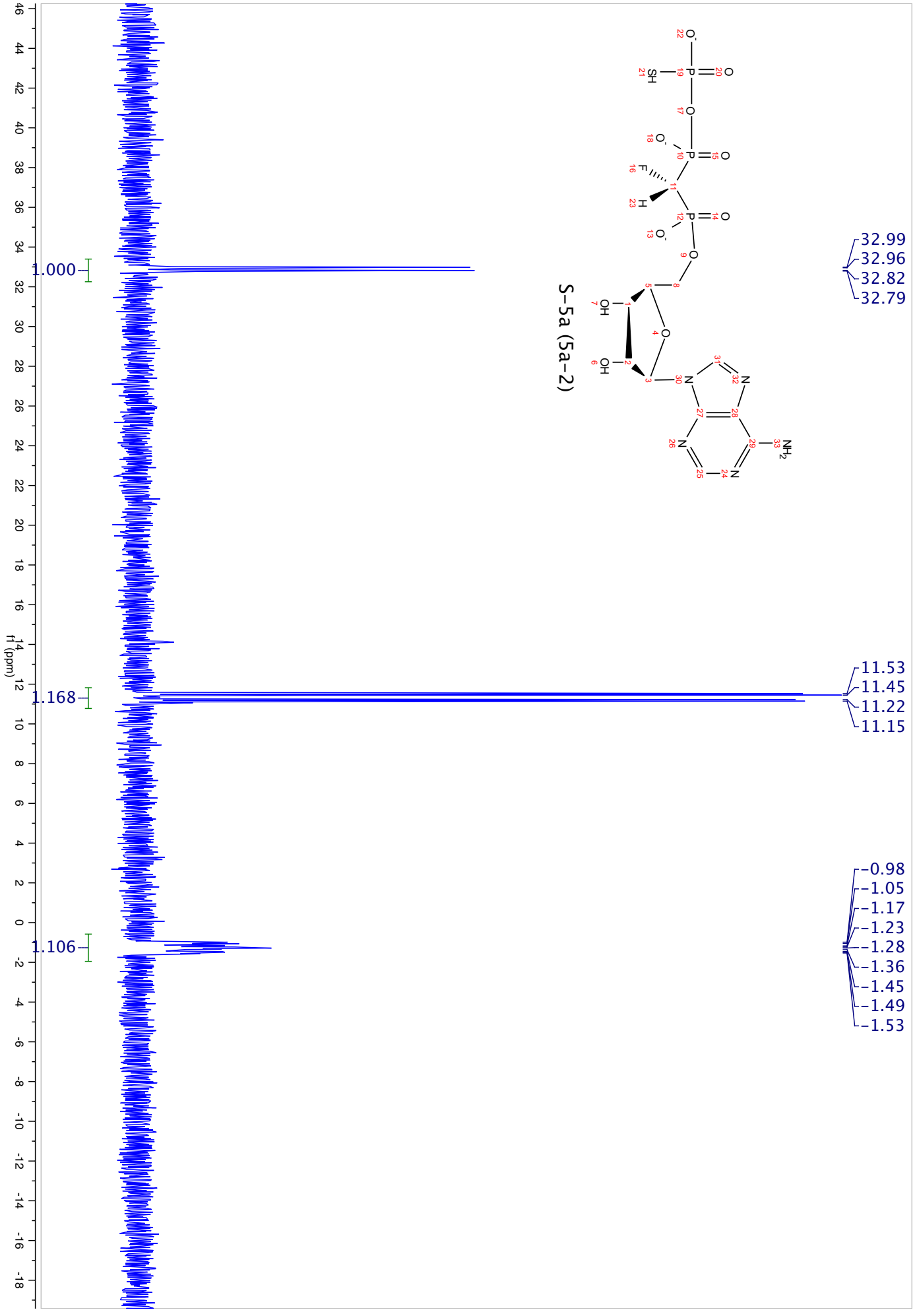
R-5a (5a-1)


32.95
32.79
11.44
11.37
11.14
11.06
1.00
1.08
1.17
1.25
1.31
1.38
1.47
1.55
R-5a (5a-1)
1.000
1.210
1.140
f1 (ppm)


8.45
8.14
6.02
6.01
5.32
5.30
5.30
5.27
5.23
5.21
5.21
5.18
4.48
4.48
4.47
4.27
4.27
4.26
4.20
4.19
4.19
4.18
4.18
4.17
4.17
4.16
4.14
4.14
4.13
4.12
4.11
4.10
S-5a (5a-2)
1.000
1.169
1.276
1.900
1.074
1.052
1.726
f1 (ppm)


-219.23
-219.33
-219.36
-219.46
-219.49
-219.59
0.044
1.000
-214.0
-214.5
-215.0
-215.5
-216.0
-216.5
-217.0
-217.5
-218.0
-218.5
-219.0
-219.5
-220.0
-220.5
-221.0
-221.5
-222.0
-222.5
-223.0
-223.5
-224.0
-224.5
-225.0
f1 (ppm)

S-5a (5a-2)


1.000
1.168
1.106
32.99
32.96
32.82
32.79
11.53
11.45
11.22
11.15
-0.98
-1.05
-1.17
-1.23
-1.28
-1.36
-1.45
-1.49
-1.53
f1 (ppm)
S-5a (5a-2)