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Article

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Stereocontrolled access to thioisosteres of nucleoside di- and triphosphates

In the format provided by the authors and unedited

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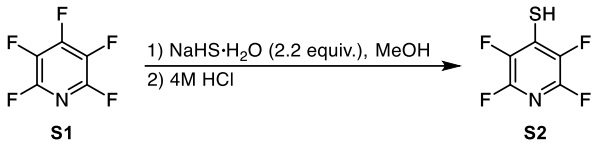
1. General Experimental

Tetrahydrofuran (THF), *N*,*N*-dimethylformamide (DMF), acetonitrile (MeCN), and dichloromethane (DCM) were obtained by passing the previously degassed solvents through an activated alumina column. Deionized water was used in all the reactions, unless otherwise stated. 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) was purchased from Sigma Aldrich (33482-50ML-F). All reagents were purchased at the highest commercial quality and used without further purification unless otherwise stated. Isolated yields refer to chromatographically and spectroscopically (^{1}H NMR and ^{31}P NMR) homogeneous material, unless otherwise stated. Reactions were monitored by thin layer chromatography (TLC), ^{31}P NMR and LC/MS. TLC was performed on 0.25 mm E. Merck silica plates (60F-254), using short-wave UV light as the visualizing agent, and p-anisaldehyde, or KMnO4 and heat as developing agents. Normal phase column chromatography was performed using Merck silica gel (60, particle size 0.043–0.063 mm). Automated reverse phase flash chromatography was performed on Teledyne ISCO CombiFlash NextGen 300+ using RediSep Gold® Reverse-phase C18 cartridge. Ion exchange chromatography was performed using Cytiva DEAE Sephadex A-25. NMR spectra were recorded on Bruker DRX-600, DRX-500, and AMX-400 instruments and are calibrated using residual undeuterated solvent ($CHCl_3$, MeOH, DMSO, acetone, HDO at 7.26, 3.31, 2.50, 2.05, 4.79 ppm for ^{1}H NMR, respectively, and 77.16, 49.00, 39.52, 29.84 ppm for ^{13}C NMR, respectively). The ^{31}P NMR chemical shifts are proton decoupled referenced to the deuterium solvent calibrated in ^{1}H NMR. High-resolution mass spectra (HRMS) were recorded on an Agilent LC/MSD TOF mass spectrometer by electrospray ionization time of flight reflectron experiments. Melting points were recorded on a Fisher-Johns 12-144 melting point apparatus and are uncorrected. Optical rotations were recorded on a Rudolph Research Analytical Autopol III Automatic Polarimeter. HPLC analyses were conducted on a Waters Autopurification LC with a Waters XBridge C18 column (4.6x150 mm, 3.5 μm). The enantiomeric ratios were determined with Waters UPC2 SFC equipped with a photodiode array detector. The single-crystal X-ray diffraction studies were carried out on a Bruker D8 Venture Ultra diffractometer equipped with Mo K_α radiation (λ = 0.71073 Å).

2. Synthesis of new Ψ* reagent

2.1. Step 1. Preparation of tetrafluoropyridine-4-thiol (S2)



Tetrafluoropyridine-4-thiol (**S2**) was prepared according to the procedure published by Dilman *et al*[1].

Caution: All steps should be performed under well ventilated fume hood due to the large amount of H_2S liberated during the reaction.

A 250 mL round bottom flask equipped with a stir bar was charged with sodium hydrosulfide hydrate (48.2 g, 660 mmol, 2.2 equiv.), followed by the addition of MeOH (100 mL). The resulting suspension was stirred at room temperature until most of the solid dissolved. The flask was immersed into ice/water bath and pentafluoropyridine (**S1**) (33.0 mL, 300 mmol, 1.0 equiv.) was slowly added, maintaining internal reaction temperature below 30 °C. The resulting viscous solution was stirred for 5 min, after which the volatile components were evaporated under reduced pressure. The residue was carefully treated with 4M HCl solution (180 mL), and the product was extracted with hexanes (100 mL, then 2 x 50 mL). The combined organic phases were dried using $MgSO_4$, filtered and the solvent was evaporated under reduced pressure (>100 mbar, temp. 30 °C) to afford thiol **S2** (52.2 g) as a colorless liquid, which solidified upon storage at 0 °C (**Yield = 95%**).

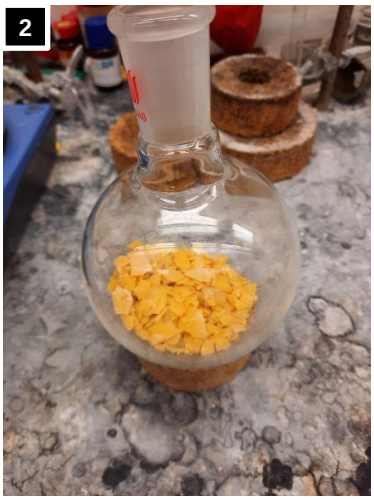
^{19}F NMR (376 MHz, $CDCl_3$) δ -93.4 – -93.7 (m, 2F), -142.4 – -142.6 (m, 2F).

Note 1: We have found that solubility of NaHS hydrate in MeOH is strongly dependent on its crystalline form, which in turn can affect the outcome of the reaction. In our experience the reaction proceeds well only when using starting material in form of yellow flakes (*see graphical procedure*). In the case of other crystalline forms (i.e. white crystals) the solubility in MeOH was significantly lower and the reaction provided < 10% of the product.

Note 2: Addition of pentafluoropyridine to sodium hydrosulfide solution is very exothermic. It is crucial to keep the temperature of the reaction mixture < 30 °C during this step to avoid formation of side products.

Note 3: Thiol **S2** is volatile under reduced pressure.

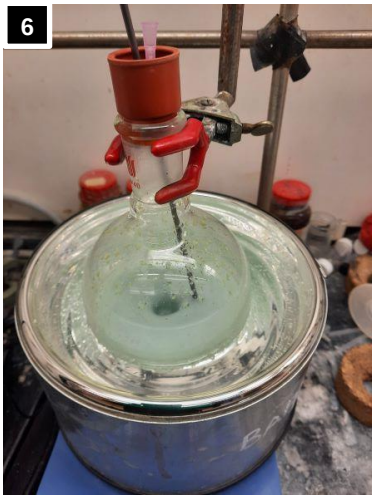
2.2. Graphical Guide for Step 1



1: Different crystalline forms of NaHS hydrate (*top* – white crystals; low solubility in MeOH; <10% yield of product) (*bottom* – yellow flakes; well soluble in MeOH; 95% yield of product).

2: Round bottom flask charged with NaHS hydrate (48.2 g).

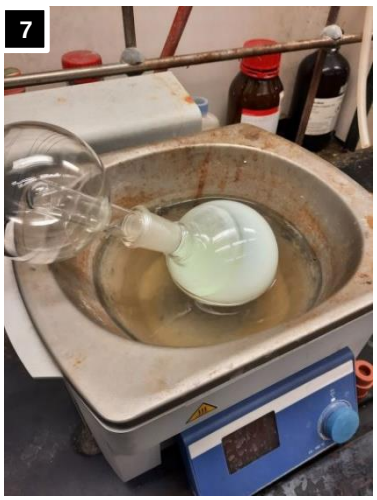
3: NaHS solution in MeOH (100 mL) after 30 min of stirring (most of the solid dissolved).



4: The reaction flask is immersed in ice/water bath (thermocouple is inserted via septum to monitor temperature of the solution).

5: Pentafluoropyridine (**S1**) (33.0 mL) is added slowly via syringe, maintaining the reaction temperature below 30 °C.

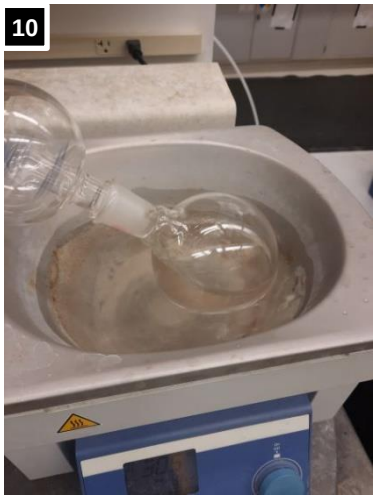
6: Reaction mixture after the addition of pentafluoropyridine (note the color change).

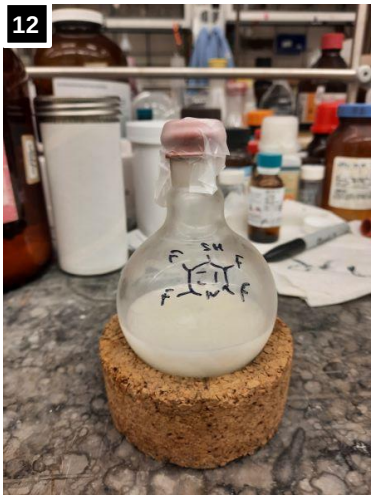


7: The resulting viscous solution is concentrated under reduced pressure.

8: The residue is treated with 4M HCl (180 mL) (haze solution with yellow oil on the bottom).

9: The product is extracted with hexanes (top layer).



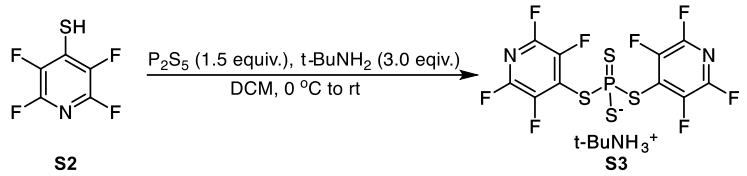


10: Organic layers are combined, dried over $MgSO_4$, filtered and the solvent is removed under reduced pressure (>100 mbar, 30 °C).

11: Tetrafluoropyridine-4-thiol (**S2**) obtained as colorless liquid (52.2 g) (**Yield = 95%**).

12: Tetrafluoropyridine-4-thiol (**S2**) solidified upon storage at 0 °C.

2.3. Step 2. Preparation of compound S3



A flame dried 1 L round bottom flask, equipped with a stir bar, was charged with phosphorus pentasulfide (17.0 g, 75 mmol, 1.5 equiv.), followed by addition of anhydrous DCM (135 mL). The batch was made inert by flushing with argon for 2 min. Subsequently, tetrafluoropyridine-4-thiol (**S2**) (21.0 g, 115 mmol, 2.3 equiv.) was added and the reaction flask was immersed in ice/water bath. *tert*-Butylamine (18.4 mL, 150 mmol, 3.0 equiv.) was carefully added to the reaction mixture (***Caution: reaction very exothermic***). The resulting suspension was warmed to room temperature and stirred for 16 h under argon atmosphere. The reaction was carefully quenched with water (135 mL) (***Caution: H_2S is evolved during this step***), followed by the addition of hexanes (135 mL). The resulting slurry was stirred for 30 min, after which precipitate was filtered off and washed consecutively with water (60 mL), DCM/hexanes (1:1; 3x 60 mL) and hexanes (60 mL). The filter cake was dried under reduced pressure overnight to provide 18.4 g of compound **S3** as a white crystalline solid (**Yield = 60%**).

m.p. 152-153 °C

^{1}H NMR (600 MHz, $(CD_3)_2CO$) δ 7.93-7.65 (m, 3H), 1.55 (s, 9H).

^{13}C NMR (150 MHz, $(CD_3)_2CO$) δ 145.2-144.9 (m), 144.7-144.4 (m), 143.6-143.3 (m), 143.0-142.6 (m), 131.4-131.0 (m), 54.7, 27.7.

^{19}F NMR (376 MHz, CD_3CN) δ -96.5 – -96.7 (m, 4F), -135.9 – -136.0 (m, 4F).

^{31}P NMR (162 MHz, CD_3CN) δ 92.5.

HRMS (ESI-TOF) m/z: calculated for $C_{10}F_8N_2PS_4$ $[M-H]^-$: 458.8559, found: 458.8562.

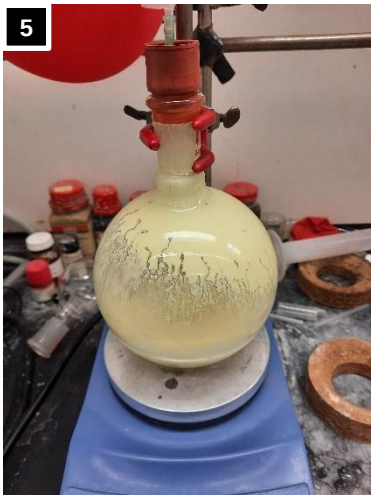
Note: Compound **S3** decompose under prolonged exposure to moisture and should be stored under protective gas atmosphere.

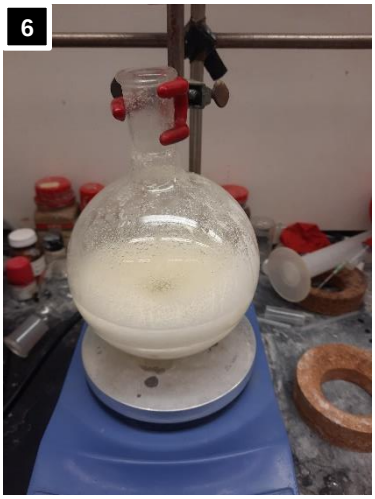
2.4. Graphical Guide for Step 2

1: Flame dried 1 L round bottom flask charged with P_2S_5 (17.0 g).

2: P_2S_5 in suspended in anhydrous DCM (135 mL), followed by addition of tetrafluoropyridine-4-thiol (**S2**) (21.0 g).

3: The reaction flask is immersed in ice/water bath and *tert*-butylamine (18.4 mL) is added carefully (***very exothermic step***).





4: Heterogeneous reaction mixture after addition of *tert*-butylamine (18.4 mL).

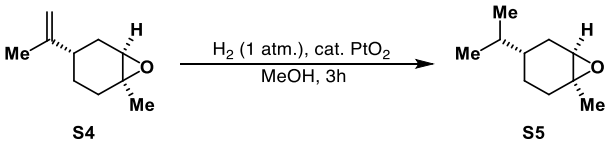
5: Reaction mixture after 16 h.

6: The reaction is quenched with water (135 mL) (***H_2S is evolved***), followed by addition of hexanes (135 mL). Resulting slurry is stirred for 30 min.

7: White precipitate is filtered off and washed consecutively with water (60 mL), DCM/hexanes (1:1; 3 x 60 mL) and hexanes (60 mL).

8: Compound **S3** (18.4 g) obtained as a white crystalline solid (**Yield = 60%**).

2.5. Step 3. Reduction of *cis*-limonene oxide (S5)



S4 S5

250 mL round bottom flask equipped with a stir bar was charged with (-)-*cis*-limonene oxide (**S4**)[2] (12.2 mL, 75 mmol, 1.0 equiv.) under argon atmosphere. MeOH (50 mL) was added, followed by PtO_2 (surface area ≥ 60 m^2/g; 84 mg, 0.37 mmol, 0.5 mol%). The atmosphere in the flask was exchanged for H_2 and the reaction vessel was equipped with a H_2 balloon. The reaction mixture was stirred at room temperature for 3 h, after which TLC indicated full conversion of the starting material (*see Note 1 below*). The crude reaction mixture was filtered through a Celite pad, followed by DCM (~200 mL). The resulting solution was concentrated under reduced pressure to ~12 mL (> 100 mbar, temp. 35 °C) and used in the next step without further purification.

Enantiomer of compound **S5** was obtained via analogous procedure starting from (+)-*cis*-limonene oxide.

Note 1: It is important to achieve full conversion of the starting material **S4**, otherwise the next step leads to inseparable mixture of Ψ reagents. The progress of the reaction can be monitored by TLC chromatography (EtOAc/Hex; 1:10) (*see graphical guide*).

Note 2: Epoxide **S5** is volatile under reduced pressure.

2.6. Graphical Guide for Step 3

1: Round bottom flask charged with *cis*-(-)-limonene oxide (**S4**) (12.2 mL).

2: Solution of *cis*-(-)-limonene oxide (**S4**) in MeOH (50 mL).

3: PtO_2 (84 mg) is added to the solution and the atmosphere is exchanged for H_2.

4: Reaction mixture before full conversion of the starting material (black suspension).

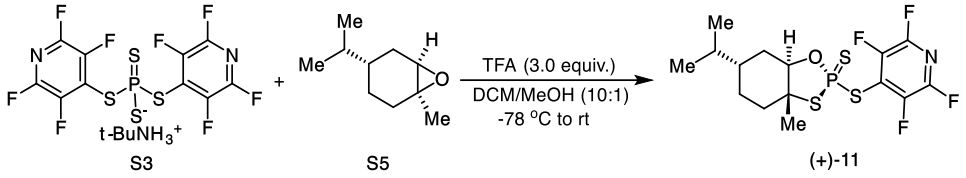
5: Reaction mixture after 3 h (note: most of the catalyst coagulates and solution becomes clearer).

6: Reaction progress is monitored by TLC (EtOAc/Hex; 1:10) (*left lane* – (-)-*cis*-limonene oxide (**S4**); *right line* – reaction mixture after full conversion of the starting material) (*note*: starting material can be spotted using cold $KMnO_4$, while the product spot requires heating to develop on TLC plate).

7: Reaction mixture is filtered through a pad of celite.

8: Crude compound **S5** (~12 mL) after concentration under reduced pressure.

2.7. Step 4. Synthesis of (+)-Ψ* reagent (11)



Round bottom flask, equipped with a stir bar was charged with compound **S3** (20.0 g, 37.5 mmol, 1.0 equiv.), followed by anhydrous DCM (75 mL). The reaction batch was made inert by flushing with argon for 2 min, and the resulting suspension was cooled to -78 °C. Subsequently, MeOH (7.5 mL), crude epoxide **S5** (12 mL; 2.0 equiv.) and TFA (12 mL; 3.0 equiv.) were added consecutively and the resulting clear solution was stirred for 5 min. The cooling bath was removed, and the reaction mixture was stirred for 1 h. After that time ^{31}P NMR indicated full conversion of the staring material **S3** (*see below*). The reaction mixture was diluted with hexanes (150 mL) and washed consecutively with water (75 mL), 10% aq. K_2HPO_4 (75 mL), and 10% aq. KH_2PO_4 (75 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude solid was redissolved in the minimal amount of DCM and the resulting solution was diluted with MeOH (100 mL). Crystals appeared after addition of MeOH, and the solution was left for 1 h at room temperature to complete crystallization. The resulting slurry was filtered, and the filter cake was washed with cold MeOH. After being dried under vacuum, compound (+)-**11** was obtained as a white crystalline solid (8.9 g, d.r. > 99:1, *ee* > 99:1, **Yield = 55%**).

Note: Diastereomeric ratio of the crude reaction mixture by ^{31}P NMR should be >20:1 (major isomer ~96 ppm, minor isomer ~102 ppm). We have found that addition of MeOH is essential to achieve high diastereoselectivity of the reaction.

m.p. 131 °C

$[\alpha]_D^{25}$ = +315.3 (*c* 1.01, $CHCl_3$)

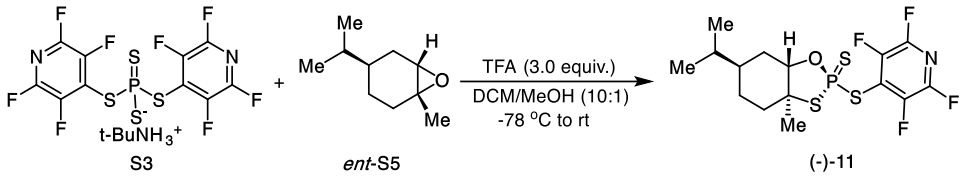
^{1}H NMR (600 MHz, $CDCl_3$) δ 4.51 (ddd, *J* = 12.8, 6.1, 3.7 Hz, 1H), 2.28-2.23 (m, 1H), 2.02 (td, *J* = 13.0, 4.2 Hz, 1H), 1.97-1.93 (m, 1H), 1.84-1.76 (m, 3H), 1.67 (s, 3H), 1.67-1.58 (m, 2H), 1.03 (d, *J* = 6.6 Hz, 3H), 0.97 (d, *J* = 6.6 Hz, 3H).

^{13}C NMR (150 MHz, $CDCl_3$) δ 144.7-144.5 (m), 143.9-143.5 (m), 143.1-142.8 (m), 142.1-141.8 (m), 124.6-124.2 (m), 86.6 (d, *J* = 3.3 Hz), 66.2, 40.9, 33.2 (d, *J* = 8.8 Hz), 27.9 (d, *J* = 14.9 Hz), 27.0, 23.4, 22.4, 22.0, 21.1.

^{19}F NMR (376 MHz, $CDCl_3$) δ -91.1 – -91.3 (m, 2F), -135.1 – -135.3 (m, 2F).

^{31}P NMR (162 MHz, $CDCl_3$) δ 96.4.

HRMS (ESI-TOF) m/z: calculated for $C_{15}H_{19}F_4NOPS_3$ $[M+H]^+$: 432.0303, found: 432.0290.



S3 *ent*-S5 (-)-11

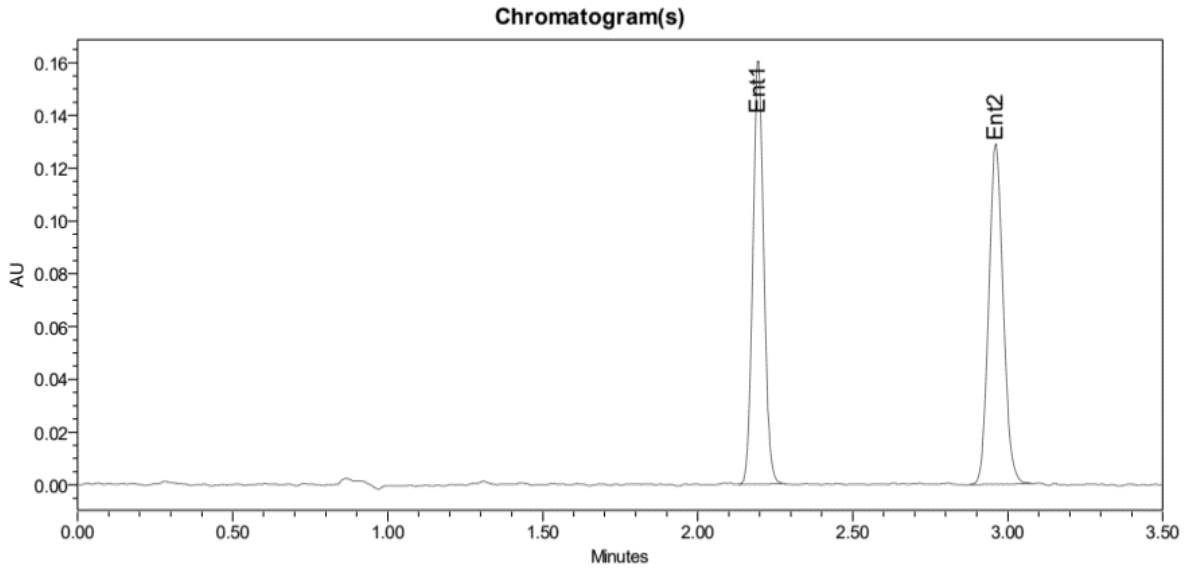
Compound (–)-**11** was obtained via analogous procedure starting from (+)-*cis*-limonene oxide. All characterization data were identical, except of the optical rotation.

$[\alpha]_D^{25}$ = -314.7 (*c* 1.01, $CHCl_3$)

2.8. Determination of enantiomeric excess

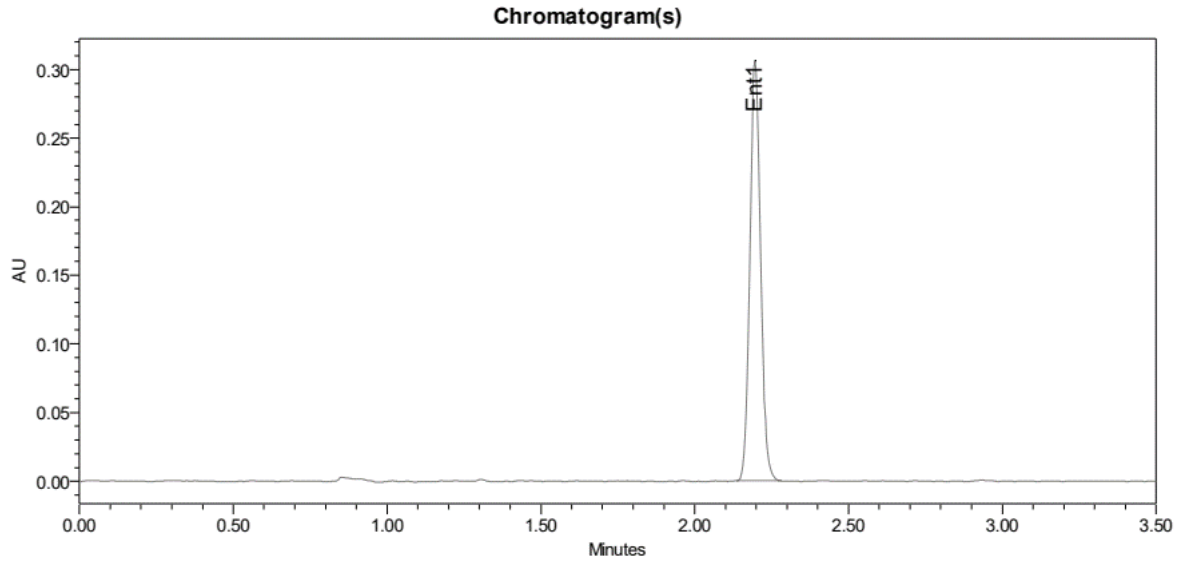
Enantiomeric excess was determined using supercritical fluid chromatography on a Waters UPC2 SFC with a Diacel IBN column (3 µm, 4.6 x 250 mm) under isocratic conditions (3.3 mL/min, 15% MeOH/CO_2, 1600 psi backpressure) at 30 °C. The enantiomers were detected by UV detector at 212 nm.

*Mixture of (–)-**11** and (+)-**11***



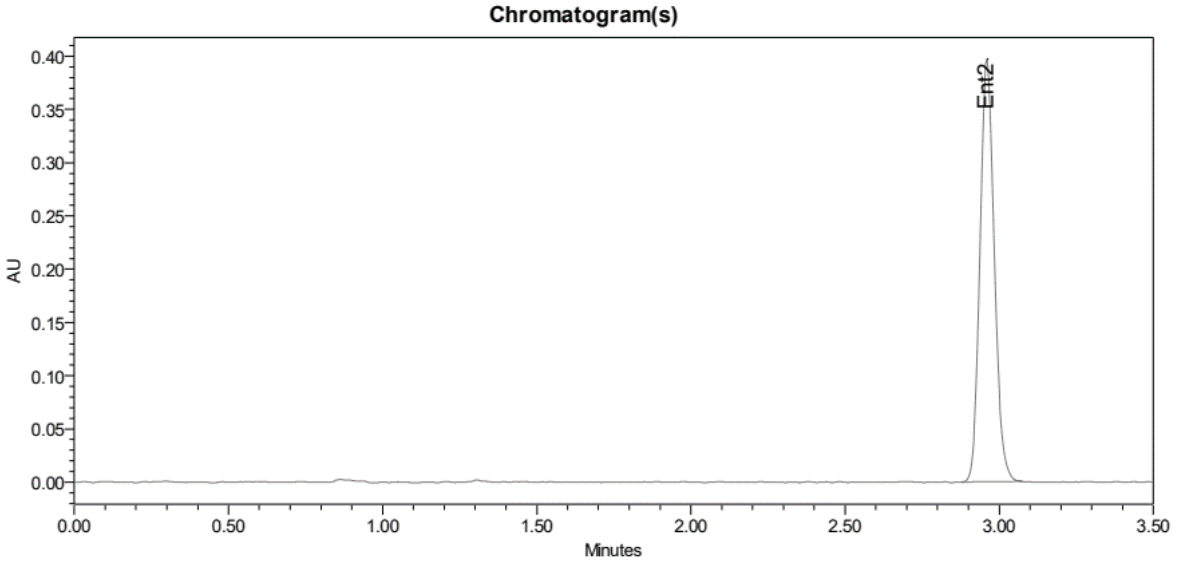
Enantiomer	RT (min)	Area	Area percent
Ent-1 (-)-11	2.19	394634	48.56
Ent-2 (+)-11	2.96	418118	51.44

*Compound (–)-**11***



Enantiomer	RT (min)	Area	Area percent
Ent-1 (-)-11	2.20	741129	100.0
Ent-2 (+)-11	2.96	0	0

*Compound (+)-**11***



Enantiomer	RT (min)	Area	Area percent
Ent-1 (-)-11	2.19	0	0
Ent-2 (+)-11	2.96	1282265	100.0

2.9. X-ray structure of the compound (–)-11

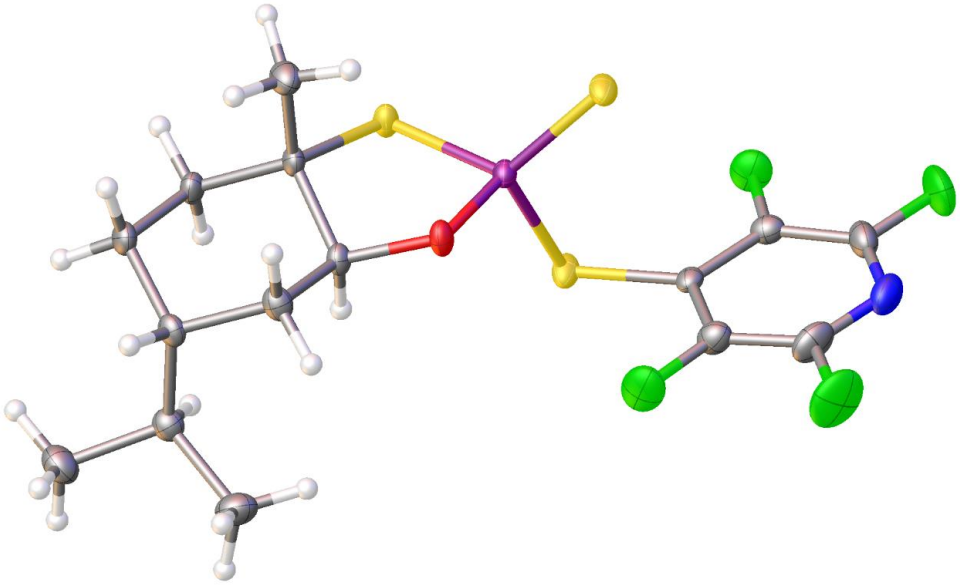


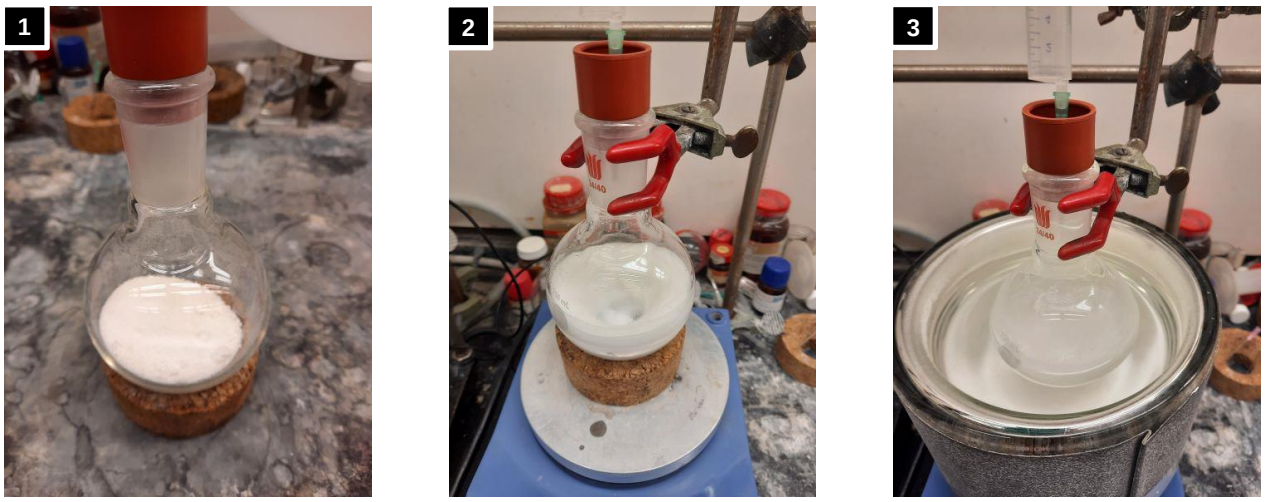
Figure S1. X-ray structure of the compound (–)-**11**

Table S1. Crystal structure data and refinement for the compound (–)-**11**

CCDC deposition number	2172688	
Empirical formula	C15 H18 F4 N O P S3	
Molecular formula	C15 H18 F4 N O P S3	
Formula weight	431.45	
Temperature	100.00 K	
Wavelength	0.71073 Å	
Crystal system	Orthorhombic	
Space group	$P2_12_12_1$	
Unit cell dimensions	a = 6.4680(3) Å	α= 90°.
	b = 15.1839(7) Å	β= 90°.
	c = 19.3616(8) Å	γ = 90°.
Volume	1901.49(15) $Å^3$	
Z	4	
Density (calculated)	1.507 Mg/m^3	
Absorption coefficient	0.515 mm^{-1}	
F(000)	888	
Crystal size	0.26 x 0.2 x 0.16 mm^3	
Crystal color, habit	colorless block	
Theta range for data collection	2.495 to 26.725°.	

Index ranges	-8<=h<=8, -19<=k<=19, -24<=l<=24
Reflections collected	46739
Independent reflections	4041 [R(int) = 0.0454]
Completeness to theta = 25.242°	99.9 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.7455 and 0.7075
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	4041 / 0 / 229
Goodness-of-fit on F^2	1.062
Final R indices [I>2sigma(I)]	R1 = 0.0199, wR2 = 0.0483
R indices (all data)	R1 = 0.0213, wR2 = 0.0489
Absolute structure parameter	0.04(2)
Largest diff. peak and hole	0.207 and -0.165 e.$Å^{-3}$

2.10. Graphical Guide for the Preparation of (+)-11



1: Round bottom flask charged with compound **S3** (20.0 g).

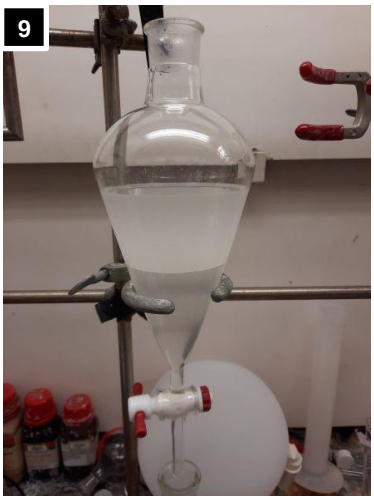
2: Compound **S3** suspended in anhydrous DCM (75 mL).

3: The reaction flask is immersed in acetone/dry ice bath and the suspension is cooled to -78 °C.

4: MeOH (7.5 mL) is added to the cooled suspension via syringe.

5: Crude epoxide **S5** (~12.0 mL) (obtained from (–)-*cis*-limonene oxide) is added via syringe.

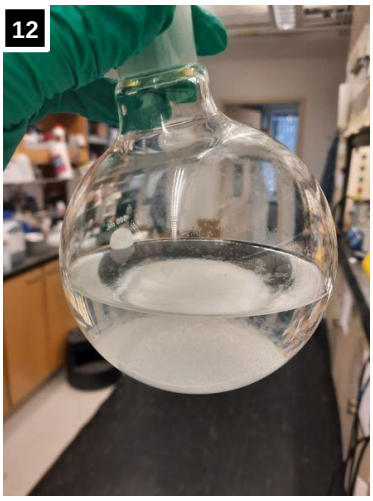
6: TFA (12.0 mL) is added to the reaction mixture.



7: After 5 min the cooling bath is removed, and the reaction mixture is warmed to room temperature.

8: The clear solution is stirred for 1 h.

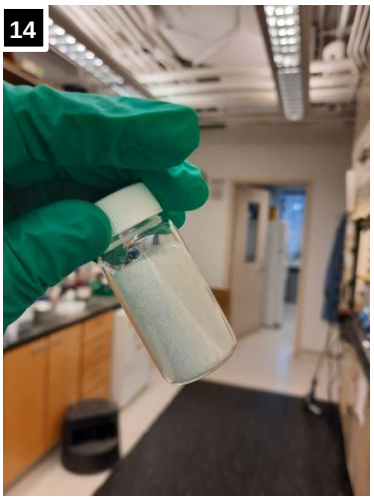
9: The reaction mixture is diluted with hexanes (150 mL) and washed consecutively with water (75 mL), 10% aq. K_2HPO_4 (75 mL) and 10% aq. KH_2PO_4 (75 mL) (organic layer on top).



10: The organic layer is dried over $MgSO_4$, filtered and the volatiles are removed under reduced pressure.

11: Crude solid obtained after evaporation of the solvent.

12: The solid is dissolved in minimal amount of DCM and the resulting solution is diluted with MeOH (100 mL). The resulting solution is left for 1 h at room temperature to complete crystallization.

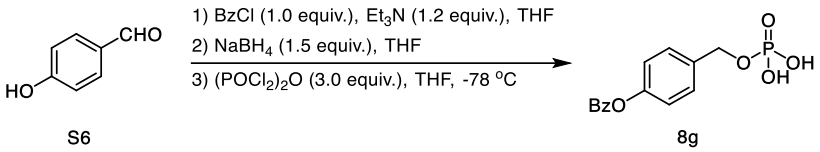


13: Obtained crystals are filtered off and washed with cold MeOH.

14: Compound (+)-**11** obtained as white crystalline solid (8.9 g, d.r. > 20:1, *ee* > 99:1, **Yield = 55%**). Compound (–)-**11** can be obtained following analogous procedure starting from reduced (+)-*cis*-limonene oxide.

3. Preparation of the Phosphate Donors

3.1. Synthesis of Monophosphate Donor 8g



S6 8g

Step 1. Benzoylation

Flame dried round bottom flask, equipped with a stir bar was charged with 4-hydroxybenzaldehyde (**S6**) (24.4 g, 200 mmol, 1.0 equiv.). The solid substrate was dissolved in anhydrous THF (200 mL), followed by the addition of Et_3N (33.5 mL, 240 mmol, 1.2 equiv.). The reaction flask was immersed in ice/water bath and benzoyl chloride (23.2 mL, 200 mmol, 1.0 equiv.) was added over 3 min. The reaction mixture was warmed to room temperature overnight. Subsequently, the mixture was diluted with EtOAc (200 mL) and filtered via a pad of Celite. The filtrate was washed with saturated aq. NH_4Cl, the organic layer was dried over Na_2SO_4, filtered and the volatiles were removed *in vacuo* to provide crude 4-formylphenyl benzoate (46 g), which was used in the next step without any additional purification.

Step 2. Reduction

Crude 4-formylphenyl benzoate (46 g) was dissolved in THF (200 mL), and the reaction mixture was cooled to 0 °C. $NaBH_4$ (11.3 g, 300 mmol, 3.0 equiv.) was added in 3 portions and the mixture was warmed to room temperature over 2 h. Subsequently, the reaction was carefully quenched with saturated aq. NH_4Cl and diluted with EtOAc (200 mL). Organic phase was separated and the aqueous fraction was extracted with EtOAc (100 mL). Combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure to provide crude 4-(hydroxymethyl)phenyl benzoate (48 g), which was used in the next step without any additional purification.

Step 3. Phosphorylation

Crude 4-(hydroxymethyl)phenyl benzoate (24 g) was dissolved in anhydrous THF (200 mL) and cooled to -78 °C. Pyrophosphoryl chloride (13.8 mL, 300 mmol, 3.0 equiv.) was added dropwise over 10 min. The reaction mixture was stirred at -78 °C for 4 h, after which the reaction was quenched with water. The pH of the resulting solution was carefully adjusted to 8 with saturated aq. $NaHCO_3$. Subsequently, 37% aq. HCl was added dropwise to the resulting suspension until the solution became clear. The reaction mixture was extracted with EtOAc (2 x 200 mL), and the combined organic fractions were washed with water. Organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo* to provide crude solid of compound **8g**. The solid was suspended in DCM, filtered, and the filter cake was washed with DCM. After being dried under reduced pressure, phosphate was obtained as a white crystalline solid (17.0 g, 55 mmol; **Yield = 55% over 3 steps**).

m.p. 140-142 °C

^{1}H NMR (600 MHz, CD_3OD) δ 8.20-8.16 (m, 2H), 7.72-7.67 (m, 1H), 7.59-7.54 (m, 2H), 7.50 (d, *J* = 8.5 Hz, 2H), 7.25 (d, *J* = 8.5 Hz, 2H), 5.05 (d, *J* = 7.4 Hz, 2H).

^{13}C NMR (150 MHz, CD_3OD) δ 166.6, 155.2, 136.2 (d, *J* = 7.8 Hz), 135.0, 131.04, 131.02, 130.7, 129.9 (d, *J* = 3.1 Hz), 122.9, 68.6 (d, *J* = 5.0 Hz).

^{31}P NMR (162 MHz, CD_3OD) δ -0.1.

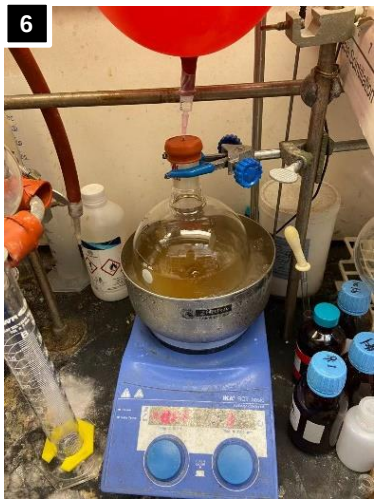
HRMS (ESI-TOF) m/z: calculated for $C_{14}H_{12}O_6P$ $[M-H]^-$: 307.0371, found: 307.0361.

3.2. Graphical Guide for the Preparation of Monophosphate 8g

1: All reagents used for the synthesis of **8g** (from left: 4-hydroxybenzaldehyde, triethylamine, benzoyl chloride, $NaBH_4$, pyrophoshoryl chloride).

2: Flame dried flask charged with 4-hydroxybenzaldehyde (**S6**) (24.4 g).

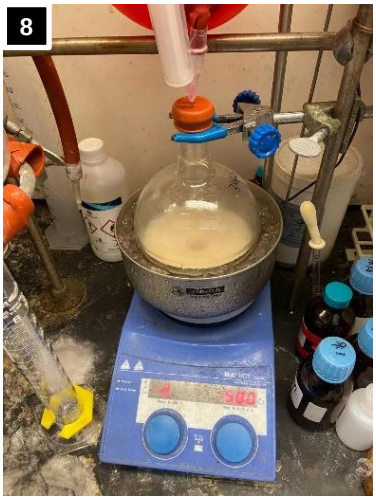
3: Anhydrous THF from the solvent purification system is used for all the steps.

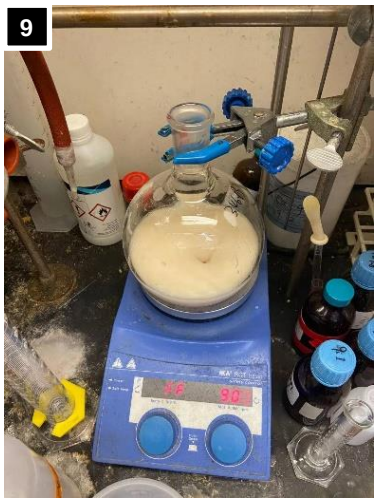


4: 4-hydroxybenzaldehyde (**S6**) dissolved in anhydrous THF (200 mL).

5: Triethylamine (33.5 mL) is added.

6: Reaction mixture is cooled to 0 °C in an ice/water bath.





7: Benzoyl chloride (23.2 mL) is added dropwise via syringe over 3 min.

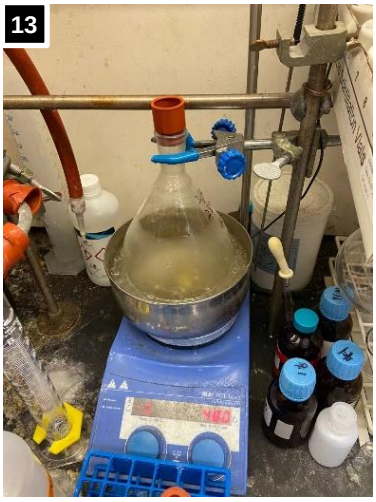
8: The reaction mixture is warmed to room temperature overnight.

9: The resulting suspension is diluted with EtOAc.

10: The reaction mixture is filtered through a pad of Celite.

11: The filtrate is washed with saturated aq. NH_4Cl solution.

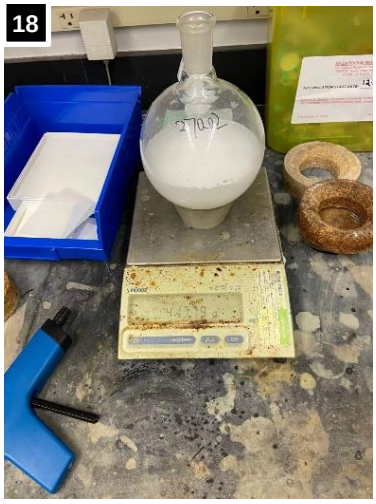
12: Organic fraction is dried over Na_2SO_4, filtered and concentrated under reduced pressure to provide crude 4-formylphenyl benzoate (46 g).



13: The crude solid is redissolved in anhydrous THF (200 mL).

14: $NaBH_4$ (11.3 g) is added carefully in 3 portions.

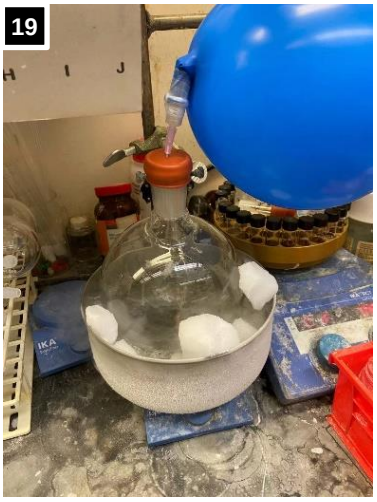
15: The reaction mixture is warmed to room temperature over 2 h.

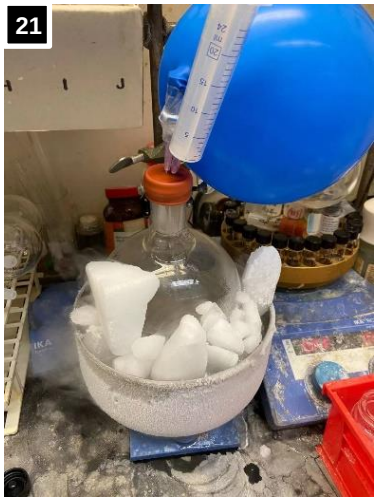


16: The reaction is carefully quenched with saturated aqueous NH_4Cl solution.

17: The product is extracted with EtOAc.

18: Organic phase is dried over Na_2SO_4, filtered and the solvent is evaporated under reduced pressure to provide crude 4-(hydroxymethyl)phenyl benzoate (48 g).

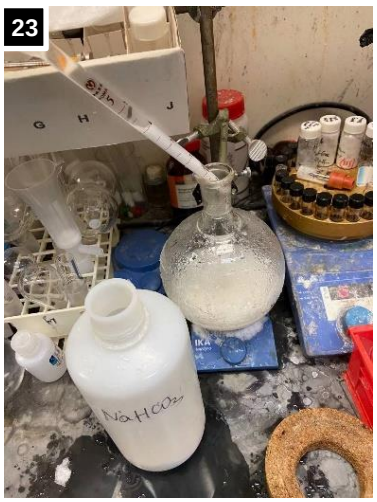




19: Crude product of the previous step (24 g; half of the obtained amount) is redissolved in anhydrous THF and cooled to -78 °C.

20: Pyrophosphoryl chloride (13.8 mL) is added dropwise over 10 min.

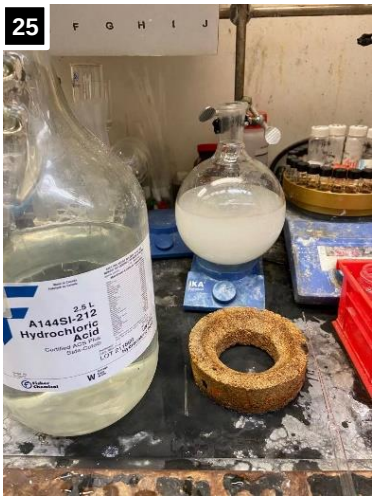
21: The reaction mixture is stirred under argon atmosphere for 4 h at -78 °C.



22: The reaction is quenched by the addition of water.

23: The pH of the solution is carefully adjusted to 8 with saturated aq. $NaHCO_3$.

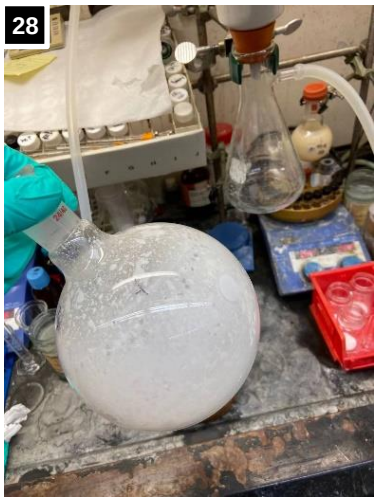
24: The suspension formed during addition of aq. $NaHCO_3$.

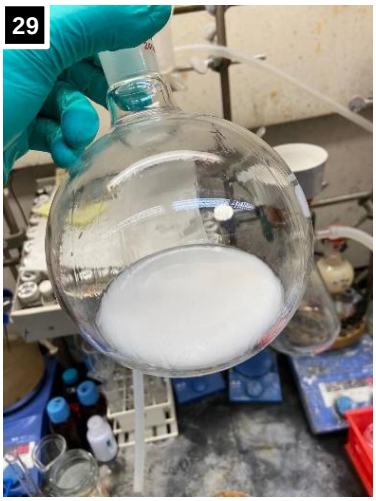


25: Concentrated aq. HCl is added dropwise until the solution become clear.

26: The reaction mixture is extracted with EtOAc (2 times).

27: Combined organic layers are washed with water, dried over Na_2SO_4, filtered and the solvent is evaporated under reduced pressure.

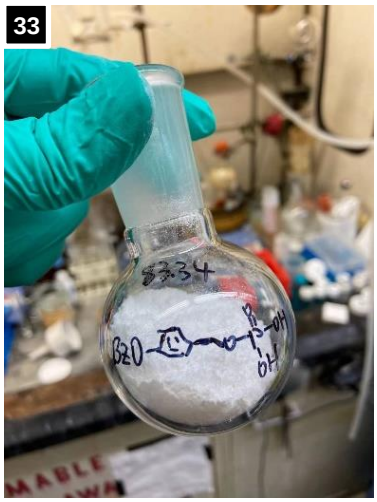




28: Crude phosphate **8g** after evaporation of the solvent.

29: Crude solid is suspended in DCM.

30: The solid is filtered and washed with DCM.

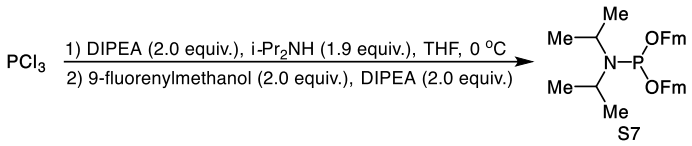


31: Monophosphate **8g** isolated as white solid after filtration.

32: Product is dried under reduced pressure.

33: Obtained product **8g** (17.0 g, **Yield = 55% over 3 steps**).

3.3. Preparation of i-$Pr_2NP(OFm)_2$ (S7)



S7

i-$Pr_2NP(OFm)_2$ (**S7**) was prepared according to the reported procedure[3].

500 mL flame dried round bottom flask, equipped with a stir bar, was evacuated, backfilled with argon (3 times) and capped with a septum. Subsequently, anhydrous THF (160 mL) was added, followed by PCl_3 (4.0 mL, 46 mmol, 1.0 equiv.). The resulting solution was cooled to 0 °C and DIPEA (16.0 mL, 92 mmol, 2.0 equiv.) was added. Anhydrous diisopropylamine (12.0 mL, 87 mmol, 1.9 equiv.) was then added dropwise over 10 min and the resulting suspension was stirred for 1 h at 0 °C. After that time, another portion of DIPEA (16.0 mL, 92 mmol, 2.0 equiv.) was added, followed by 9-fluorenylmethanol (17.9 g, 92 mmol, 2.0 equiv.). The reaction mixture was warmed to room temperature and stirred overnight under argon atmosphere. The resulting suspension was filtered via a pad of Celite, and the filtrate was concentrated under reduced pressure. The residue was diluted with DCM, loaded on a pad of silica gel (18 x 4 cm) and flushed with hexane/EtOAc/Et_3N (100:5:1) (*Note: It is important to perform this step as fast as possible, because the compound **S7** decomposes on silica gel; see graphical procedure for more details*). Fractions containing pure product (as determined by ^{31}P NMR), were combined and concentrated under reduced pressure. After being dried under high vacuum, compound **S7** was obtained as a yellow semisolid (12.4 g; **Yield = 52%**), which was used in the next step without any further purification. Compound **S7** should be stored under argon atmosphere at -20 °C.

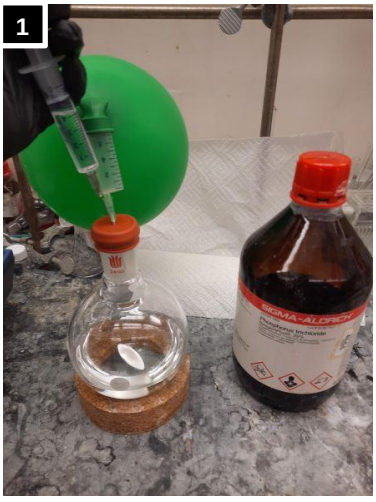
^{1}H NMR (600 MHz, $CDCl_3$) δ 7.76-7.73 (m, 4H), 7.67-7.64 (m, 4H), 7.40-7.35 (m, 4H), 7.31-7.26 (m, 4H), 4.18 (t, *J* = 6.9 Hz, 2H), 4.01 (dt, *J* = 9.9, 6.8 Hz, 2H), 3.81 (dt, *J* = 9.9, 7.3 Hz, 2H), 3.66 (hept, *J* = 6.8 Hz, 2H), 1.16 (d, *J* = 6.8 Hz, 12H).

^{13}C NMR (150 MHz, $CDCl_3$) δ 145.1, 144.8, 141.5, 141.4, 127.54, 127.50, 127.0, 126.9, 125.6, 125.3, 120.0, 119.9, 66.1 (d, *J* = 17.1 Hz), 49.3 (d, *J* = 7.7 Hz), 43.2 (d, *J* = 12.1 Hz), 24.8 (d, *J* = 7.2 Hz).

^{31}P NMR (162 MHz, $CDCl_3$) δ 146.0.

The NMR data was consistent with previously reported[3].

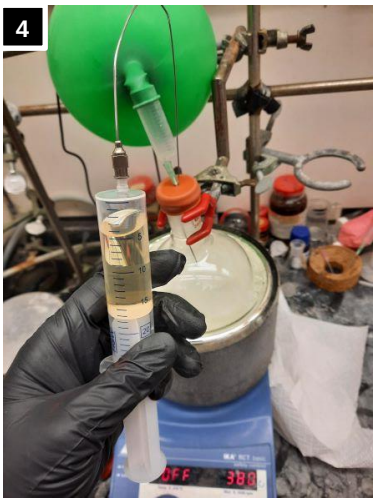
3.4. Graphical Guide for the Preparation of i-$Pr_2NP(OFm)_2$ (S7)

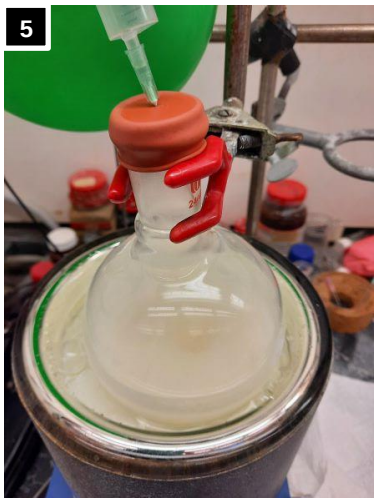


1: PCl_3 (4.0 mL) is added to a flask containing anhydrous THF (160 mL).

2: The solution is cooled to 0 °C in an ice/water bath.

3: DIPEA (16.0 mL) is added via syringe.

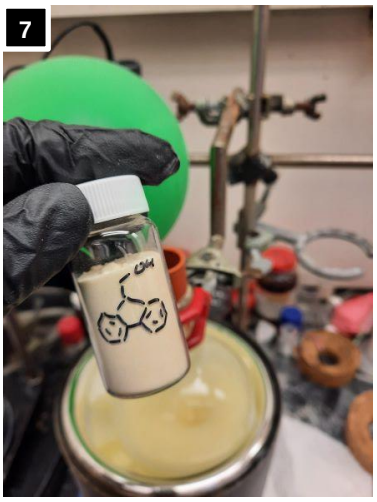




4: Anhydrous diisopropylamine (12.0 mL) is added via syringe.

5: The cloudy suspension is stirred for 1 h at 0 °C.

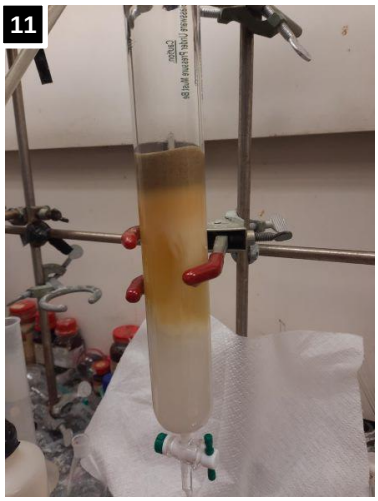
6: Another portion of DIPEA (16.0 mL) is added via syringe.



7: Solid 9-fluorenylmethanol (17.9 g) is added.

8: The reaction mixture is stirred at room temperature overnight.

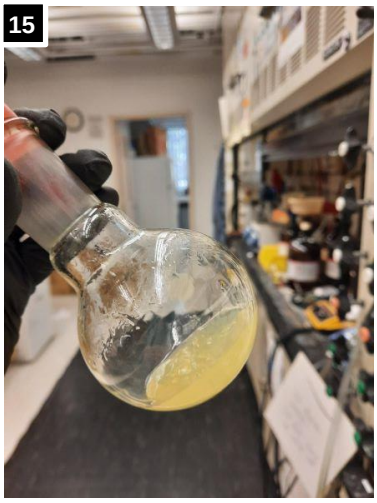
9: The suspension is filtered via a pad of Celite.



10: Filtrate is concentrated under reduced pressure and diluted with DCM.

11: The crude is loaded on a pad of silica (18 cm height, 4 cm inner diameter) and flushed with hexanes/EtOAc/NEt_3 (100:5:1) (~750 mL) (*this step should be performed in less than 10 min to minimize loss of material due to decomposition on silica*).

12: Eluate was collected in 60 mL test tubes - first 10 tubes were collected, and the solvent was evaporated under reduced pressure.

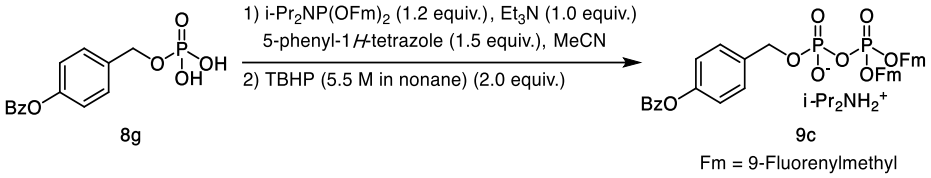


13: Concentrated fractions containing i-$Pr_2NP(OFm)_2$ (**S7**).

14: The product is dried under high vacuum.

15: i-$Pr_2NP(OFm)_2$ (**S7**) obtained as a yellow semisolid (12.4 g; **Yield = 52%**).

3.5. Synthesis of Diphosphate Donor 9c

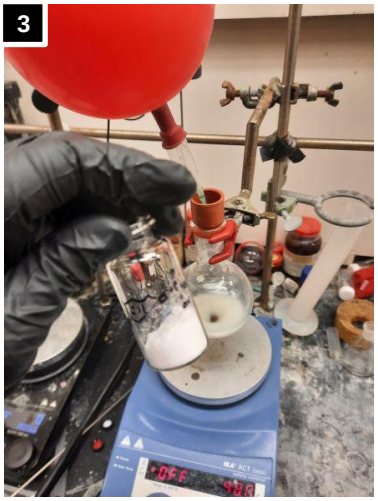


Flame dried round bottom flask, equipped with a stir bar was charged with freshly prepared i-Pr_2NP(OFm)$_2$ (**S7**) (4.0 g, 7.7 mmol, 1.2 equiv.), followed by addition of anhydrous MeCN (20 mL). To the resulting suspension were added consecutively monophosphate **8g** (2.0 g, 6.4 mmol, 1.0 equiv.) and triethylamine (0.89 mL, 6.4 mmol, 1.0 equiv.). After 2 min of stirring 5-phenyl-1*H*-tetrazole (1.4 g, 9.6 mmol, 1.5 equiv.) was added and the resulting mixture was stirred under argon atmosphere for 1 h. Subsequently, *tert*-butyl hydroperoxide (5.5 M in nonane; 2.3 mL, 12.8 mmol, 2.0 equiv.) was added and the reaction was stirred for another 1 h. The suspension was filtered through a pad of celite and washed with EtOAc. The filtrate was loaded directly on the chromatography column packed with silica gel (18 x 4 cm) and the column was flushed with 400 mL of EtOAc. The eluent was changed to MeOH/DCM (1:15) and the chromatography was continued until the product was eluted from the silica (as indicated by TLC: MeOH/DCM, 1:4, R_f = 0.8). Fractions containing product were combined and concentrated under reduced pressure (temp. < 35 °C). The residue was treated with DCM and the resulting suspension was filtered. The filter cake was washed with DCM, the filtrate was concentrated under reduced pressure (temp. < 35 °C) and the residual MeOH was removed by co-evaporation with DCM. After being dried under high vacuum, compound **9c** was obtained as a white foam (3.5 g, **Yield = 65%**). Pyrophosphate **9c** can be stored for several months at -20 °C, without any appreciable loss of purity.

Note 1: The compound **9c** is heat sensitive and should not be subjected to temp. > 35 °C for a prolonged time.
Note 2: For a prolonged storage of the product **9c** it is crucial to remove 5-phenyltetrazolate salts during column chromatography (*see graphical guide*). We have observed that pyrophosphate **9c** contaminated by these impurities decompose after ~1 week at -20 °C.

^{1}H NMR (600 MHz, $CDCl_3$) δ 8.86-8.78 (m, 2H), 8.18-8.16 (m, 2H), 7.68 (ddt, *J* = 8.7, 7.6, 1.0 Hz, 4H), 7.65-7.62 (m, 1H), 7.52-7.49 (m, 6H), 7.37-7.29 (m, 6H), 7.20 (tdd, *J* = 7.4, 4.7, 1.2 Hz, 4H), 7.09-7.06 (m, 2H), 4.97 (d, *J* = 8.0 Hz, 2H), 4.32-4.23 (m, 4H), 4.14 (t, *J* = 7.1 Hz, 2H), 3.12 (hept, *J* = 6.5 Hz, 2H), 1.21 (d, *J* = 6.5 Hz, 12H).
^{13}C NMR (150 MHz, $CDCl_3$) δ 165.3, 150.6, 143.5, 143.4, 141.5, 135.8 (d, *J* = 7.5 Hz), 133.8, 130.4, 129.7, 129.0, 128.8, 127.96, 127.95, 127.2, 125.51, 125.45, 121.65, 120.08, 120.06, 69.6 (d, *J* = 5.8 Hz), 67.9 (d, *J* = 5.8 Hz), 48.0 (d, *J* = 8.1 Hz), 46.8, 19.1 (*Two signals missing due to the overlap in the aromatic region*).
^{31}P NMR (162 MHz, CD_3OD) δ -11.6 (d, *J* = 20.0 Hz), -13.4 (d, *J* = 20.0 Hz).
HRMS (ESI-TOF) m/z: calculated for $C_{44}H_{33}O_9P_2$ $[M-H]^-$: 743.1600, found: 743.1628.

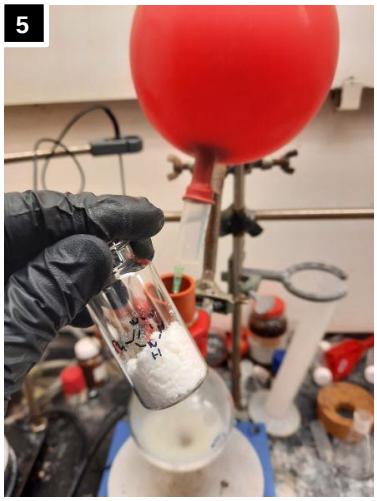
3.6. Graphical Guide for the Preparation of Pyrophosphate 9c



1: Flame dried round bottom flask charged with i-Pr_2NP(OFm)$_2$ (**S7**) (4.0 g).

2: Suspension of i-Pr_2NP(OFm)$_2$ in anhydrous MeCN (20 mL) (*after 15 min of stirring*).

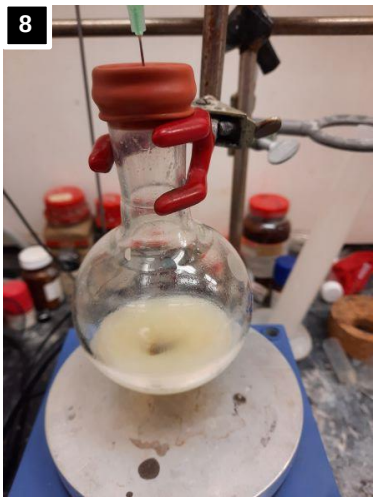
3: Solid monophosphate **8g** (2.0 g) is added.



4: Anhydrous Et_3N (0.89 mL) is added via syringe.

5: 5-Phenyltetrazole (1.4 g) is added after 2 min of stirring.

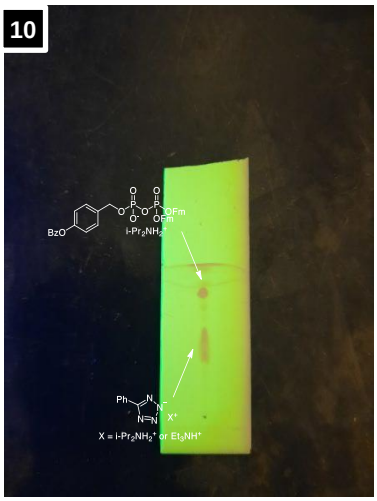
6: The reaction mixture is stirred for 1 h under argon atmosphere.

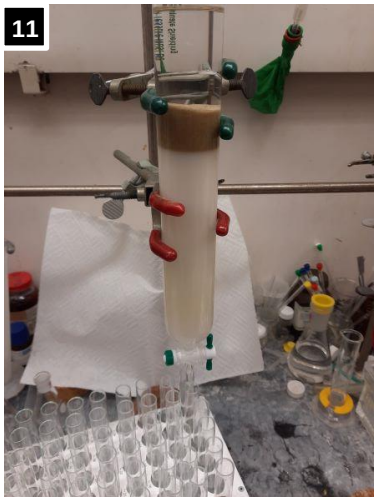


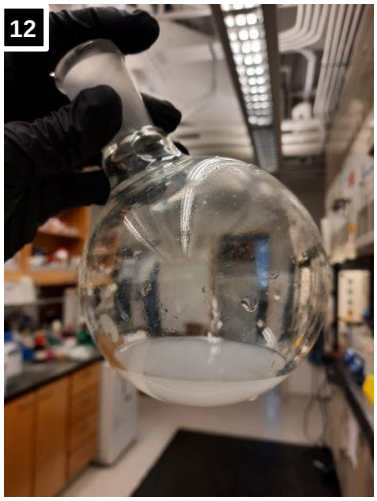
7: *tert*-Butyl hydroperoxide (2.3 mL; 5.5 M in nonane) is added via syringe.

8: The reaction mixture is stirred for 1 h.

9: The suspension is filtered through a pad of Celite.



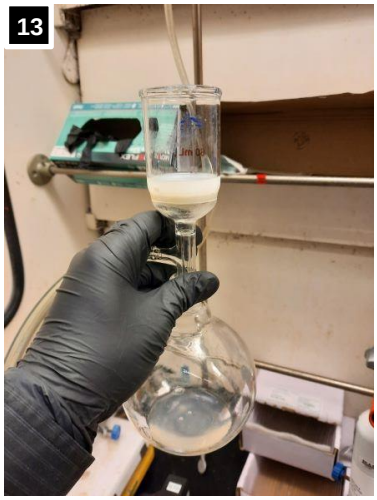


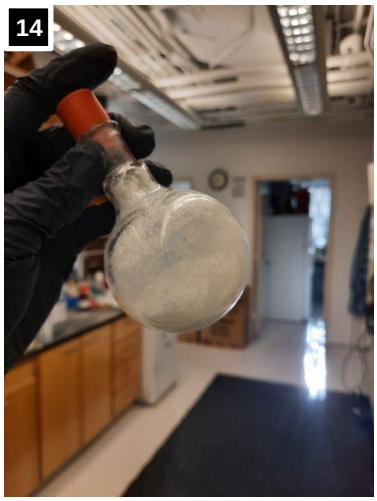


10: TLC of a crude reaction mixture (DCM/MeOH, 4:1).

11: The product is purified by silica gel chromatography (400 mL EtOAc, then DCM/MeOH, 15 :1) (*If the product is still contaminated with 5-phenyltetrazolate salts after this step, it is advised to repeat chromatography*).

12: Fractions containing product are pooled, concentrated under reduced pressure, and treated with DCM to form a suspension.



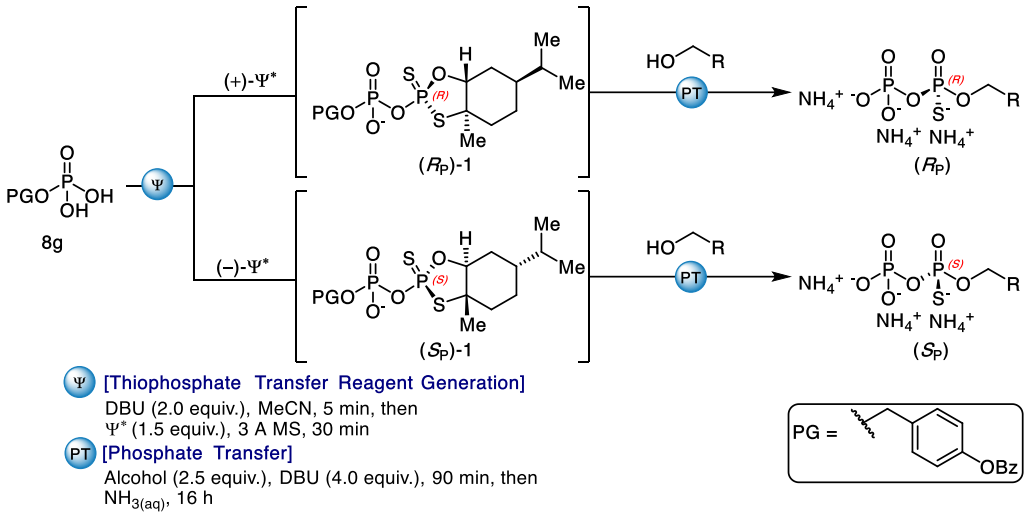


13: The solid residue is filtered off on a Schott or Büchner funnel and the filtrate is concentrated under reduced pressure. The residual MeOH is removed by co-evaporation with DCM.

14: Pure compound **9c** (3.5 g; **Yield = 65%**) obtained as a white foam, after drying under high vacuum.

4. Stereocontrolled α-Thio-Di- and Triphosphorylation

4.1. General Procedure A: Stereocontrolled Synthesis of α-Thiodiphosphates

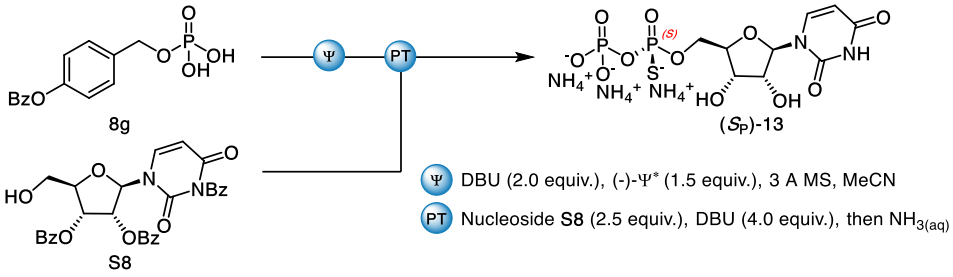


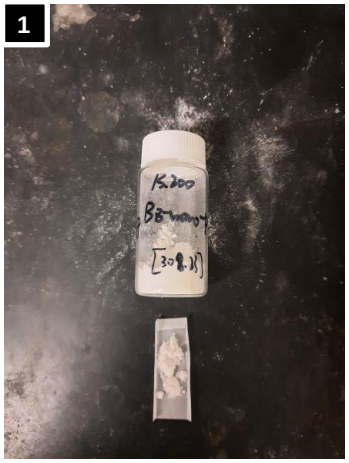
Isomer R_P of α-thiodiphosphate can be obtained from (+)-Ψ*. Isomer S_P of α-thiodiphosphate can be obtained from (–)-Ψ*.

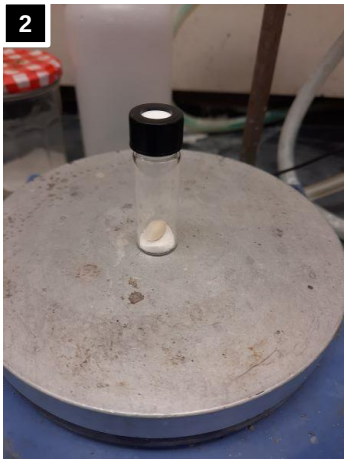
A flame dried 1-dram vial with a stir bar was charged with monophosphate **8g** (61.6 mg, 0.2 mmol, 1.0 equiv.). The vial was closed with a Teflon septum screw cap, evacuated and backfilled with argon. Anhydrous MeCN (2.0 mL) and DBU (60 µL, 0.4 mmol, 2.0 equiv.) were added and the mixture was stirred until starting material completely dissolved (~ 5 min). Subsequently, freshly flame-dried 3 Å molecular sieves (60 mg) and Ψ* reagent **11** (128 mg, 0.3 mmol, 1.5 equiv.) were added and the reaction was stirred at room temperature for 30 min. After that protected nucleoside (0.5 mmol, 2.5 equiv.) was added, followed by another portion of DBU (120 µL, 0.8 mmol, 4.0 equiv.) and the mixture was stirred for another 90 min. Upon completion of the reaction, the resulting mixture was filtered, the solid residue was washed with ~1 mL of MeCN and the filtrate was concentrated under reduced pressure to ~0.5 mL. Concentrated aq. NH_3 solution (5.0 mL) was added to the residue and the resulting mixture was stirred at room temperature (or at 40 °C, *see below*) for 16 h. Subsequently, the reaction mixture was diluted with water and washed with EtOAc and the aqueous phase was concentrated under reduced pressure (temp. ≤ 40 °C). The remaining oily residue was dissolved in 2 mL of water and added dropwise to a solution of 0.2 M $NaClO_4$ in acetone (40 mL). The resulting suspension was centrifuged at 1000 x *g* for 3 min. The supernatant was discarded, and the pellet was washed twice with acetone. The crude product was purified by ion exchange chromatography on DEAE Sephadex (gradient 1 M NH_4HCO_3/water, from 0:100 to 30:70). Fractions containing product were combined and the solvent was evaporated under reduced pressure (temp. ≤ 40 °C). The solid residue was dissolved in minimal amount of water and lyophilized to provide pure nucleoside α-thiodiphosphate.

Note: In several cases deprotection had to be performed at 40 °C to achieve full conversion (e.g. adenosine with amine group protected as benzoyl amide; *for specific examples see section 8.3.*)

4.2. Representative Graphical Procedure for Stereocontrolled Synthesis of α-Thiodiphosphates



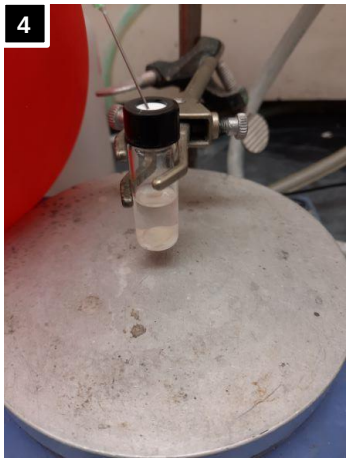


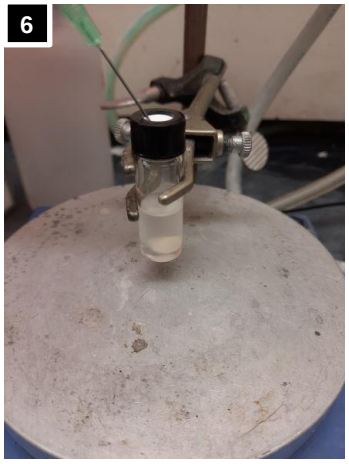


1: Weighted sample of monophosphate **8g** (62 mg).

2: 1-Dram vial charged with monophosphate **8g**.

3: The vial is evacuated and backfilled with argon.

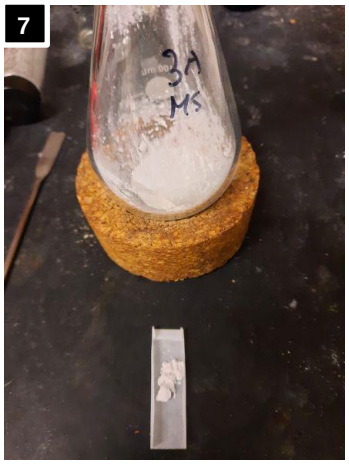


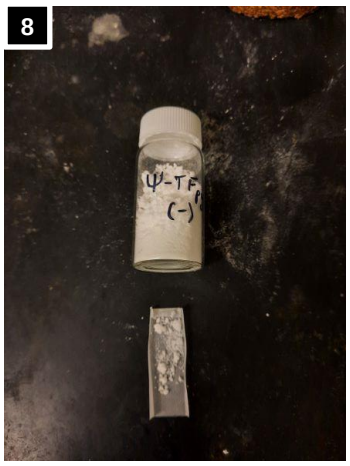


4: Anhydrous MeCN (2.0 mL) is added to the vial.

5: DBU (60 µL) is added via microsyringe.

6: After ~5 min of stirring the solution becomes clear.

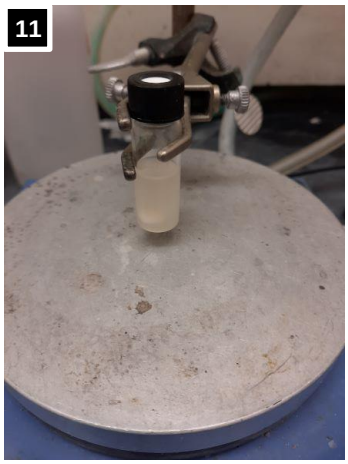


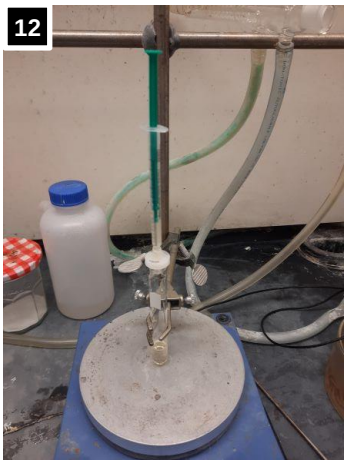


7: 3 Å molecular sieves (60 mg) are added.

8: (–)-Ψ* reagent (128 mg) is added to the suspension.

9: The reaction mixture is stirred for 30 min under argon atmosphere.

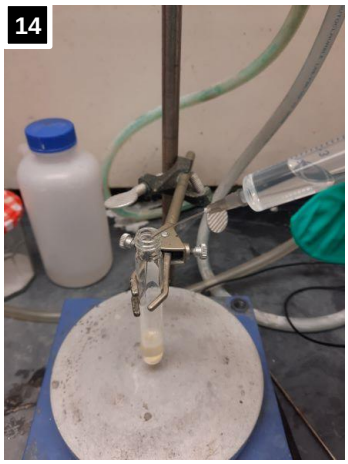


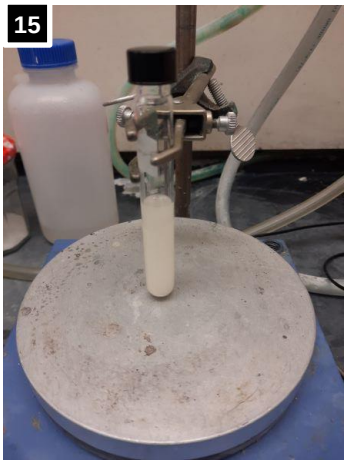


10: Protected nucleoside is added (In this case uridine derivative **S8**; 278 mg), followed by another portion of DBU (120 µL).

11: Protected nucleoside dissolves after addition of DBU and the reaction is stirred for 90 min.

12: The reaction mixture is filtered through a syringe filter.

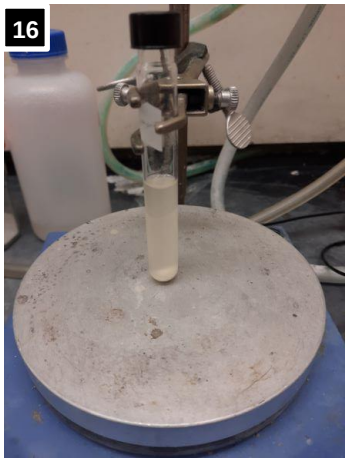




13: The residue is concentrated to ~0.5 mL under reduced pressure.

14: Concentrated aq. NH_3 (5.0 mL) is added.

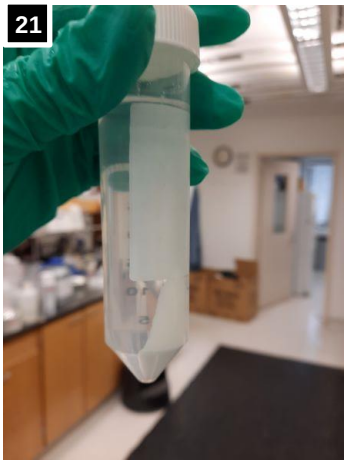
15: The resulting suspension is stirred for 16 h.



16: The reaction mixture after 16 h (*note: most of the solid dissolved*).

17: The reaction mixture is diluted with water and washed with EtOAc.

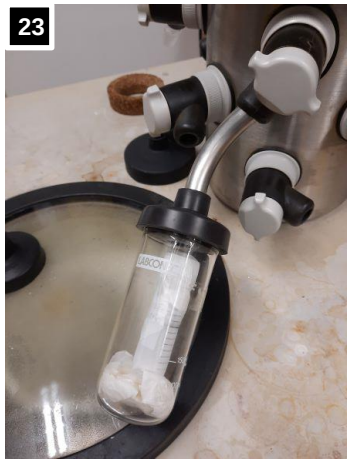
18: The aqueous fraction is concentrated under reduced pressure (< 40 °C) and diluted with ~ 2 mL of water.

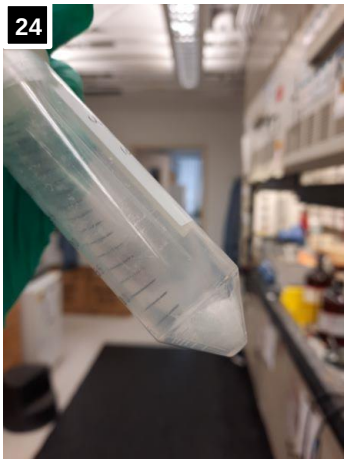


19: The aqueous solution is added dropwise to a solution of $NaClO_4$ (1.0 g) in acetone (40 mL).

20: The resulting suspension is centrifuged at 1000 x *g* for 3 min.

21: Supernatant is decanted, and the pellet is washed two times with acetone.



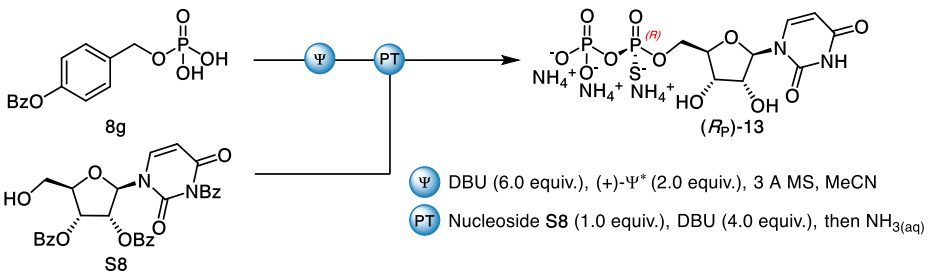


22: Crude product is purified by ion exchange chromatography on DEAE-Sephadex.

23: Fractions containing product are collected, concentrated under reduced pressure (< 40 °C) and lyophilized to remove residual water.

24: Pure sample of compound (S_P)-**13** (64 mg; **Yield = 60%**) isolated as a white fluffy solid.

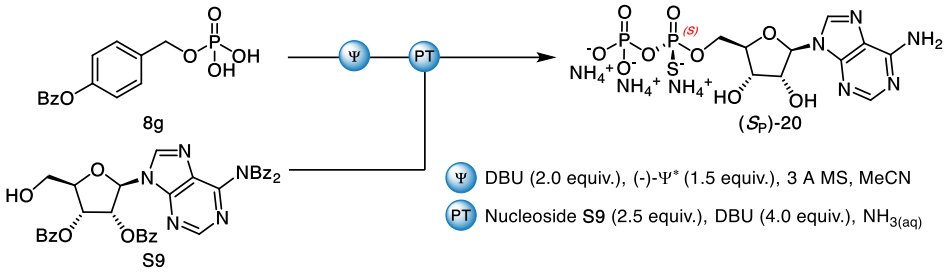
4.3. Representative Procedure for Stereocontrolled Synthesis of α-Thiodiphosphates using Nucleoside as a Limiting Reagent



A flame dried 1-dram vial equipped with a stir bar was charged with monophosphate **8g** (185 mg, 0.6 mmol, 3.0 equiv.). The vial was closed with a Teflon septum screw cap, evacuated, and backfilled with argon. Anhydrous MeCN (4.0 mL) and DBU (180 µL, 1.2 mmol, 6.0 equiv.) were added and the mixture was stirred until starting material completely dissolved (~ 5 min). Subsequently, freshly flame-dried 3 Å molecular sieves (120 mg) and (+)-Ψ* reagent (172 mg, 0.4 mmol, 2.0 equiv.) were added and the reaction was stirred at room temperature for 30 min. After that protected nucleoside (in this case uridine derivative **S8**) (111 mg, 0.2 mmol, 1.0 equiv.) was added, followed by another portion of DBU (120 µL, 0.8 mmol, 4.0 equiv.) and the mixture was stirred for another 90 min. Upon completion of the reaction, the resulting mixture was filtered and concentrated under reduced pressure to ~0.5 mL. Concentrated aq. NH_3 solution (5.0 mL) was added to the residue and the resulting mixture was stirred at room temperature for 16 h. The isolation of the product was performed as described in *General Procedure A* to afford 52 mg of compound **(R_P)-13** as a white fluffy solid (**Yield = 55%**).

*In comparison, following General Procedure A (using monophosphate **8g** as a limiting reagent and 2.5 equiv. of nucleoside **S8**) compound **(R_P)-13** was obtained in only slightly higher yield (**62%**).*

4.4. Representative Gram-Scale Procedure for Stereocontrolled Synthesis of α-Thiodiphosphates

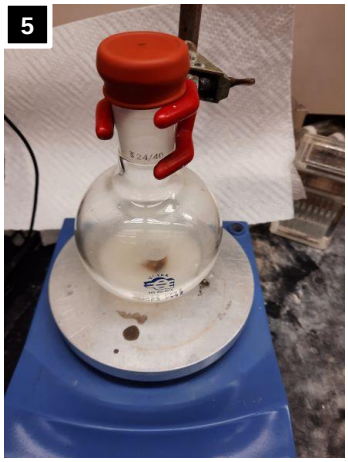


A flame dried round bottom flask with a stir bar was charged with monophosphate **8g** (0.62 g, 2.0 mmol, 1.0 equiv.) and the flask was capped with a septum. Anhydrous MeCN (20 mL) and DBU (600 µL, 4.0 mmol, 2.0 equiv.) were added and the mixture was stirred until starting material completely dissolved (~ 5 min). Subsequently, freshly flame-dried 3 Å molecular sieves (0.80 g) and (–)-Ψ* reagent **11** (1.28 g, 3.0 mmol, 1.5 equiv.) were added and the reaction was stirred at room temperature for 45 min. After that protected adenosine **S9** (3.42 g, 5.0 mmol, 2.5 equiv.) was added, followed by another portion of DBU (1.20 mL, 8.0 mmol, 4.0 equiv.), and the mixture was stirred for another 2 h. Upon completion of the reaction, the resulting mixture was filtered and concentrated under reduced pressure. Concentrated aq. NH_3 solution (50 mL) was added to the residue and the resulting mixture was stirred at 40 °C for 16 h. Subsequently, the reaction mixture was diluted with water and washed with EtOAc. Aqueous phase was concentrated under reduced pressure (temp. ≤ 40 °C) to ~20 mL. The remaining solution was partitioned between eight 50 mL centrifuge tubes containing a solution of 0.2 M $NaClO_4$ in acetone (40 mL) each. The resulting suspension was centrifuged at 1000 x *g* for 3 min. The supernatant was discarded, and the pellet was washed with acetone twice. The solid residues from each of the centrifuge tubes were redissolved in minimal amount of water, combined and purified by ion exchange chromatography on DEAE Sephadex (gradient 1 M NH_4HCO_3/water, from 0:100 to 30:70). Fractions containing the product were combined and the solvent was evaporated under reduced pressure (temp. ≤ 40 °C). The solid residue was dissolved in minimal amount of water and lyophilized to provide 620 mg of compound **(S_P)-20** as a white amorphous solid (**Yield = 63%, d.r. > 20:1**).

1: 250 mL flask charged with monophosphate **8g** (0.62 g).

2: The phosphate precursor is suspended in anhydrous MeCN (20 mL), followed by addition of DBU (600 µL).

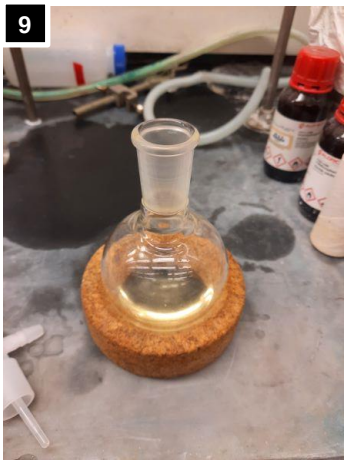
3: 3 Å molecular sieves (0.80 g) are added after 5 min of stirring.



4: (–)-Ψ* reagent (1.28 g) is added to the reaction mixture.

5: The resulting suspension is stirred at room temperature for 45 min.

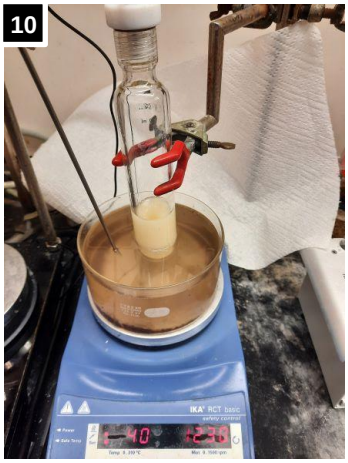
6: Protected adenosine **S9** (3.42 g) is added.



7: Second portion of DBU (1.20 mL) is added (after ~ 5 min protected adenosine **S9** fully dissolved).

8: The reaction is stirred at room temperature for 2 h.

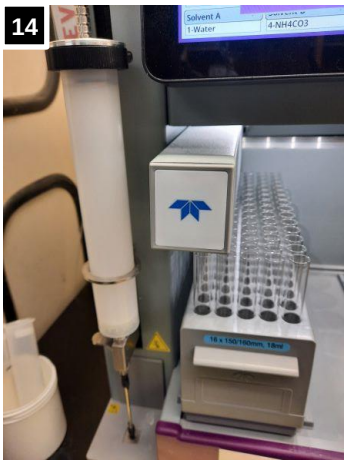
9: The reaction mixture is filtered to remove molecular sieves.

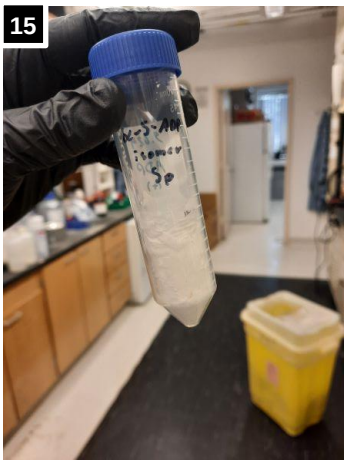


10: Concentrated filtrate is transferred to a pressure tube followed by addition of concentrated aq. NH_3. The resulting suspension is stirred at 40 °C for 16 h.

11: The reaction mixture after completed deprotection step.

12: The reaction is diluted with water and washed with EtOAc (2 times).



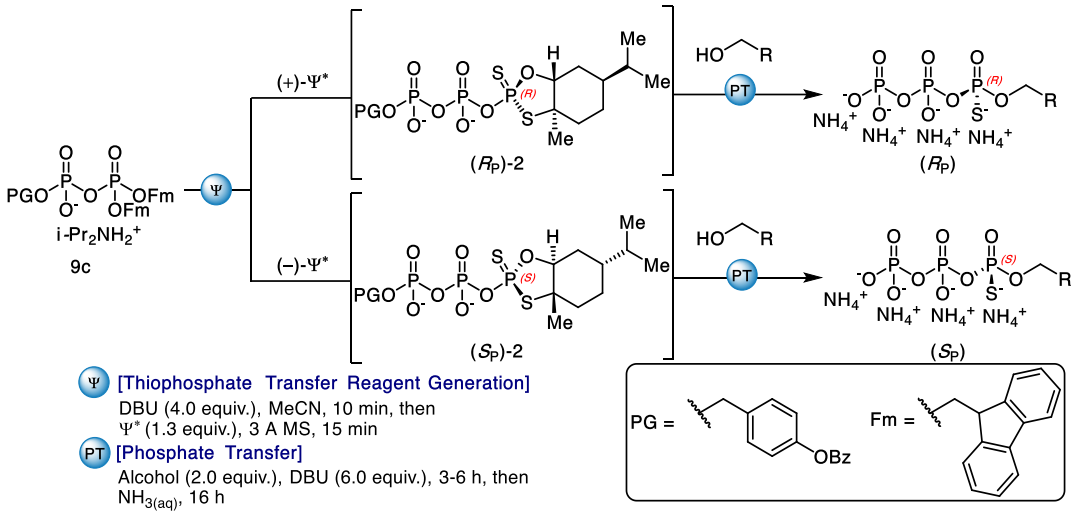


13: The crude product is precipitated from 0.2 M $NaClO_4$ in acetone (batch is partitioned between eight 50 mL centrifuge tubes).

14: Crude product is purified by ion exchange chromatography of DEAE-Sephadex.

15: Pure product **(*S*$_P$)-20** (621 mg, 1.26 mmol; **Yield = 63%**) isolated as a white solid after lyophilization.

4.5. General Procedure B: Stereocontrolled Synthesis of α-Thiotriphosphate

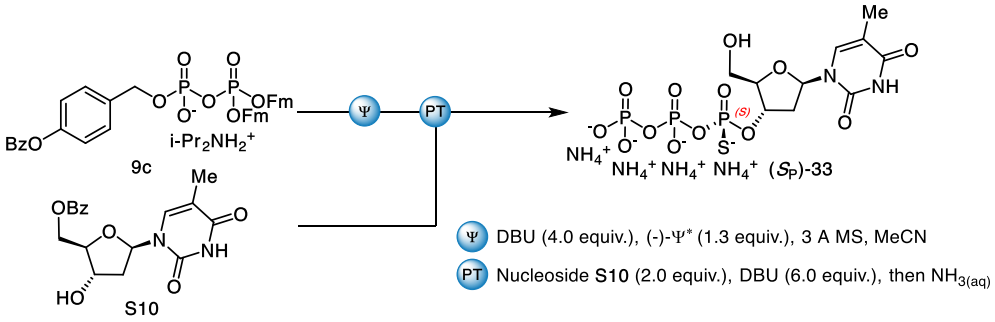


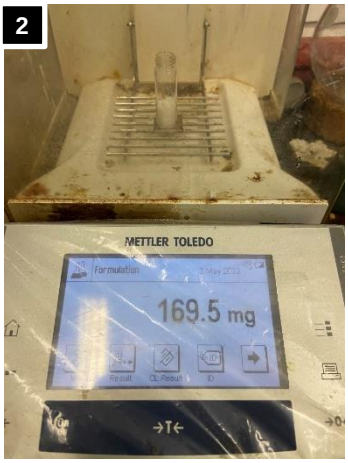
Isomer R_P of α-thiotriphosphate can be obtained from (+)-Ψ*. Isomer S_P of α-thiotriphosphate can be obtained from (–)-Ψ*.

A flame dried 1-dram vial with a stir bar was charged with pyrophosphate **9c** (169 mg, 0.20 mmol, 1.0 equiv.). The vial was closed with a Teflon septum screw cap, evacuated and backfilled with argon. Anhydrous MeCN (2.0 mL) and DBU (120 µL, 0.80 mmol, 4.0 equiv.) were added and the mixture was stirred for 10 min. Subsequently, freshly flame-dried 3 Å molecular sieves (200 mg) and Ψ* reagent **11** (112 mg, 0.26 mmol, 1.3 equiv.) were added and the reaction was stirred at room temperature for 15 min. After that protected nucleoside (0.40 mmol, 2.0 equiv.) was added, followed by another portion of DBU (180 µL, 1.2 mmol, 6.0 equiv.) and the mixture was stirred for 3 to 6 h. Upon completion of the reaction, resulting mixture was filtered, the solid residue was washed with ~1 mL of MeCN and the filtrate was concentrated under reduced pressure to ~0.5 mL. Concentrated aq. NH_3 solution (5.0 mL) was added to the residue and the reaction was stirred at room temperature (or at 40 °C, *see below*) for 16 h. Subsequently, the reaction mixture was diluted with water and washed with EtOAc, and the aqueous phase was concentrated under reduced pressure (temp. 40 °C). The remaining oily residue was dissolved in 2 mL of water and added dropwise to a solution of 0.2 M $NaClO_4$ in acetone (40 mL). The resulting suspension was centrifuged at 1000 x *g* for 3 min. The supernatant was discarded, and the pellet was washed with acetone twice. The crude product was purified by ion exchange chromatography on DEAE Sephadex (gradient 1 M NH_4HCO_3/water, from 0:100 to 50:50). Fractions containing product were combined and the solvent was evaporated under reduced pressure (temp. 40 °C). The solid residue was dissolved in minimal amount of water and lyophilized to provide pure nucleoside α-thiotriphosphate.

Note: In several cases deprotection had to be performed at 40 °C to achieve full conversion (i.e. adenosine with amine group protected as benzoyl amide; *for specific examples see section 8.3.*)

4.6. Representative Graphical Procedure for Stereocontrolled Synthesis of α-Thiotriphosphates





1: All the reagents required for the reaction. From left: Pyrophosphate precursor **9c**; (–)-Ψ*; protected nucleoside **9c**; DBU; 3 Å molecular sieves.

2: Pyrophosphate reagent **9c** (169 mg) added to a 1-dram vial.

3: Anhydrous MeCN (2.0 mL) taken from solvent purification system.

4: Pyrophosphate **9c** dissolved in anhydrous MeCN (2.0 mL).

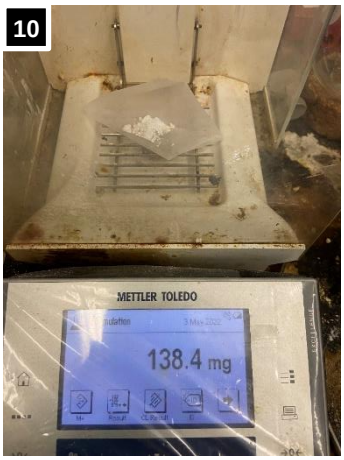
5: DBU (120 µL) is added and the solution is stirred for 10 min under argon atmosphere.

6: Weighted sample of 3 Å molecular sieves (200 mg).

7: 3 Å molecular sieves (200 mg) added to the reaction mixture.

8: Weighted sample of (–)-Ψ* (112 mg).

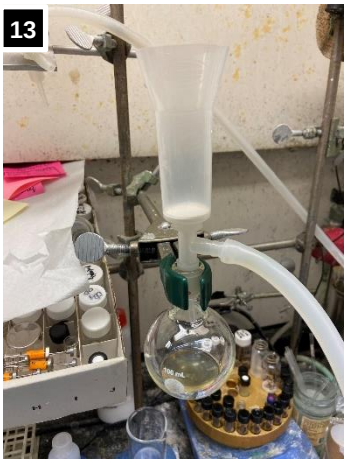
9: (–)-Ψ* (112 mg) is added to the reaction mixture and the solution is stirred for 15 min.

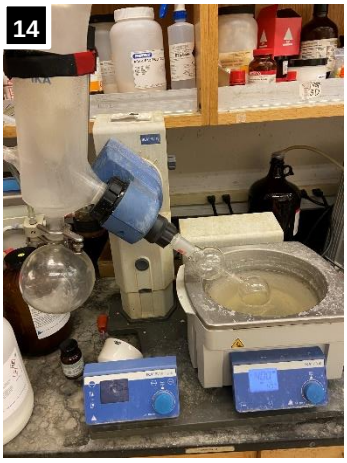


10: Weighted sample of protected nucleoside (in this case thymidine derivative **S10**; 138 mg).

11: Protected nucleoside **S10** added to the reaction mixture.

12: Second portion of DBU (180 µL) is added (**S10** fully dissolves) and the reaction mixture is stirred for 6 h.

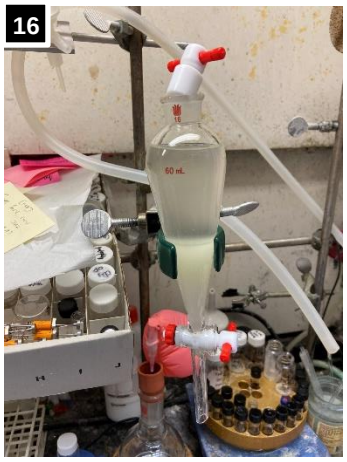


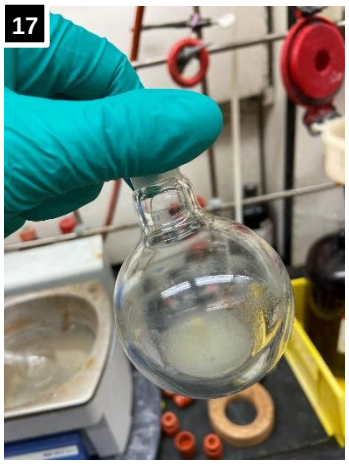


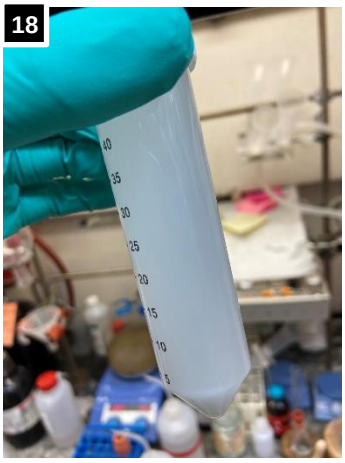
13: The reaction mixture is filtered to remove molecular sieves.

14: The filtrate is concentrated to ~0.5 mL.

15: The residue is transferred to a pressure tube. Concentrated aq. NH_3 (5.0 mL) is added, and the reaction mixture is stirred for 16 h at 40 °C.



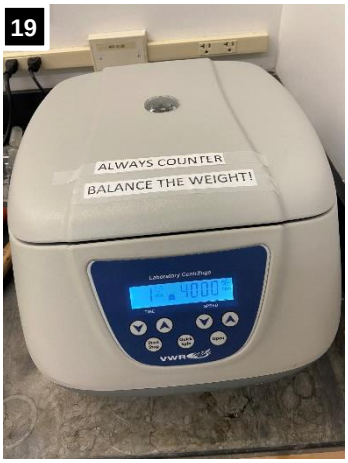


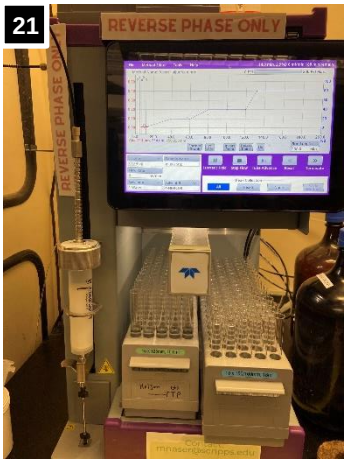


16: The reaction mixture is diluted with water and washed with EtOAc.

17: The aqueous fraction is concentrated under reduced pressure (< 40 °C) and diluted with ~2 mL of water.

18: The aqueous solution is added dropwise to a solution of $NaClO_4$ (1.0 g) in acetone (40 mL).

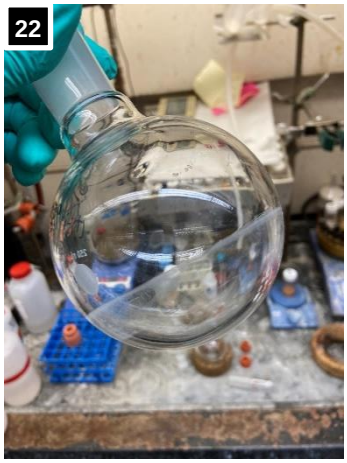




19: The resulting suspension is centrifuged at 1000 x *g* for 3 min.

20: Supernatant is decanted, and the pellet is washed two times with acetone.

21: Crude product is purified by ion exchange chromatography on DEAE-Sephadex.

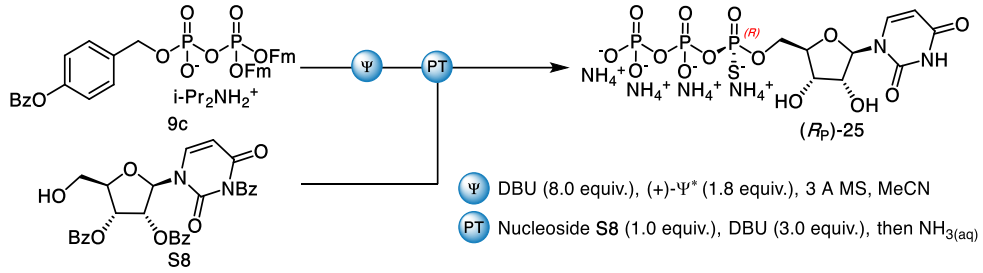


22: Fractions containing product are collected and concentrated under reduced pressure (< 40 °C).

23: Product is lyophilized to remove residual water.

24: Pure sample of compound (S_P)-**33** (60 mg; **Yield = 53%**) isolated as a white fluffy solid.

4.7. Representative Procedure for Stereocontrolled Synthesis of α-Thiotriphosphates using Nucleoside as a Limiting Reagent

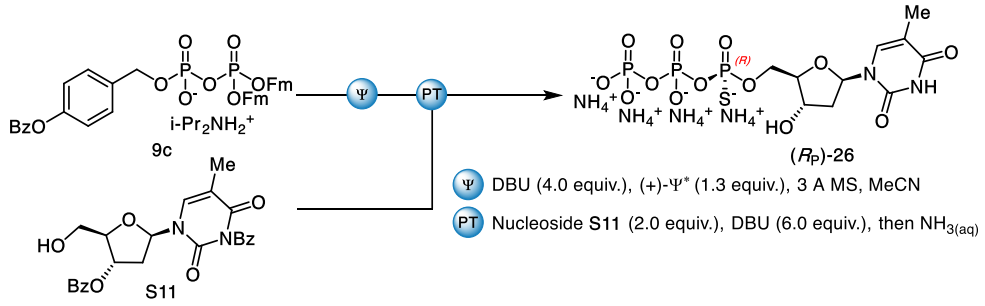


A flame dried 1-dram vial with a stir bar was charged with pyrophosphate **9c** (338 mg, 0.40 mmol, 2.0 equiv.). The vial was closed with a Teflon septum screw cap, evacuated, and backfilled with argon. Anhydrous MeCN (4.0 mL) and DBU (230 µL, 1.60 mmol, 8.0 equiv.) were added and the mixture was stirred for 10 min. Subsequently, freshly flame-dried 3 Å molecular sieves (400 mg) and (+)-Ψ* reagent **11** (154 mg, 0.36 mmol, 1.8 equiv.) were added and the reaction was stirred at room temperature for 15 min. After that protected nucleoside (in this case protected uridine derivative **S8**) (111 mg, 0.20 mmol, 1.0 equiv.) was added, followed by another portion of DBU (90 µL, 0.6 mmol, 3.0 equiv.) and the mixture was stirred for 4 h. Upon completion of the reaction, resulting mixture was filtered, the solid residue was washed with ~1 mL of MeCN and the filtrate was concentrated under reduced pressure to ~0.5 mL. Concentrated aq. NH_3 solution (5.0 mL) was added to the residue and the reaction was stirred at room temperature for 16 h. The isolation of the product was performed as described in *General Procedure B* to afford 23 mg of compound **(R_P)-25** as a white fluffy solid (**Yield = 20%**).

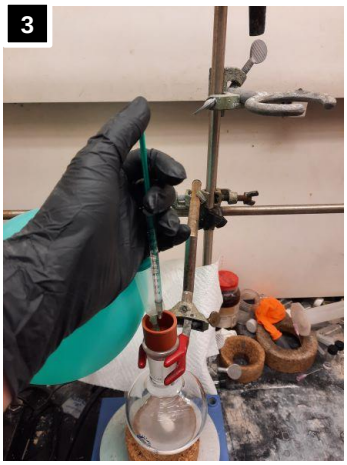
*In comparison, following General Procedure B (using pyrophosphate **9c** as a limiting reagent and 2.0 equiv. of nucleoside) compound **(R_P)-25** was obtained in 47% yield.*

In general yields of the products obtained following the above procedure (1.0 equiv. of nucleoside) are about half the value of the yields obtained following General Procedure B (2.0 equiv. of nucleoside). Therefore, the obtained yields with regards to the amount of the nucleoside used are similar.

4.8. Representative Gram-Scale Procedure for Stereocontrolled Synthesis of α-Thiotriphosphates



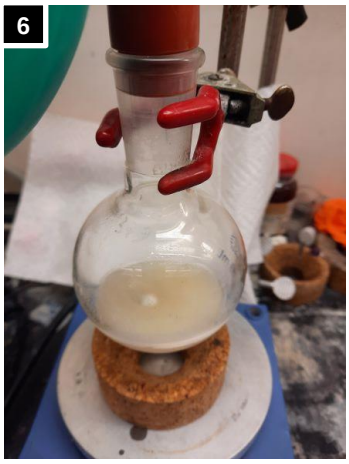
A flame dried round bottom flask with a stir bar was charged with pyrophosphate **9c** (1.69 g, 2.0 mmol, 1.0 equiv.) and the flask was capped with a septum. Anhydrous MeCN (20 mL) and DBU (1.20 mL, 8.0 mmol, 4.0 equiv.) were added and the mixture was stirred for 10 min. Subsequently, freshly flame-dried 3 Å molecular sieves (2.0 g) and (+)-Ψ* reagent **11** (1.12 g, 2.6 mmol, 1.3 equiv.) were added and the reaction was stirred at room temperature for 30 min. After that protected thymidine **S11** (1.80 g, 4.0 mmol, 2.0 equiv.) was added, followed by another portion of DBU (1.80 mL, 12.0 mmol, 6.0 equiv.) and the mixture was stirred for 4 h. Upon completion of the reaction, the resulting mixture was filtered and concentrated under reduced pressure. Concentrated aq. NH_3 solution (50 mL) was added to the residue and the reaction was stirred at 40 °C for 16 h. Subsequently, the reaction mixture was diluted with water and washed with EtOAc. Aqueous phase was concentrated under reduced pressure (temp. ≤ 40 °C) to ~20 mL. The remaining solution was partitioned between eight 50 mL centrifuge tubes containing a solution of 0.2 M $NaClO_4$ in acetone (40 mL) each. The resulting suspension was centrifuged at 1000 x *g* for 3 min. Supernatant was discarded and the pellet was washed with acetone twice. Solid residues from each of the centrifuge tubes were redissolved in minimal amount of water, combined, and purified by ion exchange chromatography on DEAE Sephadex (gradient 1 M NH_4HCO_3/water, from 0:100 to 30:70). Fractions containing product were combined and the solvent was evaporated *in vacuo* (temp. ≤ 40 °C). The solid residue was dissolved in minimal amount of water and lyophilized to provide 566 mg of compound **(R_P)-26** as a white amorphous solid (**Yield = 50%, d.r. > 20:1**).



1: 250 mL flask charged with pyrophosphate **9c** (1.69 g).

2: The phosphate precursor **9c** is dissolved in anhydrous MeCN (20 mL).

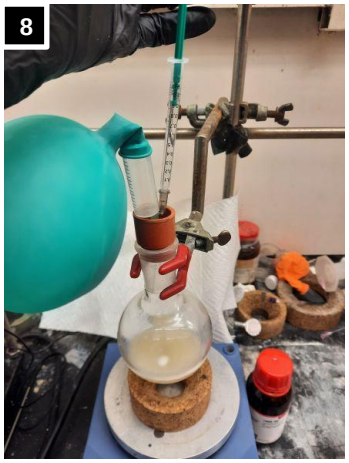
3: DBU (1.20 mL) is added.



4: 3 Å molecular sieves (2.0 g) are added after 10 min of stirring.

5: (+)-Ψ* reagent (1.12 g) is added to the reaction mixture.

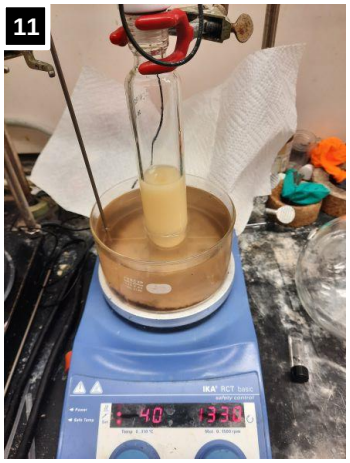
6: The resulting suspension is stirred at room temperature for 30 min.



7: Protected thymidine **S11** (1.80 g) is added.

8: Second portion of DBU (1.20 mL) is added (after ~ 5 min protected thymidine **S11** fully dissolves).

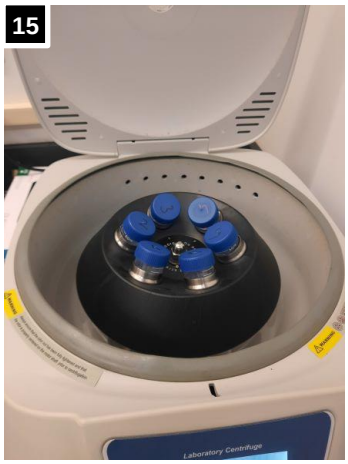
9: The reaction is stirred at room temperature for 4 h.



10: The reaction mixture is filtered to remove molecular sieves.

11: Concentrated filtrate is transferred to a pressure tube followed by addition of concentrated aq. NH_3 (50 mL). The resulting suspension is stirred at 40 °C for 16 h.

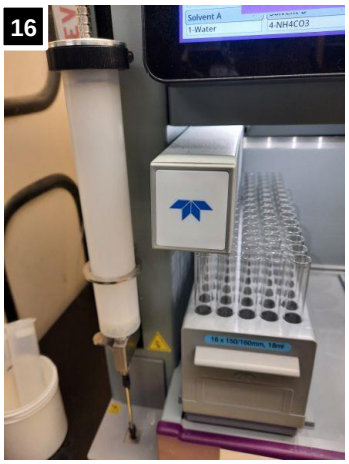
12: The reaction mixture after completed deprotection step.

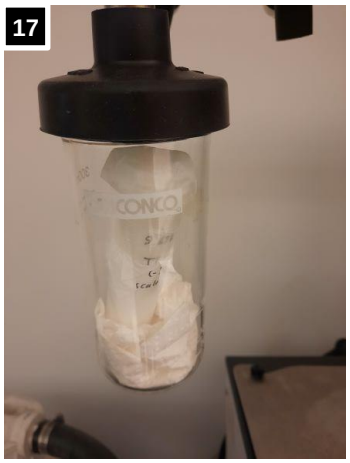


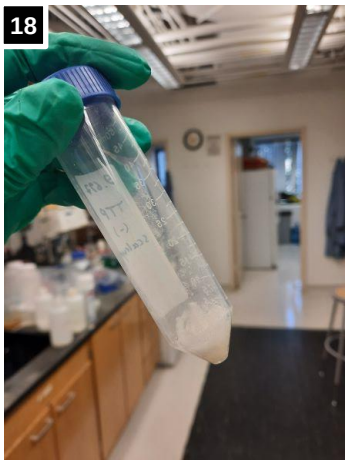
13: The reaction is diluted with water and washed with EtOAc (2 times).

14: The crude product is precipitated from 0.2 M $NaClO_4$ in acetone (batch is partitioned between eight 50 mL centrifuge tubes).

15: Tubes with the suspension are centrifuged at 1000 x *g* for 3 min, supernatant is decanted, and the pellet is washed with acetone (2 times).





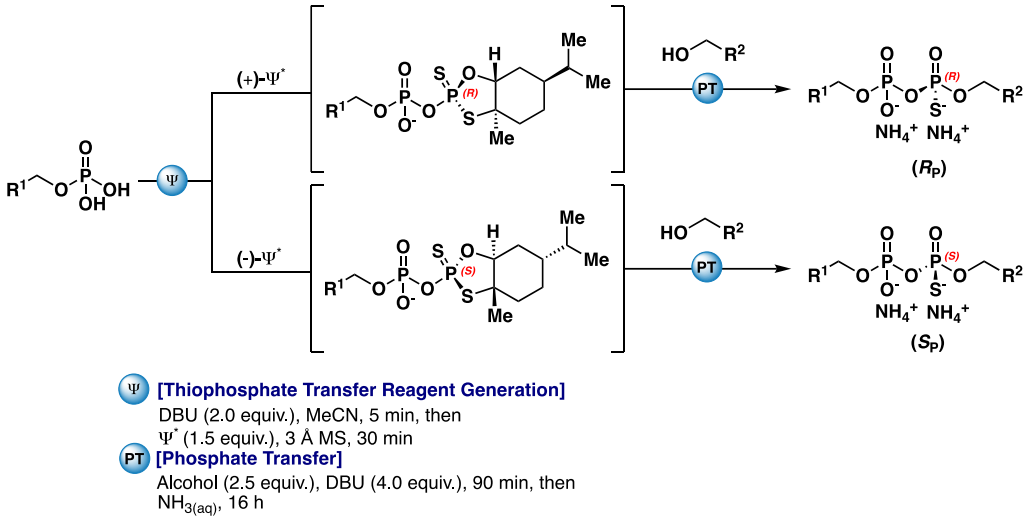


16: Crude product is purified by ion exchange chromatography on DEAE-Sephadex.

17: Fractions containing product are pooled, concentrated under reduced pressure, and lyophilized.

18: Pure product **(R_P)-26** (566 mg, 1.0 mmol; **Yield = 50%**) isolated as a white solid after lyophilization.

4.9. General Procedure C: Stereocontrolled Synthesis of Dinucleoside Thiodiphosphates

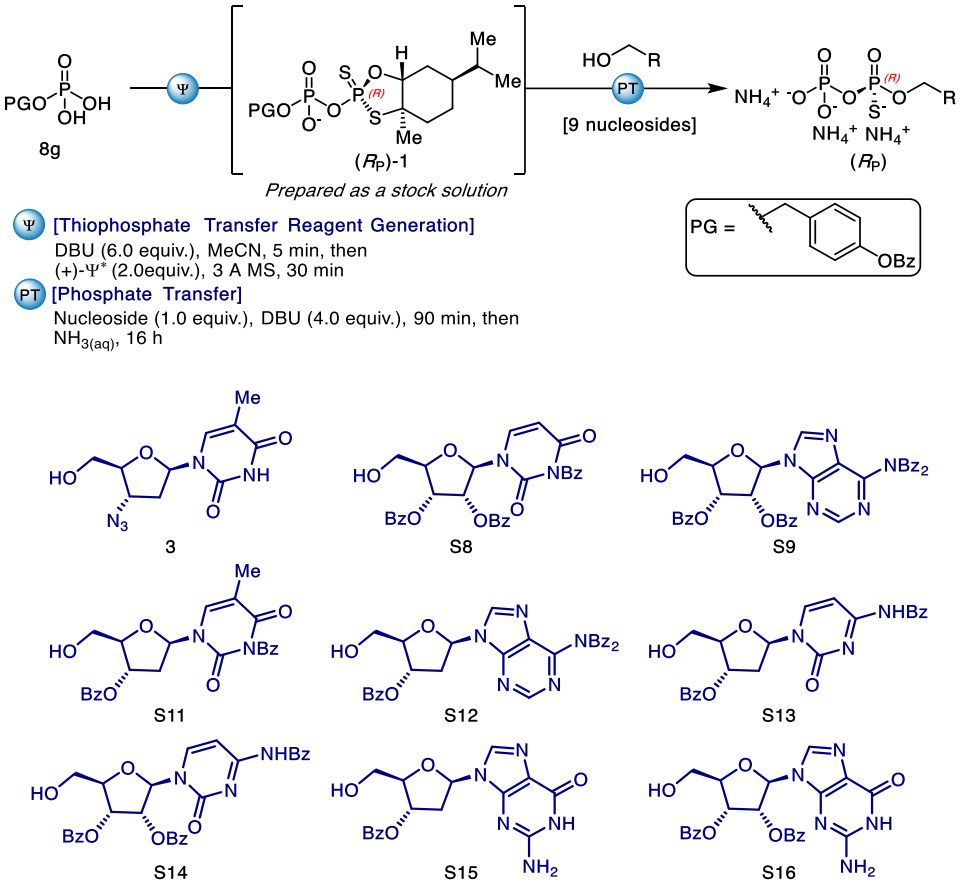


Isomer R_P of dinucleoside thiodiphosphate can be obtained from (+)-Ψ*. Isomer S_P of dinucleoside thiodiphosphate can be obtained from (–)-Ψ*.

A flame dried 1-dram vial with a stir bar was charged with protected nucleoside monophosphate (0.2 mmol, 1.0 equiv.). The vial was closed with a Teflon septum screw cap, evacuated and backfilled with argon. Anhydrous MeCN (2.0 mL) and DBU (60 µL, 0.4 mmol, 2.0 equiv.) were added and the mixture was stirred until starting material completely dissolved (~ 5 min). Subsequently, freshly flamed-dried 3 Å molecular sieves (60 mg) and Ψ* reagent **11** (128 mg, 0.3 mmol, 1.5 equiv.) were added and the reaction was stirred at room temperature for 30 min. After that protected nucleoside (0.5 mmol, 2.5 equiv.) was added, followed by another portion of DBU (120 µL, 0.8 mmol, 4.0 equiv.) and the mixture was stirred for another 2 h. Upon completion of the reaction, the resulting mixture was filtered, the solid residue was washed with ~1 mL of MeCN and the filtrate was concentrated under reduced pressure to ~0.5 mL. Concentrated aq. NH_3 solution (5.0 mL) was added to the residue and the reaction was stirred at 40 °C for 16 h. Subsequently, the reaction mixture was diluted with water and washed with EtOAc. Aqueous phase was concentrated under reduced pressure (temp. ≤ 40 °C). The remaining oily residue was dissolved in 2 mL of water and added dropwise to a solution of 0.2 M $NaClO_4$ in acetone (40 mL). The resulting suspension was centrifuged at 1000 x *g* for 3 min. Supernatant was discarded and the pellet was washed with acetone twice. The crude product was purified by ion exchange chromatography on DEAE Sephadex (gradient 1 M NH_4HCO_3/water, from 0:100 to 30:70). Fractions containing product were combined and the solvent was evaporated under reduced pressure (temp. ≤ 40 °C). The solid residue was dissolved in minimal amount of water and lyophilized to provide pure dinucleoside thiodiphosphate.

4.10. Parallel Synthesis and Chemoselectivity Screening

Parallel Synthesis

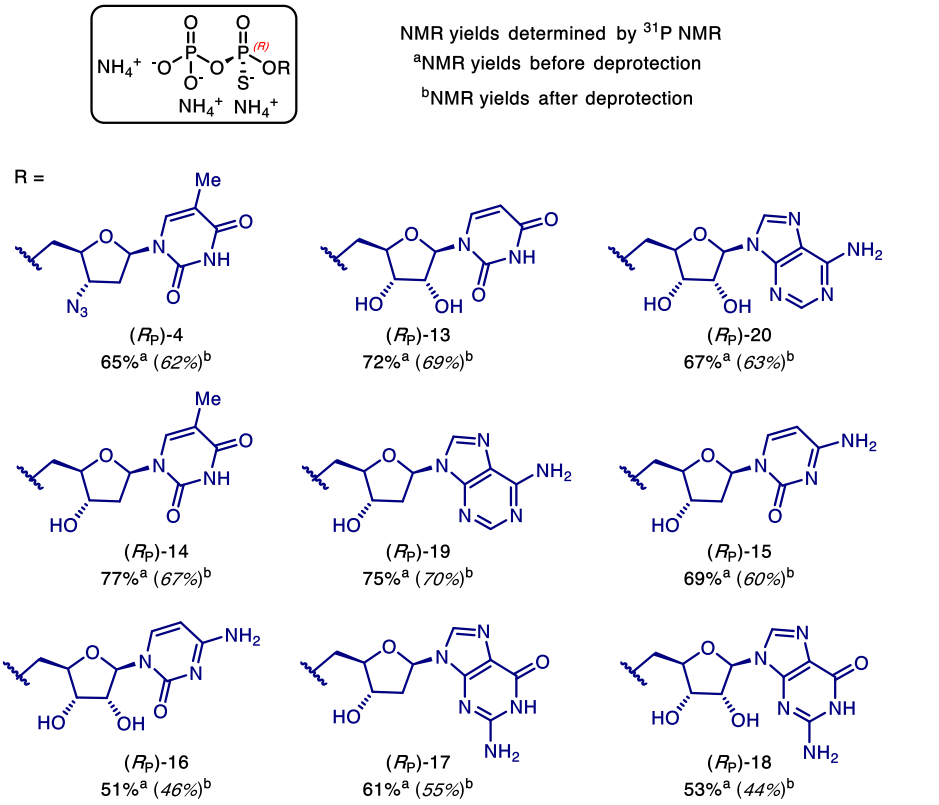


A flame dried round-bottom flask with a stir bar was charged with monophosphate **8g** (462 mg, 1.5 mmol). The flask was closed with a septum, evacuated, and backfilled with argon. Anhydrous MeCN (10 mL) and DBU (450 µL, 3.0 mmol) were added and the mixture was stirred until starting material completely dissolved (~ 5 min). Subsequently, 3 Å molecular sieves (300 mg) and (+)-Ψ* reagent (430 mg, 1.0 mmol) were added and the reaction was stirred at room temperature for 30 min.

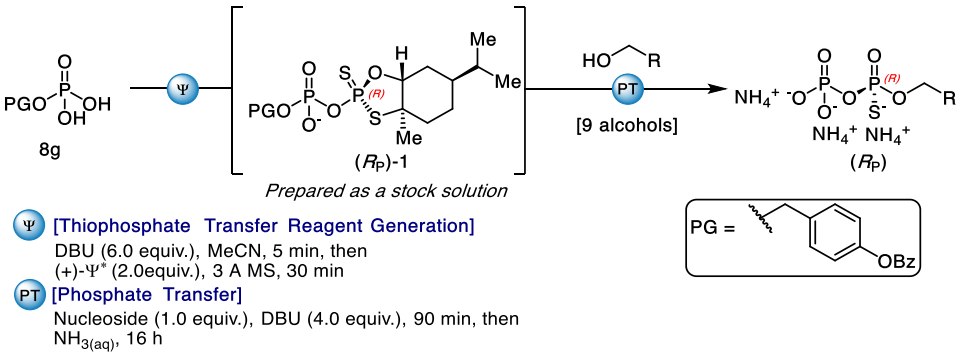
Simultaneously, to nine 1-dram vials equipped with stir bars were added respectively nucleosides **3** (13.4 mg, 0.05 mmol), **S8** (27.8 mg, 0.05 mmol), **S9** (34.2 mg, 0.05 mmol), **S11** (22.5 mg, 0.05 mmol), **S12** (28.2 mg, 0.05 mmol), **S13** (21.8 mg, 0.05 mmol), **S14** (27.8 mg, 0.05 mmol), **S15** (18.6 mg, 0.05 mmol), **S16** (24.6 mg, 0.05 mmol). 1.1 ml of the above prepared solution of thiophosphate transfer reagent **(R_P)-1** was added directly to each vial, followed by DBU (30 µL, 0.2 mmol) and the reactions were stirred for 90 min at room temperature. Subsequently, internal standard (triphenyl phosphate) (0.05 mmol) was added to each vial and the yields were determined by quantitative ^{31}P NMR.

Note: NMR yields were determined before deprotection step. To obtain deprotected α-thiodiphosphates, the solution from each vial was transferred to screw-capped 20 mL cultured tube, followed by addition of concentrated aq. NH_3 solution (5.0 mL). The resulting mixtures were stirred at 40 °C for 16 h. The yield of

deprotection was >85% in each case based on crude NMR (using methylphosphonic acid as an internal standard).



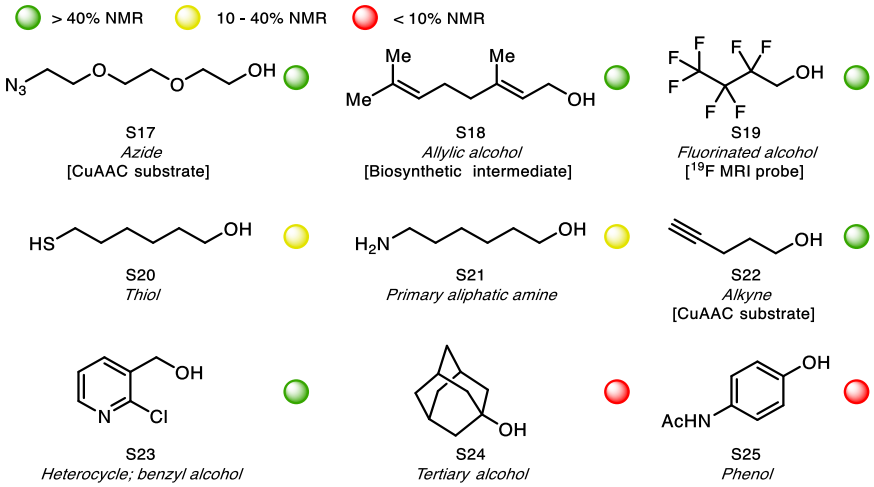
Chemoselectivity Screening



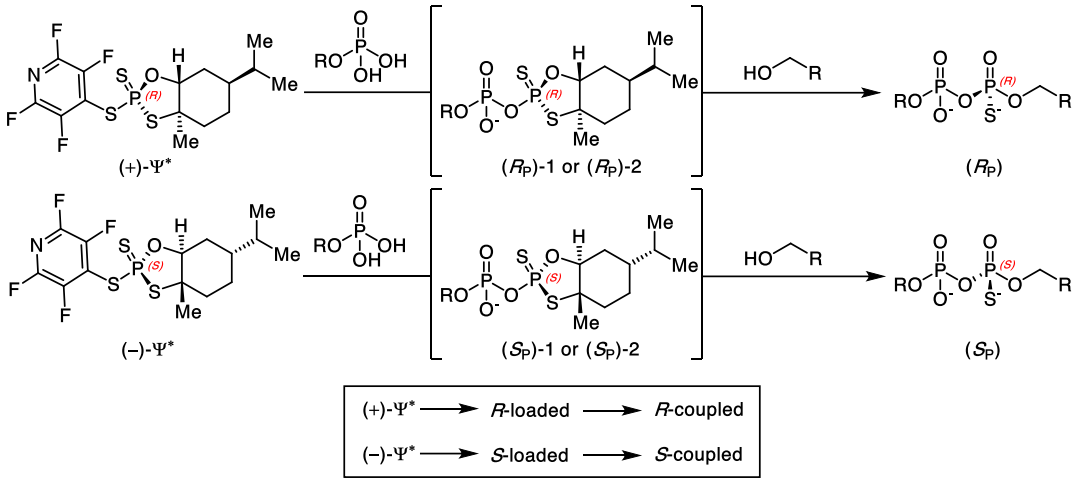
A flame dried round-bottom flask with a stir bar was charged with monophosphate **8g** (462 mg, 1.5 mmol). The flask was closed with a septum, evacuated and backfilled with argon. Anhydrous MeCN (10 mL) and DBU (450 µL, 3.0 mmol) were added and the mixture was stirred until starting material completely dissolved (~ 5 min). Subsequently, 3 Å molecular sieves (300 mg) and (+)-Ψ* reagent (430 mg, 1.0 mmol) were added and the reaction was stirred at room temperature for 30 min.

Simultaneously, to nine 1-dram vials equipped with stir bars were added respectively alcohols **S17** (8.8 mg, 0.05 mmol), **S18** (7.7 mg, 0.05 mmol), **S19** (10.0 mg, 0.05 mmol), **S20** (6.7 mg, 0.05 mmol), **S21** (5.9 mg, 0.05 mmol), **S22** (4.2 mg, 0.05 mmol), **S23** (7.2 mg, 0.05 mmol), **S24** (7.6 mg, 0.05 mmol), **S25** (7.6 mg, 0.05

mmol). 1.1 ml of the above prepared solution of thiophosphate transfer reagent **(R_P)-1** was added directly to each vial, followed by DBU (30 µL, 0.2 mmol) and the reactions were stirred for 90 min at room temperature. Subsequently, internal standard (triphenyl phosphate) (0.05 mmol) was added to each vial and the outcome of the reaction was determined by quantitative ^{31}P NMR.



4.11. Assignment of Absolute Stereochemistry



The following logic was used to determine the absolute stereochemistry outcome:

1. Absolute configuration of Ψ* reagents was determined based on X-ray crystallography.
2. Addition of nucleophile (in this case phosphate anion) to Ψ* reagent occurs with stereoretention, as described in our previous works[4,5].
3. Reaction of the phosphate transfer reagent **1** or **2** with nucleophile (in this case alcohol) occurs in a stereospecific manner, with retention of the relative configuration at the phosphorus atom (due to a pseudorotation step involved in the mechanism), as described in the literature[6,7].
4. The expected net stereochemical retention was confirmed based on the literature data[8,9] by comparing the downfield ^{1}H NMR chemical shifts of α-thio-ADP and α-thio-ATP originating from either (–)- or (+)-Ψ*.

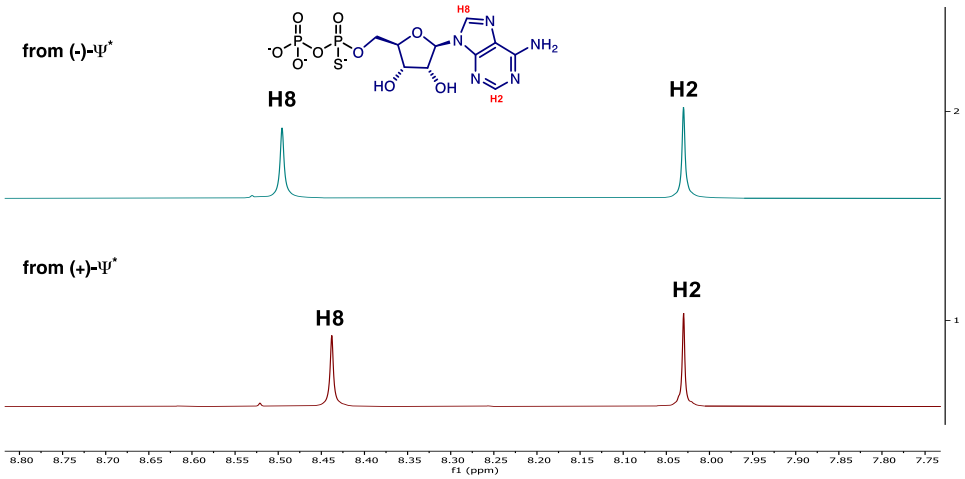


Figure S1. Comparison of ^{1}H NMR for the obtained epimers of α-thio-ADP.

α-thio-ADP from (–)-Ψ*: δ_{H8} = 8.50; δ_{H2} = 8.03. Literature data for S_P isomer: δ_{H8} = 8.51; δ_{H2} = 8.03[8].

α-thio-ADP from (+)-Ψ*: δ_{H8} = 8.44; δ_{H2} = 8.03. Literature data for R_P isomer: δ_{H8} = 8.44; δ_{H2} = 8.03[8].

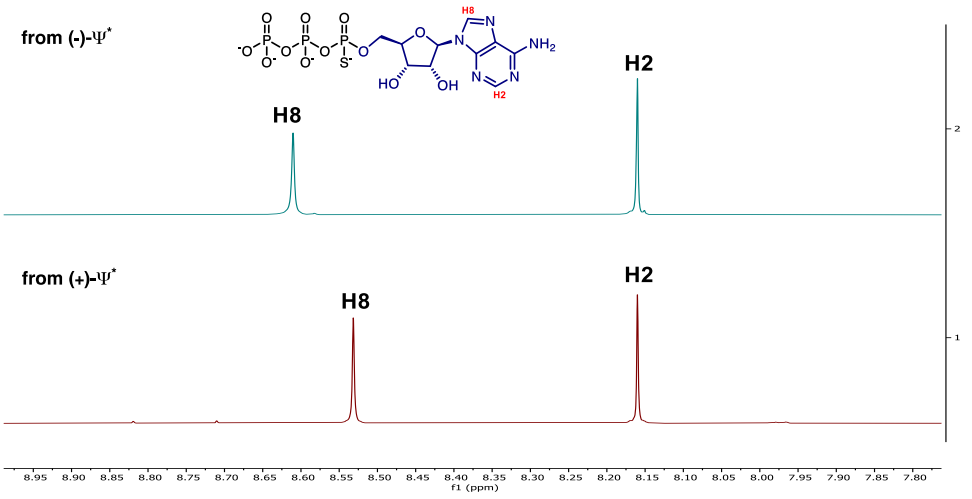


Figure S2. Comparison of ^{1}H NMR for the obtained epimers of α-thio-ATP.

α-thio-ATP from (–)-Ψ*: δ_{H8} = 8.61; δ_{H2} = 8.17. Literature data for S_P isomer: δ_{H8} = 8.60; δ_{H2} = 8.16[9]. α-thio-ATP from (+)-Ψ*: δ_{H8} = 8.54; δ_{H2} = 8.17. Literature data[9] for R_P isomer: δ_{H8} = 8.53; δ_{H2} = 8.17[9].

5. Additionally, relative chromatographic mobility of the obtained pairs of epimers is consistent with the literature data for α-thio-ADP[8] and α-thio-ATP[9] (*fast-eluting epimer: S_P; slow-eluting epimer: R_P*).

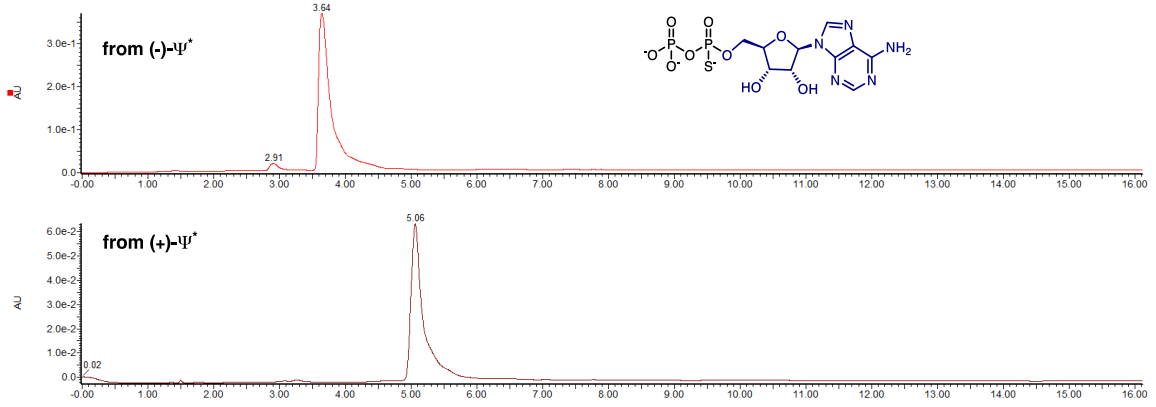


Figure S3. Comparison of chromatographic mobility for the obtained epimers of α-thio-ADP.

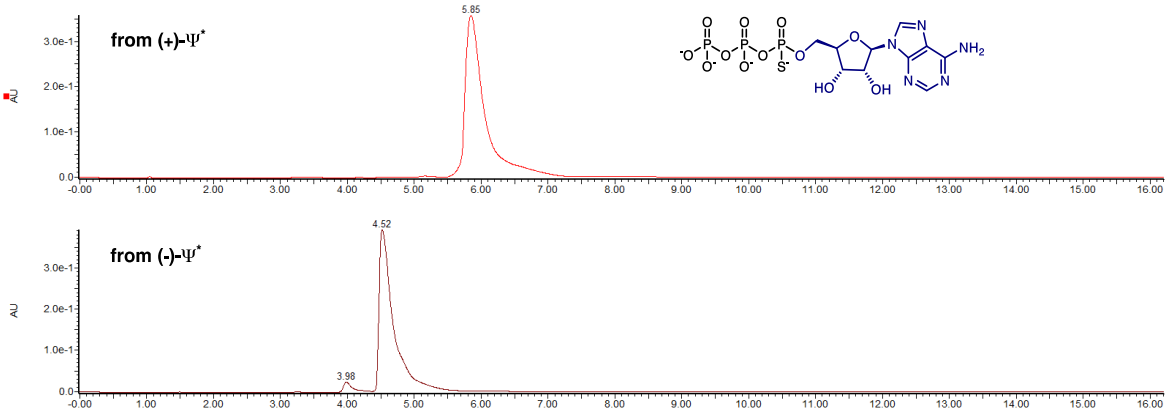


Figure S4. Comparison of chromatographic mobility for the obtained epimers of α-thio-ATP.

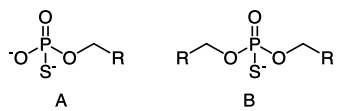
4.12. Isolation and Purification Tips

General

1. Organic-soluble impurities should be removed before ion-exchange chromatography, by precipitation of the crude product as a sodium salt from 0.2 M $NaClO_4$ solution in acetone. Otherwise, the product will likely coelute with organic impurities (e.g. 4-thiopentafloropyridine salts, DBU salts). The exception from this are guanosine derivatives, which display low solubility in water and their sodium salts are difficult to redissolve after precipitation.
2. In case of cytidine and guanosine derivatives it is crucial to adjust the pH of the solution to ~7, before loading on the DEAE Sephadex resin. We have observed that if basic solutions (pH ≥ 8) of these derivatives are loaded on the ion-exchange resin part of the product is eluted at the dead volume of the column.
3. We have observed that removal of the residual ammonium bicarbonate buffer (after ion-exchange chromatography) by multiple lyophilization leads to deterioration of the product purity due to partial hydrolysis. In our experience, it is better to decompose NH_4HCO_3 by multiple evaporation with water under reduced pressure (temp. of the water bath ≤ 40 °C) and then remove residual water by single lyophilization.
4. Removal of water by evaporation under reduced pressure should be performed at temperature ≤ 40 °C, to minimize deterioration of the product purity due to hydrolysis.
5. It is highly advised to perform ion-exchange and reverse-phase chromatography using automated chromatography system. In case of our work all separations were performed using Teledyne ISCO CombiFlash NextGen 300+. Ion-exchange chromatography DEAE Spehadex A-25 columns were prepared and loaded manually (*see below for details and graphical guide*). For reverse-phase chromatography we used prepacked Teledyne ISCO RediSep Gold® cartridges.

Nucleoside α-thiodiphosphates

1. Major impurities in the synthesis of nucleoside α-thiodiphosphates are thiophosphate **A**, resulting from the hydrolysis of phosphate anhydride bond in the product, and dimer **B** originating from double addition of nucleoside to the Ψ reagent.



2. Most of the nucleoside α-thiodiphosphates can be purified by ion-exchange chromatography on DEAE Sephadex (7 x 3 cm on 0.2 mmol scale) using 1M NH_4HCO_3/water. Impurities **A** and **B** are usually

eluted below or at 15:85 1 M NH_4HCO_3/water and the product is eluted at 20:80 1 M NH_4HCO_3/water (or higher for cytidine and guanosine derivatives) (*see below for representative chromatogram*).

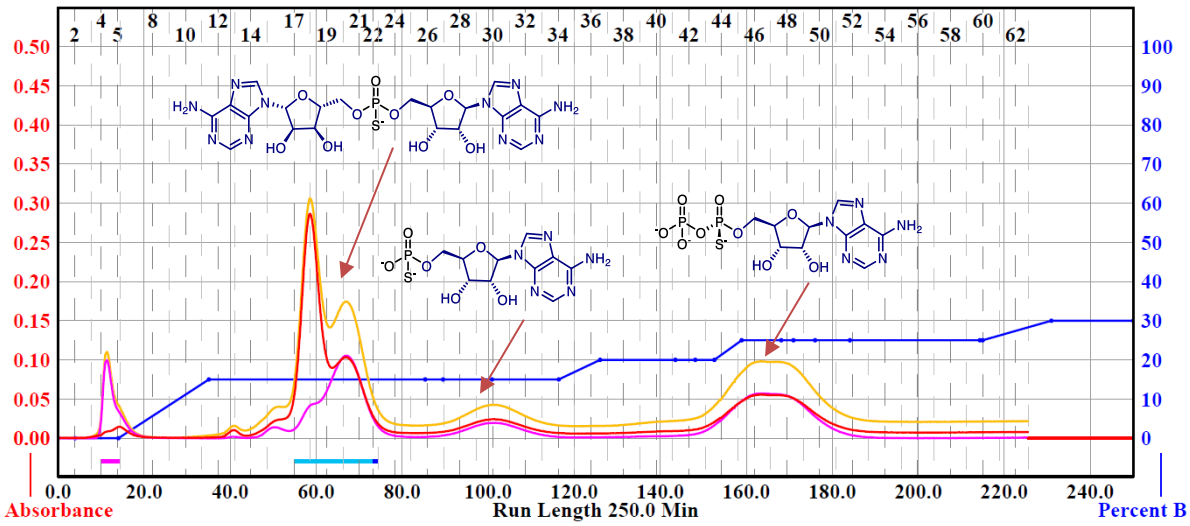
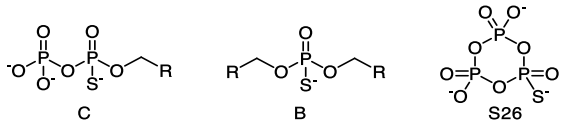


Figure S5. Purification of α-thio-ADP (isomer S_P) by ion exchange chromatography on DEAE Sephadex. The above separation was performed using Teledyne ISCO CombiFlash NextGen 300+ (solvent A: water; solvent B: 1 M aq. NH_4HCO_3). Legend: Red – absorbance at 214 nm; Pink – absorbance at 254 nm; Yellow – total absorbance at a range from 200 to 300 nm; Blue – elution gradient in % of solvent B.

Nucleoside α-thiotriphosphates

1. Major impurities in the synthesis of nucleoside α-thiotriphosphates are α-thiodiphosphates **C**, resulting from the hydrolysis of phosphate anhydride bond in the product, dimer **B** originating from double addition of nucleoside to the Ψ reagent, and cyclic compound **S26** coming from intramolecular ring closure during the phosphate transfer step.



2. Most of the nucleoside α-thiotriphosphates can be purified by ion-exchange chromatography on DEAE Sephadex (7 x 3 cm on 0.2 mmol scale) using 1M NH_4HCO_3/water. Impurities **B** and **C** are usually eluted below or at 20:80 1 M NH_4HCO_3/water and the product is eluted at 25:75 1 M NH_4HCO_3/water (or higher for cytidine and guanosine derivatives) (*see below for representative chromatogram*).
3. It is possible that the product may be contaminated with cyclic byproduct **S26** even after ion-exchange chromatography. In that case the mixture can be easily separated by reverse phase chromatography using gradient 1 M aqueous triethylammonium acetate (TEAA)/MeCN from 100:0 to 95:5. (*Note: it is not advised to concentrate the solution containing TEAA buffer under reduced pressure, as it may lead to decomposition of the product due to formation of acetic acid.*).

4. After reverse phase chromatography TEAA buffer can be removed by lyophilization and the product can be stored as a TEAA salt or converted into: ammonium salt by ion exchange chromatography; or sodium salt by precipitation from 0.2 M $NaClO_4$ in acetone.

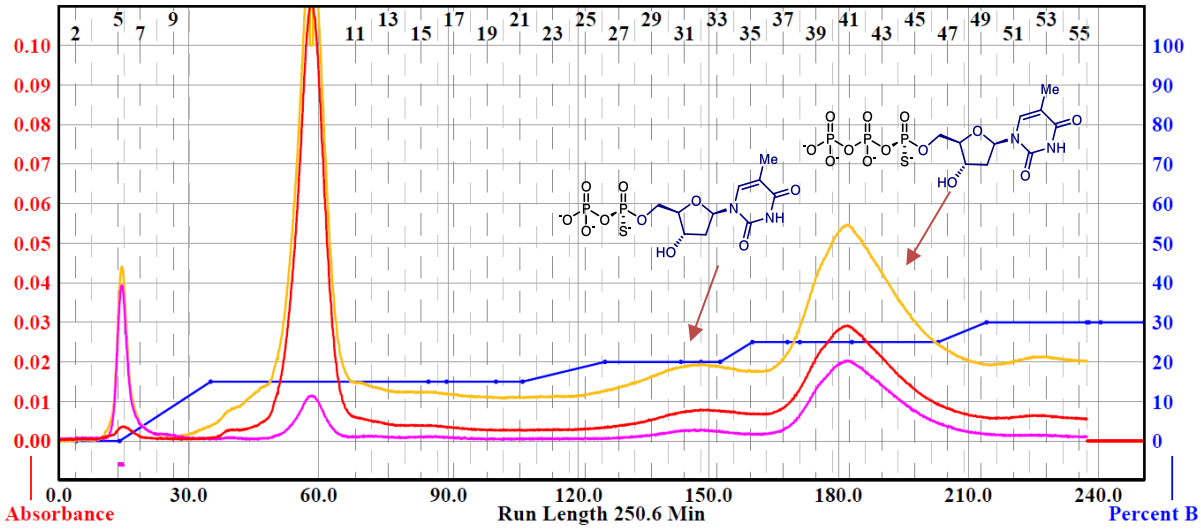
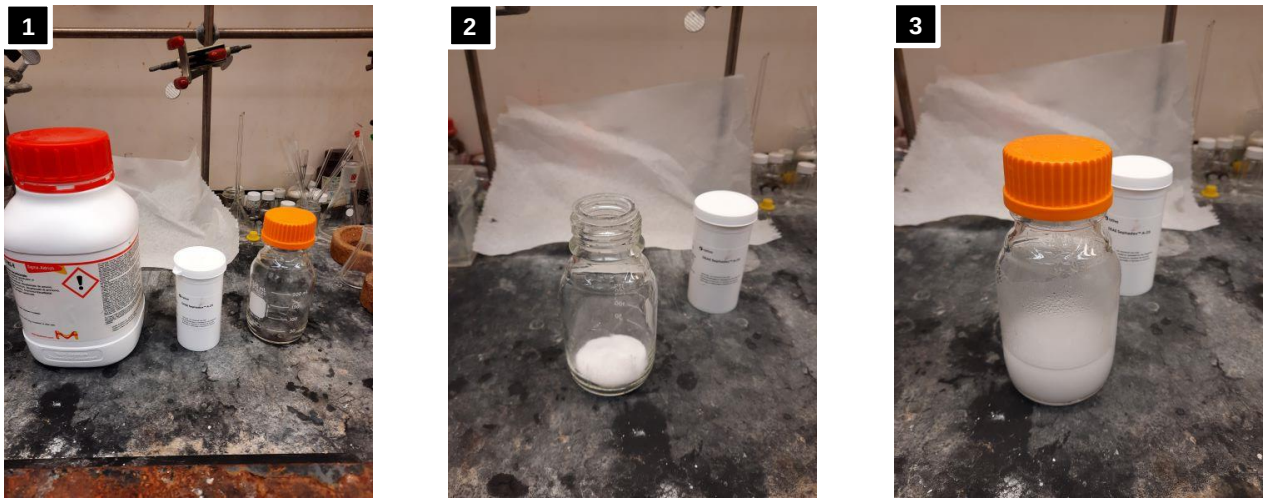


Figure S6. Purification of α-thio-TTP (isomer R_P) by ion exchange chromatography on DEAE Sephadex. The above separation was performed using Teledyne ISCO CombiFlash NextGen 300+ (solvent A: water; solvent B: 1 M aq. NH_4HCO_3). Legend: Red – absorbance at 214 nm; Pink – absorbance at 254 nm; Yellow – total absorbance at a range from 200 to 300 nm; Blue – elution gradient in % of solvent B.

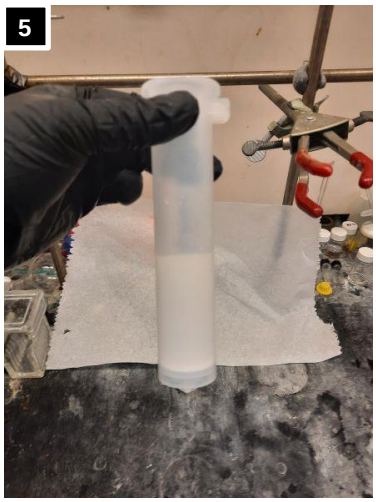
Beginner Guide for the Ion-Exchange Chromatography on DEAE-Sephadex

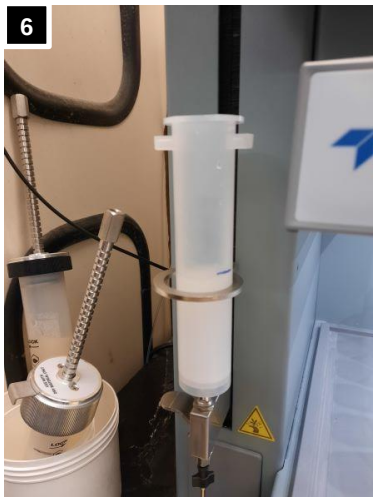


1: Left: ammonium bicarbonate; Center: Cytvia DEAE Sephadex A-25 resin; Right: empty 250 mL bottle.

2: 250 mL bottle charged with DEAE Sephadex resin.

3: The resin is suspended in 1M ammonium bicarbonate aqueous solution and left overnight in a fridge (at ~ 4 °C).

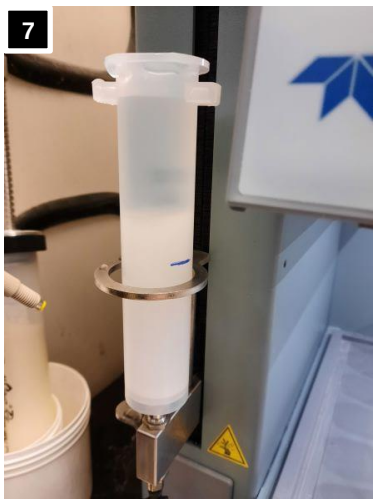


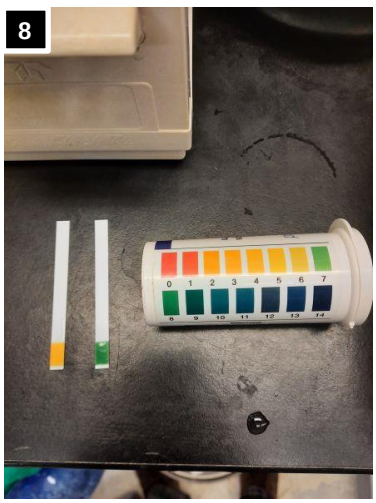


4: Empty plastic cartridge (14 x 3 cm) with a filter frit.

5: Cartridge is loaded with Sephadex resin to about half of its volume (~ 7 cm high). *Note: for large scale purifications bigger cartridges should be used e.g. for 2 mmol scale purification we have used 15 x 4 cm resin bed.*

6: The cartridge is installed in the automated chromatography system and the resin is flushed with 250 mL of deionized water (flow rate = 6 mL/min). *Note: This step can be also performed manually by allowing deionized water to pass gravitationally through the resin, however it takes significantly more time.*

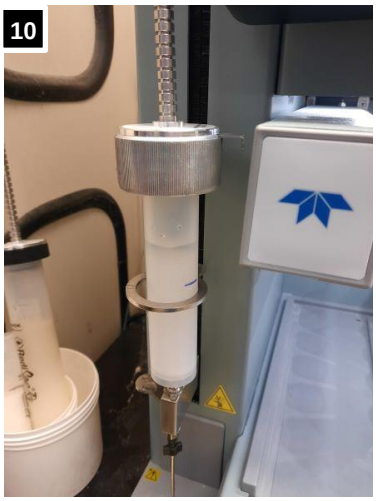


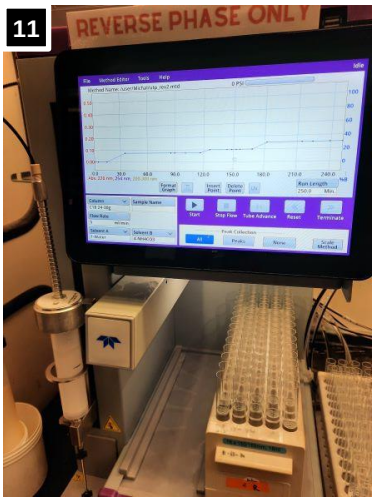


7: Sephadex resin after the water wash (*note that the volume of the resin expanded*).

8: After completed water wash the eluate should have neutral pH. Left pH test strip: after water wash pH ~ 6; right pH test strip: before water wash pH ~ 8.

9: The residual water is decanted, and the sample is loaded on the top of the resin via pipette. After the sample solution is absorbed by the resin, a layer of deionized water (~ 1 cm high) is loaded on the top of the resin.





10: The loaded cartridge is installed in an automated chromatography system and the separation is performed using gradient of 1M NH_4HCO_3 (stronger eluent) and deionized water (weaker eluent) (flow rate: 5 mL/min). *Note: although it is possible to perform ion exchange chromatography manually, the separation of α-thio-NDPs and NTPs usually require precise control of the eluent gradient over long time (few hours). Therefore, for the sake of practicality and simplicity we strongly recommend using automated chromatography system.*

11: After the separation, resin can be regenerated by flushing with 200 mL of 1M NH_4HCO_3, followed by 250 mL of water.

4.13. Troubleshooting and FAQ

Q: *How can I monitor the reaction?*

A: The progress of the reaction can be monitored by ^{31}P NMR (*see section 6 for representative NMR spectra*). Deprotection step can be monitored via RP-HPLC.

Q: *How can I purify my product?*

A: α-Thio-NDPs and NTPs are highly polar, anionic compounds, therefore preferred purification technique is ion-exchange chromatography using DEAE Sephadex as a stationary phase and aqueous ammonium bicarbonate as mobile phase. Additionally, in more difficult cases (e.g. guanosine derivatives) ion-exchange purification, can be followed by reverse-phase chromatography on C18 silica gel to afford analytically pure product (*for more details regarding isolation and purification of products see section 4.12*).

Q: *Can I use a different solvent for the reaction?*

A: We successfully performed the reaction in anhydrous MeCN, DMF and DMSO. Based on the optimization, the formation of the phosphate transfer reagent can be also performed in THF or DCM, however the choice of solvent for the subsequent phosphate transfer step is limited by the solubility of the protected nucleoside.

Q: *Can I use a different base for the reaction?*

A: Based on the optimization, formation of the phosphate transfer reagent can be also performed using tertiary amines (i.e. triethylamine, DIPEA), however addition of DBU is essential for the subsequent phosphate transfer step. Moreover, when using diphosphate donor **9c**, addition of DBU during the formation of the phosphate transfer reagent facilitates removal of 9-fluorenylmethyl protecting groups.

Q: *Can I leave the reaction running for a longer time?*

A: For the formation of the phosphate transfer reagent may be prolonged to ~1 h, however running this step longer is not recommended, as this may lead to decreased yield due to hydrolysis. In case of the subsequent steps no significant problem should arise from prolongation of the reaction unless compound is base sensitive.

Q: *How do you store Ψ* reagent and phosphate donors? Do any special precautions need to be taken during handling of these reagent?*

A: The Ψ* reagents and monophosphate donor **8g** are non-hygroscopic and bench-stable, and no special precautions need to be taken during handling of theses reagents. Pyrophosphate donor **9c** can be stored for short time at room temperature and is infinitely stable if stored at -20 °C. This reagent can be weighted out on air without any special precautions.

Q: *Is the reaction moisture or air sensitive?*

A: The reaction is not air sensitive, however, to obtain the product with optimal yield, precautions need to be taken to exclude moisture. Standard reaction setup includes the use of oven-dried glassware, inert atmosphere, anhydrous solvent, and addition of 3 Å molecular sieves.

Q: *Can I isolate any of the phosphate transfer reagents?*

A: Due to low stability of these reagents we were unable to isolate any of them in a pure form, therefore it is recommended to generate these compounds *in situ*.

Q: *My nucleoside is very precious. Can I run the reaction using it as a limiting reagent?*

A: In case of the stereocontrolled synthesis of α-thiodiphosphates it is possible to run the reaction with reversed stoichiometry with only slight decrease in the product yield (*see section 4.3 for representative example*). In example compounds **34** and **35** were obtained using nucleoside as limiting reagent (*for details see section 8.3*). In case of α-thiotriphosphates reaction using nucleoside as a limiting reagent under unoptimized conditions provide a product in diminished, but still workable yield (~ 20-30% decrease in isolated yield) (*see section 4.7 for representative example*).

Q: *My yield is low; how can I optimize the reaction for my substrate?*

A: Usually the yield of the reaction can be significantly affected either by hydrolysis of Ψ* reagent or incomplete addition of alcohol to the phosphate transfer reagent. Monitoring of the reaction by ^{31}P NMR can help to determine the source of the problem. If hydrolysis is the cause of a low yield, it is important to exclude any source of moisture, including reagents. In the case if slow addition of alcohol to the phosphate transfer reagent is causing problems, increasing the amount of the second portion of DBU usually lead to increased yield.

Q: *How can I store nucleoside α-diphosphorothioates and α-triphosphorothioates?*

A: Ammonium or triethylammonium salts of nucleoside α-thio-NDPs and NTPs can be stored as solids at -20 °C under argon atmosphere for several months without any appreciable loss of purity. We have observed that generally ammonium salts of these compounds are more resistant to hydrolysis than sodium salts. Therefore, for long-term storage it is recommended to convert the final product into ammonium form. Aqueous solutions of nucleoside α-thio-NDPs and NTPs are stable only for short amount of time (< 24 h) if stored at -20 °C. For prolonged storage in solution, it is necessary to add buffer (e.g. 1M Tris buffer) to minimize deterioration of purity due to the hydrolysis.

Q: *Can I run the reaction with unprotected nucleoside? Which protecting groups are compatible with the reaction?*

A: Reaction with unprotected nucleoside is not recommended as it leads to a complex mixture resulting from phosphorylation of all the available hydroxyl groups. On the other hand, the phosphate transfer reagents **1** and **2** are highly selective for oxygen nucleophiles and protection of amine groups (e.g. in guanosine, adenosine, cytosine) is not necessary. Final products are relatively stable in the range of pH from 2.5 to 10, therefore any protecting groups that are resistant to base used in the reaction (DBU), but can be later removed under basic or mildly acidic conditions, are most likely to be compatible with the reaction. In our experience, protection of hydroxyl groups as benzoyl esters is preferred as it also increases solubility of a nucleosides in organic solvents, which is beneficial for the reaction. We have also successfully performed the reaction using acetyl ester, TBS, DMTr and methylorthoformate protecting groups.

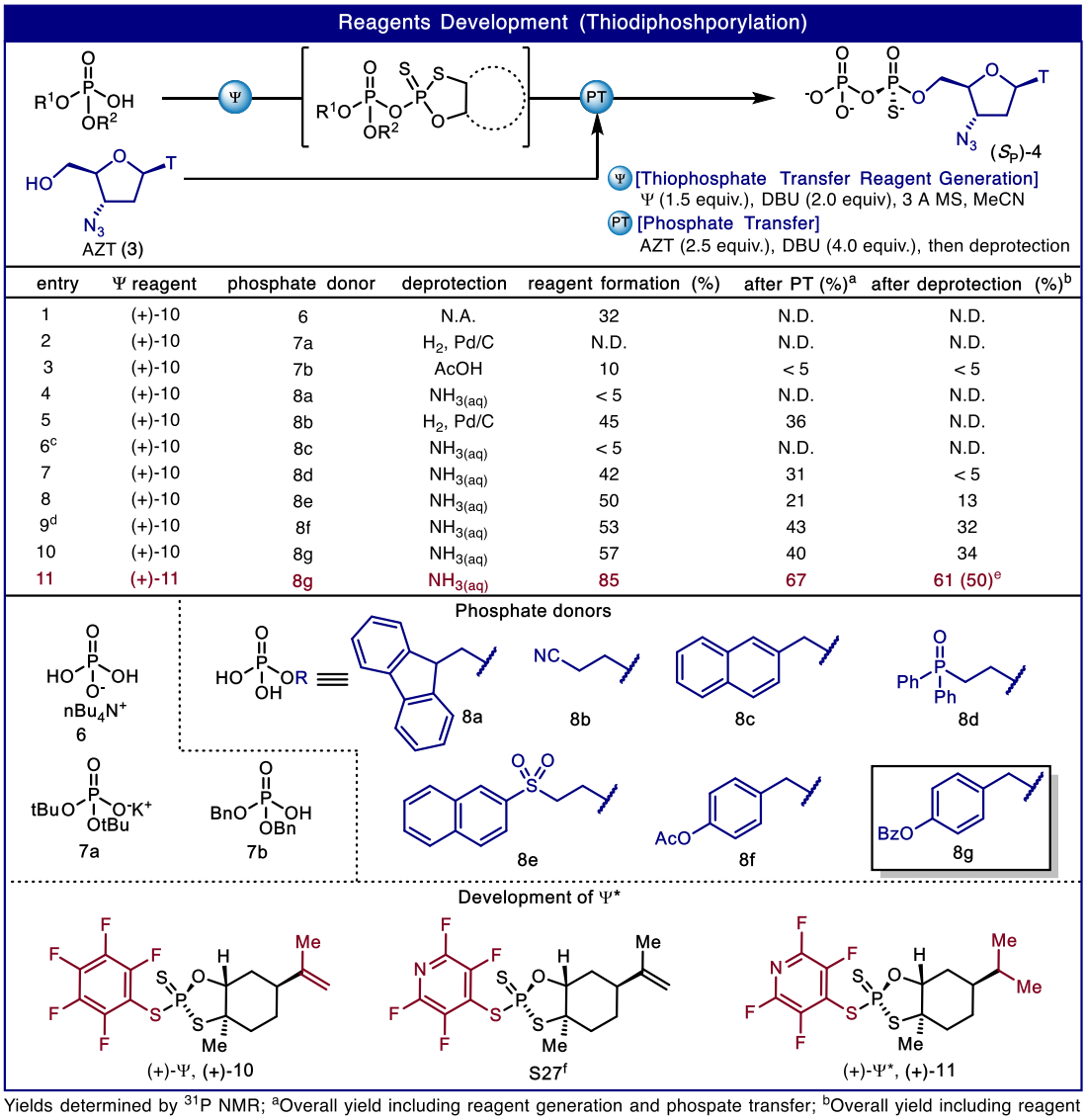
Q: *What are the current limitations of the method?*

A: The limitations of this method stem from lower nucleophilicity of sterically hindered substrates. In example the reaction does not work with tertiary alcohols, and in case of neopentyl-type nucleosides yields are usually < 10%. The reaction also does not work well with phenols, likely due to increased susceptibility of the product to hydrolysis.

Q: *Can I use commercially available Ψ reagent (**10**) instead of Ψ* (**11**)?*

A: The reactions work with commercially available Ψ reagent (**10**), albeit providing products in lower yields as compared with the new reagent **11**. Moreover, the biggest advantage of the new reagent is much cleaner reaction profile, which greatly simplifies purification of products (*for comparison of crude NMRs using Ψ and Ψ* reagents see section 5*).

5. Reagent Development



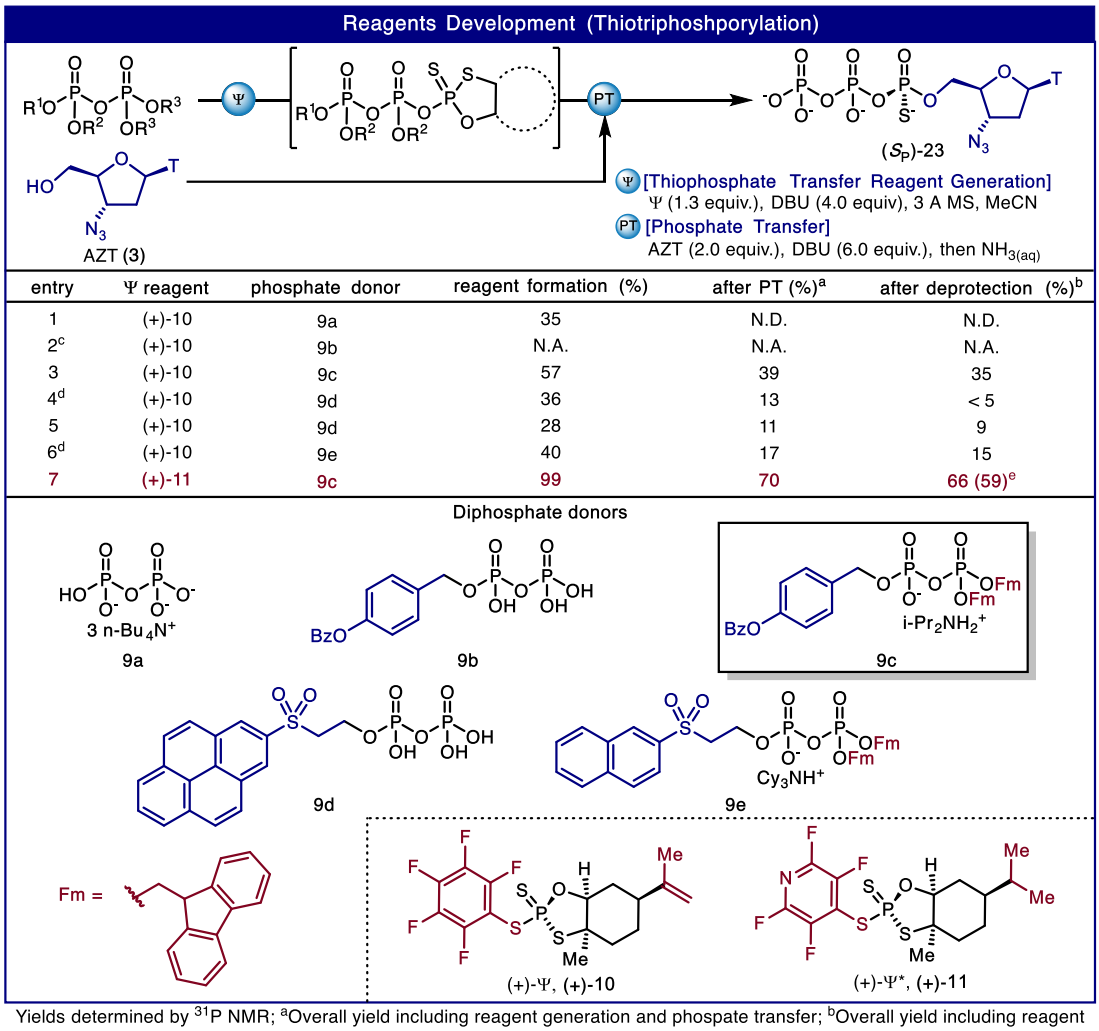
entry	Ψ reagent	phosphate donor	deprotection	reagent formation (%)	after PT (%)[a]	after deprotection (%)[b]
1	(+)-10	6	N.A.	32	N.D.	N.D.
2	(+)-10	7a	H_2, Pd/C	N.D.	N.D.	N.D.
3	(+)-10	7b	AcOH	10	< 5	< 5
4	(+)-10	8a	$NH_{3(aq)}$	< 5	N.D.	N.D.
5	(+)-10	8b	H_2, Pd/C	45	36	N.D.
6[c]	(+)-10	8c	$NH_{3(aq)}$	< 5	N.D.	N.D.
7	(+)-10	8d	$NH_{3(aq)}$	42	31	< 5
8	(+)-10	8e	$NH_{3(aq)}$	50	21	13
9[d]	(+)-10	8f	$NH_{3(aq)}$	53	43	32
10	(+)-10	8g	$NH_{3(aq)}$	57	40	34
11	(+)-11	8g	$NH_{3(aq)}$	85	67	61 (50)[e]

Yields determined by ^{31}P NMR; [a]Overall yield including reagent generation and phospate transfer; [b]Overall yield including reagent generation, phosphate transfer and deprotecion; [c]Used as dipyridinium salt; [d]Used as triethylammonium salt; [e]Overall isolated yield; [f]Difficult to isolate as a pure enantiomer

Although the NMR yields obtained using either **8f** or **8g** are similar, from the standpoint of practicality, we have chosen compound **8g**. Monophosphate **8f** is moisture-sensitive, viscous oil, while benzoylated derivative **8g** is bench-stable, non-hygroscopic solid.

Employment of newly developed Ψ* reagent **11** (bearing more reactive leaving group) resulted in cleaner reaction with weak nucleophiles providing product in higher yield and significantly facilitating purification (*see below for comparison of crude NMR profiles*).

Reduction of the double bond in the limonene backbone is crucial to facilitate isolation of compound **11** as a pure diastereoisomer. Derivative **S27** shows significantly higher solubility in MeOH and was difficult to separate from diastereomeric impurities even after multiple crystallizations.



entry	Ψ reagent	phosphate donor	reagent formation (%)	after PT (%)[a]	after deprotection (%)[b]
1	(+)-10	9a	35	N.D.	N.D.
2[c]	(+)-10	9b	N.A.	N.A.	N.A.
3	(+)-10	9c	57	39	35
4[d]	(+)-10	9d	36	13	< 5
5	(+)-10	9d	28	11	9
6[d]	(+)-10	9e	40	17	15
7	(+)-11	9c	99	70	66 (59)[e]

Yields determined by ^{31}P NMR; [a]Overall yield including reagent generation and phospate transfer; [b]Overall yield including reagent generation, phosphate transfer and deprotecion; [c]Not isolated in pure form; [d]2.0 equiv. of Ψ used. [e]Overall isolated yield.

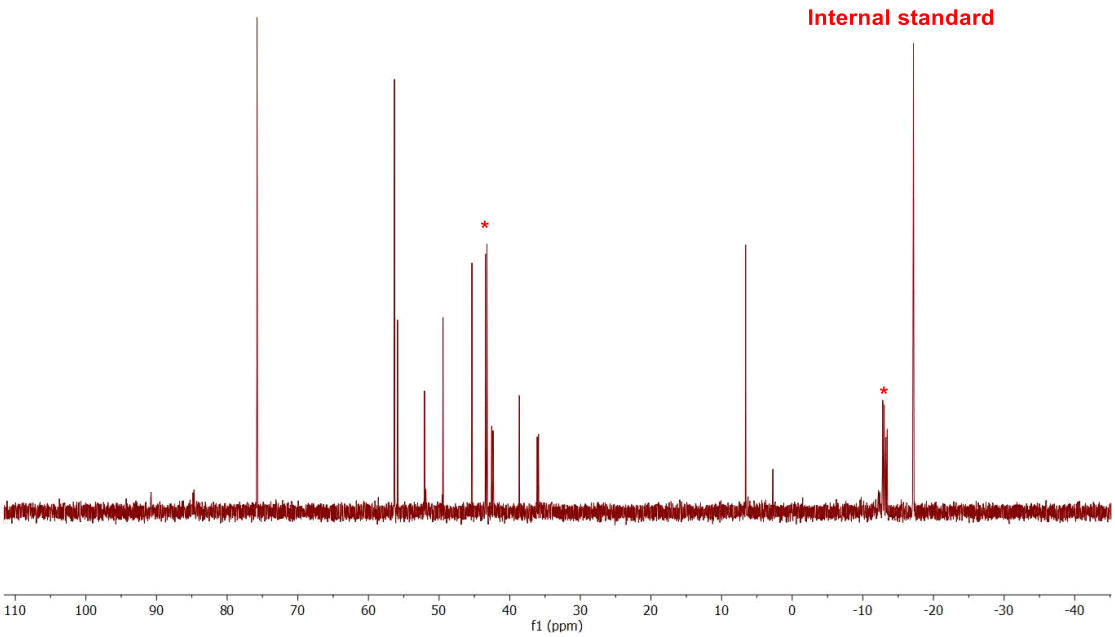


Figure S7. Representative ^{31}P NMR (162 MHz) spectrum of a crude reaction mixture (thiodiphosphorylation) using previously developed Ψ reagent **10** (product peaks marked by *).

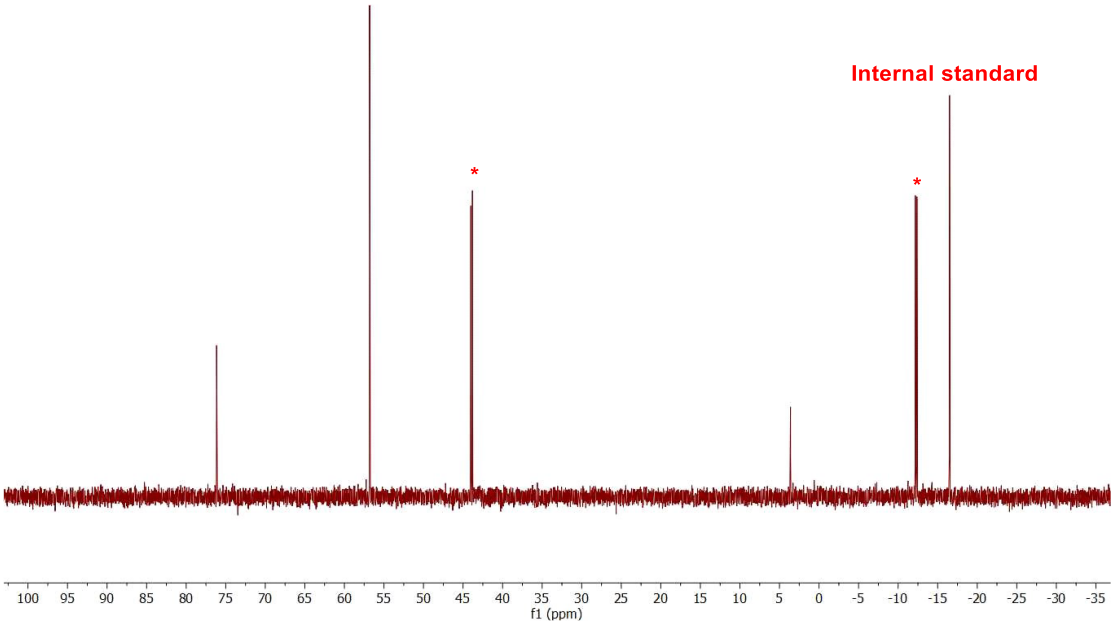
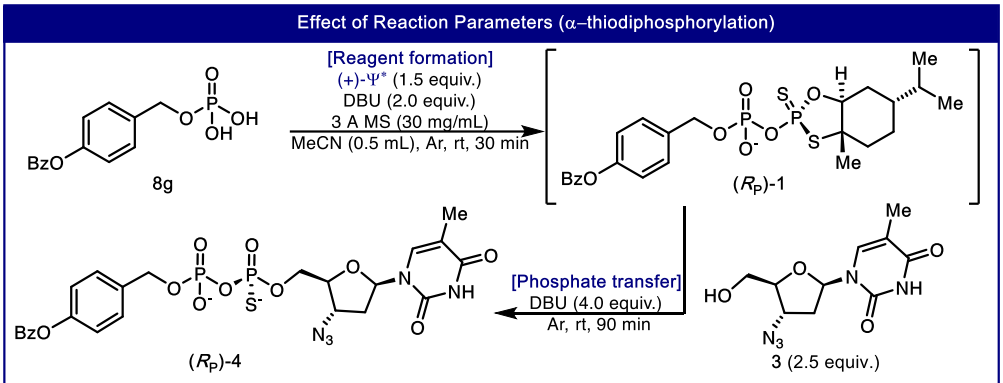


Figure S8. Representative ^{31}P NMR (162 MHz) spectrum of a crude reaction mixture (thiodiphosphorylation) using new Ψ* reagent **11** (product peaks marked by *).

6. Optimization details

All optimization reactions were carried out on a 0.05 mmol scale. The crude reaction mixture was analyzed by quantitative ^{31}P NMR.

Table S2. Optimization of the stereocontrolled synthesis of α-thiodiphosphates.



Reagent formation			Phosphate transfer		
entry	deviation from above	yield (%)[a]	entry	deviation from above	overall yield (%)[a]
1	none	**85**	1	none	67 (**53**)[b]
2	2.0 equiv. of Ψ*	84	2	3.0 equiv. of nucleoside	68
3	1.3 equiv. of Ψ*	73	3	2.0 equiv. of nucleoside	59
4	1.0 equiv. of Ψ*	46	4	1.5 equiv. of nucleoside	44
5	3.0 equiv. of DBU	83	5	3.0 equiv. of DBU	56
6	1.5 equiv. of DBU	55	6	2.5 equiv. of DBU	34
7	TEA instead of DBU	74	7	TEA instead of DBU	N.D.
8	*N*-methylimidazole instead of DBU	8	8	*N*-methylimidazole instead of DBU	N.D.
9	DABCO instead of DBU	25	9	DABCO instead of DBU	<5
10	DCM instead of MeCN	83	10	DCM instead of MeCN	8
11	THF instead of MeCN	76	11	THF instead of MeCN	<5
12	DMF instead of DCM	86	12	DMF instead of MeCN	59
13	*c* of **8g** = 0.20 M	85	13	time = 120 min	65
14	*c* of **8g** = 0.05 M	84	14	time = 60 min	60
15	time = 15 min	37	15	time = 30 min	32
16	time = 60 min	85	16	no 3 A MS	49
17	no 3 A MS	63	17	no DBU	N.D.
18	no DBU	N.D.	18	non-anhydrous MeCN	44
19	non-anhydrous MeCN	57	19	under air	56
20	under air	79	20	reversed stoichiometry[c]	65

[a]Yields determined by ^{31}P NMR; [b]Overall isolated yield after deprotection ($NH_{3(aq)}$, rt, 16h). [c]Monophosphate donor **8g** (3.0 equiv.), Ψ* reagent (2.0 equiv.), nucleoside **3** (1.0 equiv.).

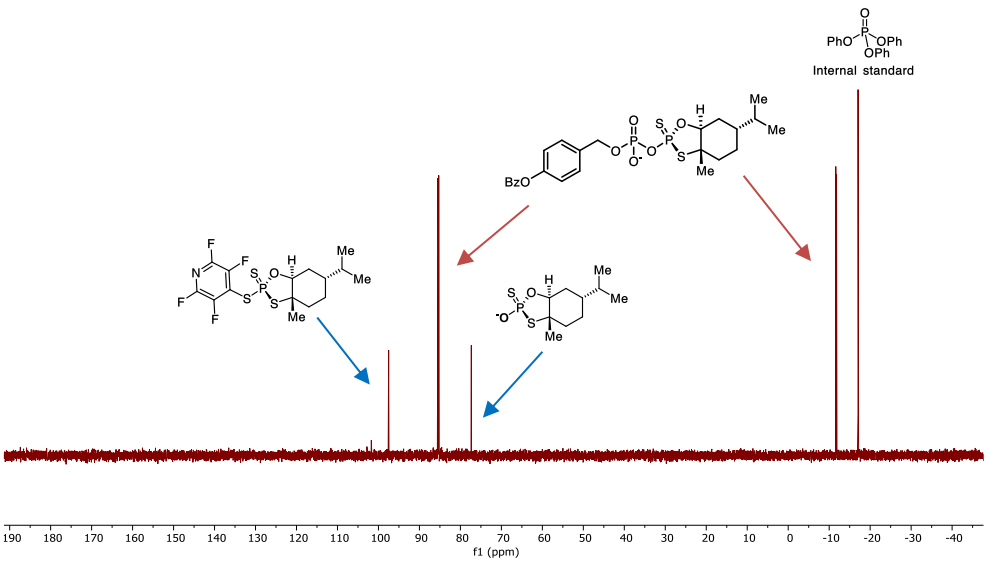


Figure S9. Representative ^{31}P NMR of the thiophosphate transfer reagent formation step (thiodiphosphorylation)

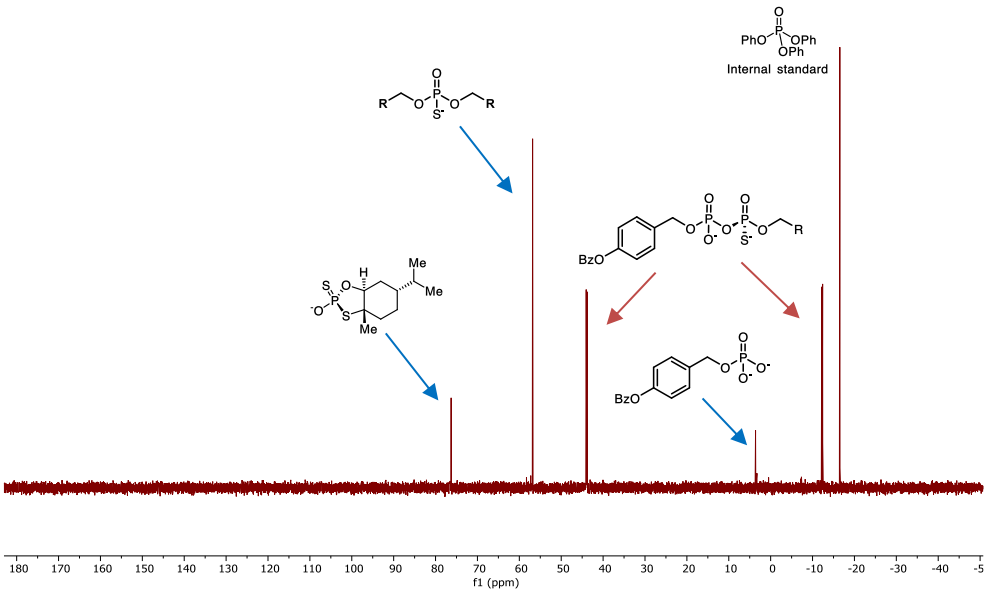


Figure S10. Representative ^{31}P NMR of the phosphate transfer step (thiodiphosphorylation; before deprotection).

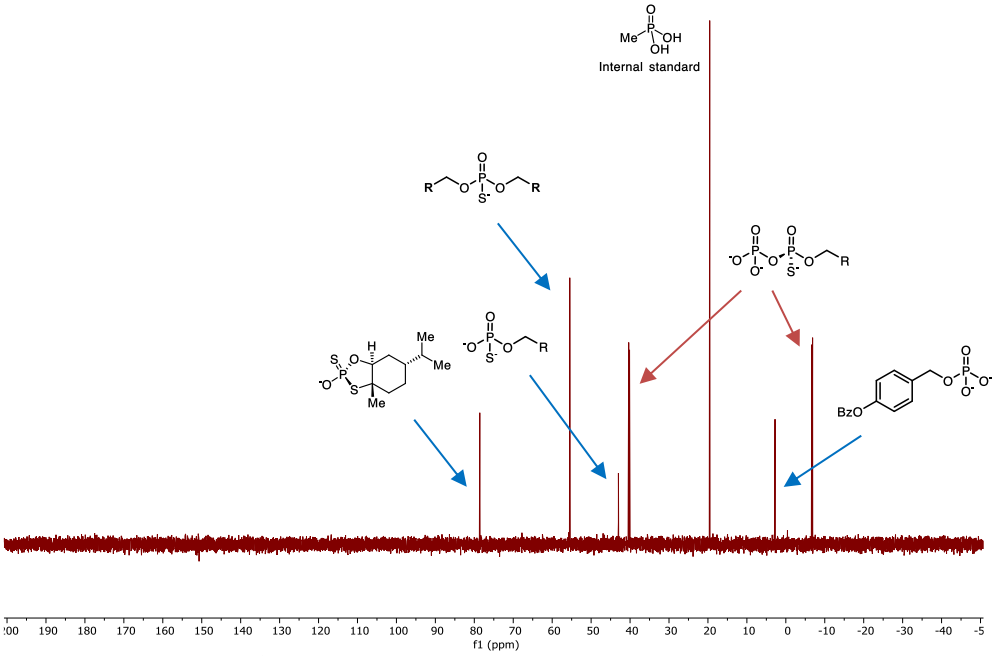
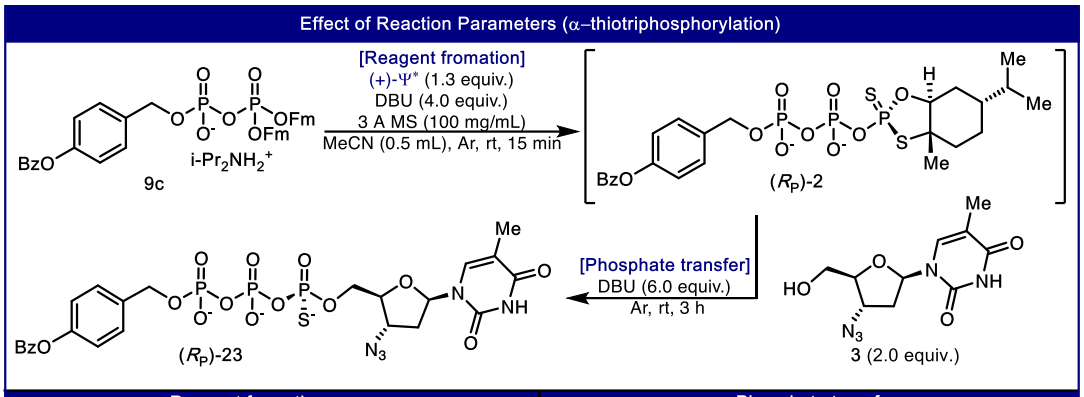


Figure S11. Representative ^{31}P NMR of the phosphate transfer step (thiodiphosphorylation; after deprotection).

Table S3. Optimization of the stereocontrolled synthesis of α-thiotriphosphates.



Reagent formation			Phosphate transfer		
entry	deviation from above	yield (%)[a]	entry	deviation from above	overall yield (%)[a]
1	none	99	1	none	70 (59)[b]
2	1.5 equiv. of Ψ*	99	2	2.5 equiv. of nucleoside	71
3	1.1 equiv. of Ψ*	86	3	1.5 equiv. of nucleoside	53
4	5.0 equiv. of DBU	99	4	8.0 equiv. of DBU	70
5	3.0 equiv. of DBU	48	5	4.0 equiv. of DBU	62
6	TEA instead of DBU	<5	6	TEA instead of DBU	N.D
7	*N*-methylimidazole instead of DBU	N.D.	7	*N*-methylimidazole instead of DBU	N.D.
8	DMF instead of MeCN	96	8	DMF instead of MeCN	60
9	DMSO instead of MeCN	90	9	DMSO instead of MeCN	62
10	3 A MS (50 mg/mL)	90	10	no 3 A MS	42
11	no 3 A MS	84	11	no DBU	N.D.
12	no DBU	N.D.	12	non-anhydrous MeCN	39
13	non-anhydrous MeCN	65	13	under air	65
14	under air	94	14	reversed stoichiometry[c]	42

[a]Yields determined by ^{31}P NMR; [b]Overall isolated yield after deprotection ($NH_{3(aq)}$, rt, 16h). [c]Diphosphate donor **9c** (2.0 equiv.), Ψ* reagent (2.0 equiv.), nucleoside **3** (1.0 equiv.).

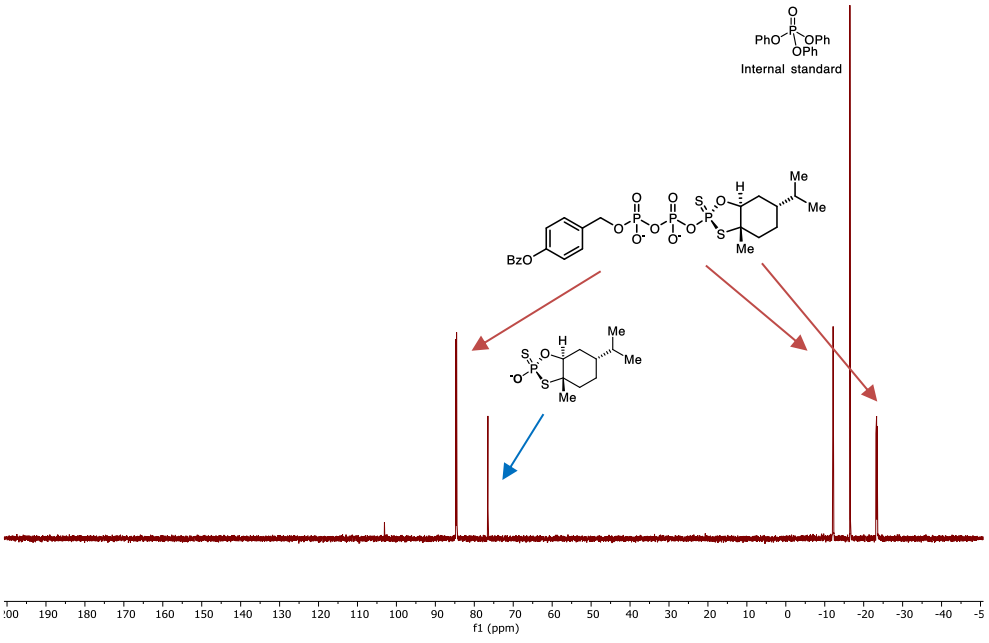


Figure S12. Representative ^{31}P NMR of the thiophosphate transfer reagent formation step (thiotriphosphorylation).

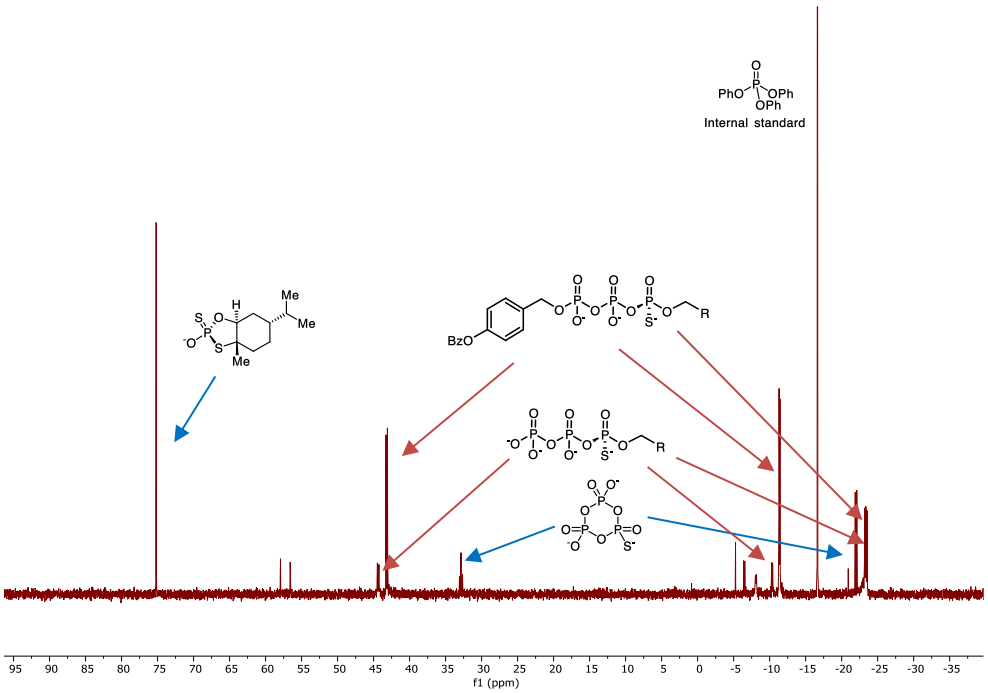


Figure S13. Representative ^{31}P NMR of the phosphate transfer step (thiotriphosphorylation; before deprotection) (*Note: small amount of unprotected product already formed during the phosphate transfer step*).

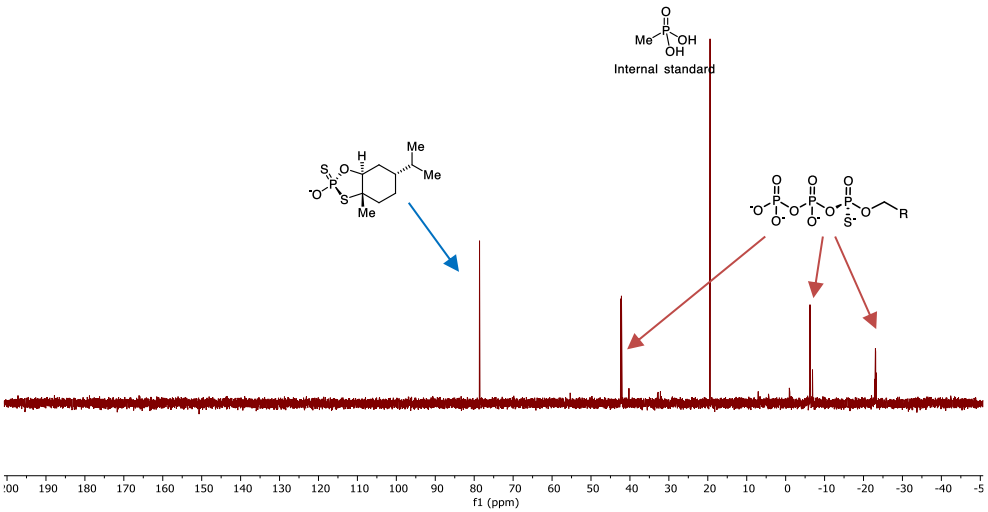


Figure S14. Representative ^{31}P NMR of the phosphate transfer step (thiotriphosphorylation; after deprotection).

Quantitative ^{31}P NMR details

The NMR yields were determined based on measurement of the relative concentration of the product and internal standard: triphenyl phosphate (>99% purity, Sigma-Aldrich) for measurements in organic solvents or methylphosphonic acid (99% purity, Sigma-Aldrich) for experiments in aqueous solutions. In a typical experiment, accurately weighted amount of the internal standard (usually ~ 0.05 mmol) was added to the crude reaction mixture. After 2 min of stirring an aliquot of the solution was transferred to an NMR tube. The NMR spectrum was recorded on a Bruker 400 MHz instrument using 1D-pulse sequence with inverse gated decoupling and following measurement parameters: pulse width p1 = 7.45 μs; relaxation delay d1 = 30 s; number of scans ns = 8; dummy scans ds = 0. The relative concentration of the product and the internal standard was determined by comparing integrals of their respective signals.

The NMR yield was calculated using following formula:

$$Y_{NMR} = (I_{prod} \bullet n_{sub})/(I_{is} \bullet n_{is})$$

Y_{NMR} – NMR yield [%]

I_{prod} – product signal integral

I_{is} – internal standard signal integral

n_{sub} – amount of the limiting reagent used in the reaction [mmol]

n_{is} – amount of internal standard added to the crude reaction mixture [mmol]

7. HPLC methods

Analytical method

HPLC analyses were conducted on a Waters Autopurification LC with a Waters XBridge C18 column (4.6x150 mm, 3.5 µm).

Solvent A: 0.1 M triethylammonium acetate (TEAA) in H_2O
Solvent B: MeCN
Flow rate: 1.5 mL/min
Temperature: 25 °C

Table S4. HPLC method gradient (*Method 1*).

time (min)	Solvent A (%)	Solvent B (%)
0	98	2
15	92	8
17	5	90

Table S5. HPLC method gradient (*Method 2*).

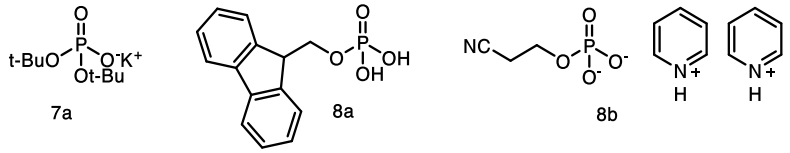
time (min)	Solvent A (%)	Solvent B (%)
0	99	1
4	99	1
15	95	5
17	5	90

Table S6. HPLC method gradient (*Method 3*).

time (min)	Solvent A (%)	Solvent B (%)
0	99	1
4	99	1
15	92	5
17	5	90

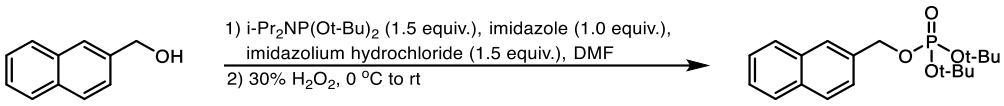
8. Experimental Procedures and Characterization Data

8.1. Preparation of Phosphate Donors



Compounds **7a**,[10] **8a**,[11] and **8b**[12] were prepared following literature procedures.

Compound S29



S28 **S29**

An oven-dried 100 mL round-bottom flask was charged with 2-naphthalenemethanol (**S28**) (3.16 g, 20 mmol, 1.0 equiv.), imidazolium hydrochloride (3.15 g, 30 mmol, 1.5 equiv.) and imidazole (1.37 g, 20 mmol, 1.0 equiv.). The mixture was dissolved in anhydrous DMF (20 mL) and i-$Pr_2NP(Ot\text{-}Bu)_2$ (9.5 mL, 30 mmol, 1.5 equiv.) was added dropwise over 5 min. The reaction was stirred for 1 h at room temperature under argon atmosphere. Subsequently, the mixture was cooled to 0 °C, followed by addition of 30% aq. H_2O_2 (5.7 mL), and the reaction warmed to room temperature over 1 h. The reaction mixture was cooled to 0 °C and quenched by addition of saturated aq. $Na_2S_2O_3$ (10 mL). The resulting solution was extracted with EtOAc. The organic layer was washed with brine (2 times), dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude residue was purified by silica gel chromatography (EtOAc/Hexanes/DCM; 5:45:50) to provide 5.54 g of compound **S29** (**Yield = 79%**).

Physical state: colorless oil

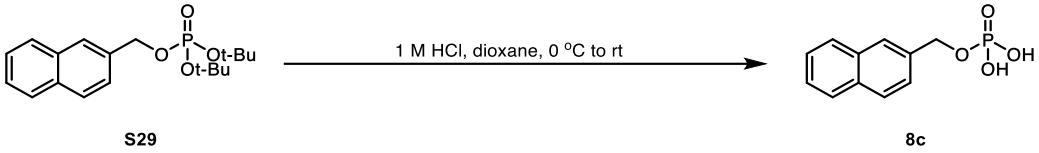
^{1}H NMR (600 MHz, $CDCl_3$) δ 7.85-7.82 (m, 4H), 7.52-7.46 (m, 3H), 5.17 (d, *J* = 7.3 Hz, 2H), 1.49 (s, 18H).

^{13}C NMR (150 MHz, $CDCl_3$) δ 134.4 (d, *J* = 7.9 Hz), 133.3, 133.2, 128.3, 128.1, 127.8, 126.7, 126.34, 126.25, 125.6, 82.6 (d, *J* = 7.5 Hz), 68.6 (d, *J* = 5.8 Hz), 30.0 (d, *J* = 4.3 Hz).

^{31}P NMR (162 MHz, $CDCl_3$) δ -9.7.

HRMS (ESI-TOF) m/z: calculated for $C_{19}H_{27}NaO_4P$ $[M+Na]^+$: 373.1539, found: 373.1554.

Compound 8c



S29 **8c**

An oven-dried 100 mL round-bottom flask was charged with compound **S29** (5.50 g, 15.7 mmol, 1.0 equiv.). The reaction vessel was submerged in ice/water bath and 1 M HCl in dioxane (50 mL) was added over 5 min. The reaction warmed to room temperature and stirred for 4 h under argon atmosphere. Subsequently, the volatiles were removed under reduced pressure and the residue was treated with DCM. The resulting precipitate was filtered off, washed with DCM (2 times) and hexanes (3 times). After being dried under reduced pressure compound **8c** was obtained as a white solid (2.44 g; **Yield = 65%**).

*Note: Compound **8c** should be stored under argon atmosphere at 0 °C.*

Physical state: white solid

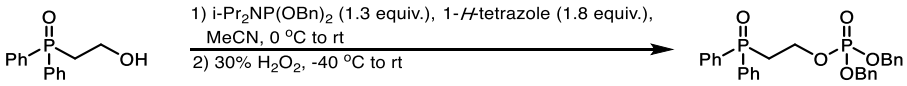
^{1}H NMR (600 MHz, DMSO-d_6) δ 9.50 (br s, 2H), 7.94-7.87 (m, 4H), 7.55-7.49 (m, 3H), 5.07 (d, *J* = 7.2 Hz, 2H).

^{13}C NMR (150 MHz, DMSO-d_6) δ 135.1 (d, *J* = 7.8 Hz), 132.8, 132.6, 128.0, 127.8, 127.6, 126.4, 126.2, 125.9, 125.5, 66.9 (d, *J* = 4.9 Hz).

^{31}P NMR (162 MHz, DMSO-d_6) δ -1.0

HRMS (ESI-TOF) m/z: calculated for $C_{11}H_{10}O_4P$ $[M-H]^-$: 237.0322, found: 237.0320.

Compound S31



S30 **S31**

Flame-dried round bottom flask was charged with alcohol **S30**[13] (0.44 g, 1.8 mmol, 1.0 equiv.) and 1-*H*-tetrazole (0.23 g, 3.2 mmol, 1.8 equiv.). Substrates were dissolved in anhydrous MeCN (10 mL), and the mixture was cooled down to 0 °C. i-$Pr_2NP(OBn)_2$ (0.75 mL, 2.3 mmol, 1.3 equiv.) was added dropwise to the resulting mixture and the reaction was stirred for 3 h, at room temperature under argon atmosphere. Subsequently, the reaction mixture was cooled down to -40 °C, followed by addition of 30% aq. H_2O_2 (1.7 mL) and the reaction was warmed to room temperature over 2 h. The resulting solution was diluted with DCM and the organic phase was sequentially washed with saturated aq. $NaHCO_3$ and brine. The organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude residue was purified by silica gel chromatography (MeOH/EtOAc/DCM; 2:30:68) to provide 0.73 g of compound **S31** (**Yield = 80%**).

Physical state: colorless semi-solid

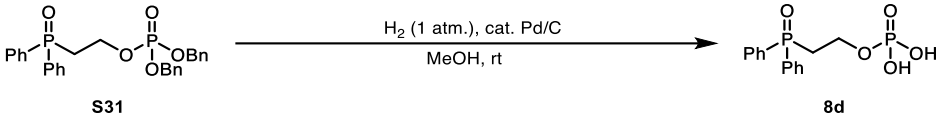
^{1}H NMR (600 MHz, $CDCl_3$) δ 7.71-7.66 (m, 4H), 7.54-7.49 (m, 2H), 7.47-7.43 (m, 4H), 7.34-7.30 (m, 6H), 7.30-7.26 (m, 4H), 4.95-4.88 (m, 4H), 4.36-4.29 (m, 2H), 2.71-2.65 (m, 2H).

^{13}C NMR (150 MHz, $CDCl_3$) δ 135.8 (d, *J* = 6.8 Hz), 132.4 (d, *J* = 100.4 Hz), 132.2 (d, *J* = 2.7 Hz), 130.8 (d, *J* = 9.6 Hz), 128.9 (d, *J* = 12.0 Hz), 128.7, 128.1, 69.5 (d, *J* = 5.6 Hz), 61.9 (d, *J* = 5.0 Hz), 31.6 (dd, *J* = 69.0, 5.9 Hz). (*One signal missing due to an overlap in the aromatic region*).

^{31}P NMR (162 MHz, $CDCl_3$) δ 27.9, -1.4.

HRMS (ESI-TOF) m/z: calculated for $C_{28}H_{29}O_5P_2$ $[M+H]^+$: 507.1485, found: 507.1509.

Compound 8d



Round bottom flask equipped with a stir bar was charged with protected phosphate **S31** (0.71 g, 1.4 mmol, 1.0 equiv.), and the atmosphere was exchanged to argon. MeOH (6.0 mL) was added, followed by Pd/C (10% wt.; 100 mg). The atmosphere in the flask was exchanged for H_2 and the reaction vessel was equipped with a H_2 balloon. The reaction mixture was stirred at room temperature for 1 h, after which TLC indicated full conversion of the starting material. The crude reaction mixture was filtered through a pad of celite, followed by a few volumes of MeOH. The volatiles were removed under reduced pressure, and the residue was co-evaporated with DCM/Hexanes (1:1) (2 times) to remove residual MeOH. After being dried under reduced pressure compound **8d** was obtained as a white solid (0.31 g; **Yield = 69%**).

Physical state: white solid

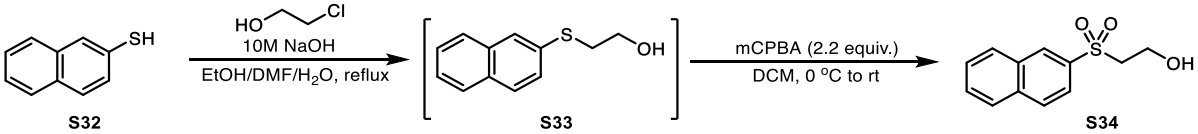
^{1}H NMR (600 MHz, CD_3OD) δ 7.82-7.77 (m, 4H), 7.64-7.60 (m, 2H), 7.58-7.54 (m, 4H), 4.23 (quint, *J* = 7.5 Hz, 2H), 2.93 (dt, *J* = 11.8, 7.5 Hz, 2H).

^{13}C NMR (150 MHz, CD_3OD) δ 133.7 (d, *J* = 2.6 Hz), 132.8 (d, *J* = 102.0 Hz), 131.8 (d, *J* = 9.9 Hz), 130.1 (d, *J* = 12.1 Hz), 61.4 (d, *J* = 5.1 Hz), 31.9 (dd, *J* = 70.1, 6.6 Hz).

^{31}P NMR (162 MHz, CD_3OD) δ 33.2, -0.3.

HRMS (ESI-TOF) m/z: calculated for $C_{14}H_{15}O_5P_2$ $[M-H]^-$: 325.0400, found: 325.0402.

Compound S34



Aqueous 10 M NaOH (5.0 mL) was added dropwise to a stirring mixture of 2-chloroethanol (4.05 mL, 60 mmol, 1.2 equiv.) and 2-naphthalenethiol (**S32**) (8.0 g, 50 mmol, 1.0 equiv.) in EtOH (90 mL). The reaction mixture was refluxed for 1 h, followed by addition of a second portion of 2-chloroethanol (2.25 mL, 33 mmol, 0.7 equiv.) and DMF (30 mL). The mixture was refluxed overnight, cooled down, and concentrated under reduced pressure. The residue was partitioned between EtOAc and water. The aqueous layer was extracted with EtOAc, and the combined organic layers were dried over $MgSO_4$, filtered, and concentrated under reduced

pressure. The crude residue was filtered through a short silica plug (EtOAc/Hexanes, 0:100, then 20:80) to provide 9.40 g of a compound **S33**, which was used in the next step without further purification.

Crude sulfide **S33** (9.40 g, 46 mmol, 1.0 equiv.) was dissolved in DCM (250 mL). The resulting mixture was cooled to 0 °C and mCPBA (22.4 g, 101 mmol, 2.2 equiv., 77% stabilized with H_2O) was added portionwise. The resulting suspension was stirred at room temperature for 3 h (until TLC indicated full consumption of sulfide and intermediate sulfoxide). The resulting mixture was quenched with saturated aq. $NaHCO_3$ and extracted with DCM. Combined organic fractions were washed successively with saturated aq. $NaHCO_3$ and brine, dried over $MgSO_4$, filtrated and concentrated under reduced pressure. The crude residue was purified by silica gel chromatography (EtOAc/Hexanes, from 10:90 to 20:80) to provide 8.68 g of compound **S34** (**Yield = 74% over 2 steps**).

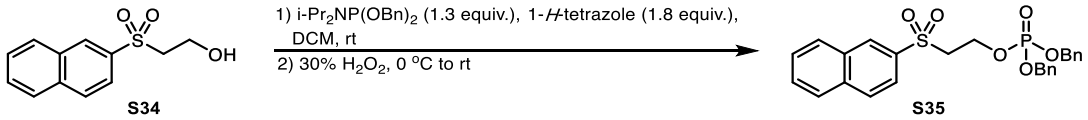
Physical state: white solid

^{1}H NMR (600 MHz, $CDCl_3$) δ 8.52 (d, *J* = 1.9 Hz, 1H), 8.03 (d, *J* = 8.6 Hz, 1H), 8.01 (d, *J* = 8.2 Hz, 1H), 7.94 (d, *J* = 8.2 Hz, 1H), 7.88 (dd, *J* = 8.6, 1.9 Hz, 1H), 7.70 (ddd, *J* = 8.2, 6.9, 1.3 Hz, 1H), 7.65 (ddd, *J* = 8.2, 6.9, 1.2 Hz, 1H), 4.04-4.01 (m, 2H), 3.45-3.42 (m, 2H), 2.81 (br s, 1H).

^{13}C NMR (150 MHz, $CDCl_3$) δ 135.9, 135.6, 132.3, 130.1, 130.0, 129.7, 129.6, 128.2, 128.1, 122.5, 58.4, 56.6.

HRMS (ESI-TOF) m/z: calculated for $C_{12}H_{13}O_3S$ $[M+H]^+$: 237.0580, found: 237.0571.

Compound S35



Flame-dried round bottom flask was charged with alcohol **S34** (3.50 g, 15 mmol, 1.0 equiv.) and 1-*H*-tetrazole (1.58 g, 22.5 mmol, 1.8 equiv.). Substrates were dissolved in anhydrous DCM (100 mL). i-$Pr_2NP(OBn)_2$ (6.5 mL, 19.5 mmol, 1.3 equiv.) was added dropwise to the resulting mixture and the reaction was stirred for 3 h, at room temperature under argon atmosphere. Subsequently, the reaction mixture was cooled down to 0 °C, followed by addition of 30% aq. H_2O_2 (15 mL) and the reaction was warmed to room temperature over 1 h. The resulting solution was diluted with DCM and the organic phase was sequentially washed with saturated aq. $NaHCO_3$ and brine. The organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude residue was purified by silica gel chromatography (EtOAc/Hexanes; 50:50) to provide 6.60 g of compound **S35** (**Yield = 89%**).

Physical state: colorless semi-solid

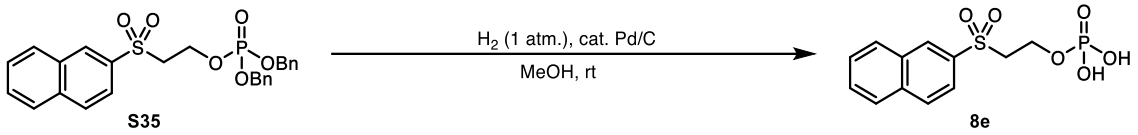
^{1}H NMR (600 MHz, $CDCl_3$) δ 8.48-8.46 (m, 1H), 7.95 (d, *J* = 8.4 Hz, 2H), 7.90 (d, *J* = 8.2 Hz, 1H), 7.83 (dd, *J* = 8.6, 1.8 Hz, 1H), 7.69-7.65 (m, 1H), 7.64-7.60 (m, 1H), 7.33-7.28 (m, 6H), 7.23-7.18 (m, 4H), 4.83 (d, *J* = 8.4 Hz, 4H), 4.32 (dt, *J* = 8.2, 6.2 Hz, 2H), 3.44 (t, *J* = 6.2 Hz, 2H).

^{13}C NMR (150 MHz, $CDCl_3$) δ 136.2, 135.6, 135.5, 132.3, 130.3, 129.8, 129.7, 129.6, 128.8, 128.7, 128.14, 128.10, 128.0, 122.8, 69.7 (d, *J* = 5.7 Hz), 60.8 (d, *J* = 5.0 Hz), 56.3 (d, *J* = 7.0 Hz).

^{31}P NMR (162 MHz, $CDCl_3$) δ -1.6.

HRMS (ESI-TOF) m/z: calculated for $C_{26}H_{26}O_6PS$ $[M+H]^+$: 497.1182, found: 497.1161.

Compound PD8



Round bottom flask equipped with a stir bar was charged with protected phosphate **S35** (6.60 g, 13.3 mmol, 1.0 equiv.), and the atmosphere was exchanged to argon. MeOH (100 mL) was added, followed by Pd/C (10% wt.; 660 mg). The atmosphere in the flask was exchanged for H_2 and the reaction vessel was equipped with a H_2 balloon. The reaction mixture was stirred at room temperature for 1 h, after which TLC indicated full conversion of the starting material. The crude reaction mixture was filtered through a pad of celite, followed by a few volumes of MeOH. The volatiles were removed under reduced pressure, and the residue was co-evaporated with DCM/Hexanes (1:1) (2 times) to remove residual MeOH. After being dried under reduced pressure compound **8e** was obtained as a white solid (4.03 g; **Yield = 96%**).

Physical state: white solid

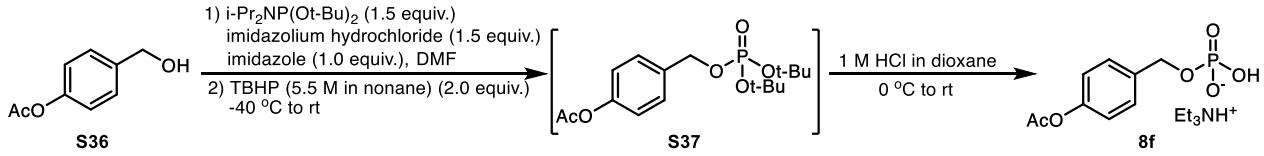
^{1}H NMR (600 MHz, CD_3OD) δ 8.56 (s, 1H), 8.11-8.07 (m, 2H), 8.00 (d, *J* = 8.2 Hz, 1H), 7.92 (dd, *J* = 8.7, 1.5 Hz, 1H), 7.73-7.68 (m, 1H), 7.68-7.64 (m, 1H), 4.30 (q, *J* = 6.2 Hz, 2H), 3.70 (t, *J* = 6.2 Hz, 2H).

^{13}C NMR (150 MHz, CD_3OD) δ 137.8, 136.9, 133.6, 131.1, 130.7, 130.6, 130.5, 129.1, 128.8, 123.8, 61.0 (d, *J* = 4.7 Hz), 57.2 (d, *J* = 7.7 Hz).

^{31}P NMR (162 MHz, CD_3OD) δ -0.7.

HRMS (ESI-TOF) m/z: calculated for $C_{12}H_{12}O_6PS$ $[M-H]^-$: 315.0097, found: 315.0100.

Compound 8f



An oven-dried 100 mL round-bottom flask was charged with 4-acetoxybenzyl alcohol (**S36**)[14] (0.80 g, 4.8 mmol, 1.0 equiv.), imidazolium hydrochloride (0.76 g, 7.2 mmol, 1.5 equiv.) and imidazole (0.33 g, 4.8 mmol, 1.0 equiv.). The mixture was dissolved in anhydrous DMF (5 mL) and i-$Pr_2NP(Ot\text{-}Bu)_2$ (2.3 mL, 7.2 mmol, 1.5 equiv.) was added dropwise over 5 min. The reaction was stirred for 1 h at room temperature under argon atmosphere. Subsequently, the mixture was cooled to -40 °C, followed by dropwise addition of *tetr*-butyl hydroperoxide (1.45 mL, 9.6 mmol; 5.5 M in nonane), and the reaction was warmed to room temperature over 2 h. The resulting mixture was extracted with EtOAc. The organic layer was washed with brine (2 times), dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude residue was filtered through a short silica plug (EtOAc/Hexanes/DCM, 15:35:50) to provide 1.05 g of a compound **S37**, which was used in the next step without further purification.

An oven-dried 100 mL round-bottom flask was charged with a crude compound **S37** (1.05 g, 2.9 mmol, 1.0 equiv.). The reaction vessel was submerged in ice/water bath and 1 M HCl in dioxane (9.0 mL) was added over 5 min. The reaction was warmed to room temperature and stirred for 3 h under argon atmosphere. Subsequently, the volatiles were removed under reduced pressure and the residue was purified by reverse phase C18-silica gel chromatography (1 M aq. TEAA/MeCN; from 100:0 to 95:5). Residual water was removed by lyophilization to provide 305 mg of compound **8f**. (**Yield = 18% over 2 steps**).

*Note: Compound **8f** should be stored under argon atmosphere at 0 °C. We have observed that removal of residual TEAA buffer by multiple lyophilization leads to partial degradation of the product.*

Physical state: viscous colorless oil

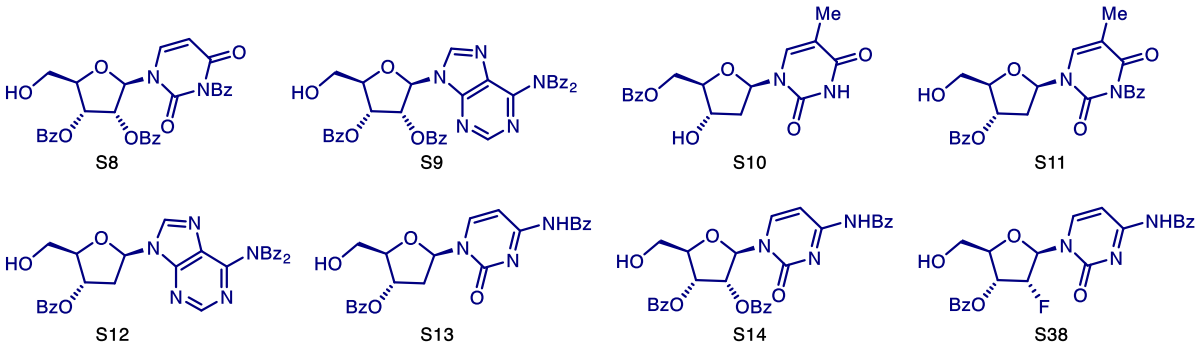
^{1}H NMR (600 MHz, D_2O) δ 7.51 (d, *J* = 8.6 Hz, 2H), 7.17 (d, *J* = 8.6 Hz, 2H), 4.94 (d, *J* = 7.1 Hz, 2H), 3.20 (q, *J* = 7.3 Hz, 6H), 2.35 (s, 3H), 1.28 (t, *J* = 7.3 Hz, 9H).

^{13}C NMR (150 MHz, D_2O) δ 173.5, 149.8, 135.8 (d, *J* = 7.5 Hz), 129.0, 121.7, 66.6 (d, *J* = 5.0 Hz), 46.7, 20.4, 8.2.

^{31}P NMR (162 MHz, D_2O) δ 0.3.

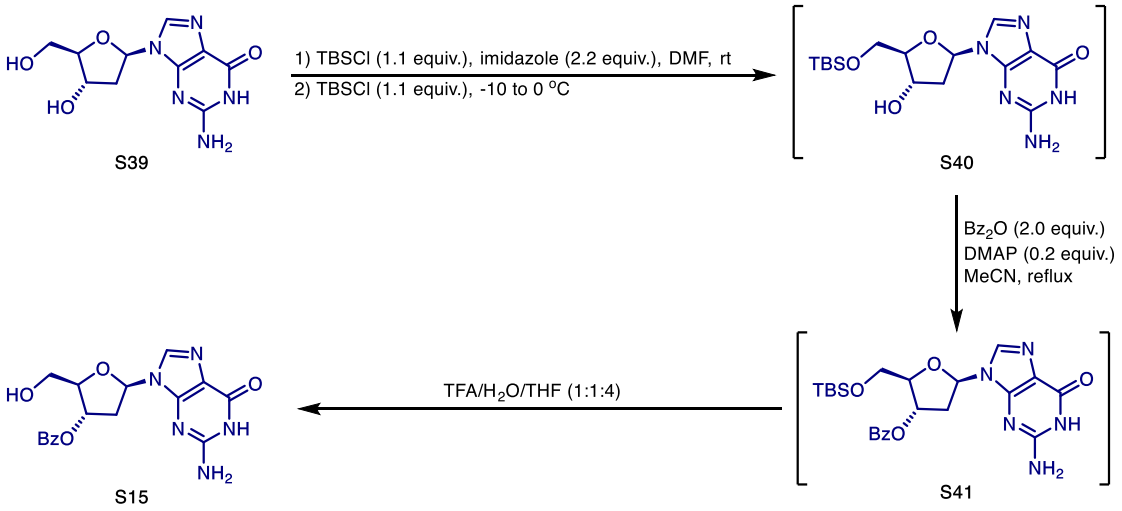
HRMS (ESI-TOF) m/z: calculated for $C_9H_{10}O_6P$ $[M-H]^-$: 245.0220, found: 245.0225.

8.2. Preparation of Non-Commercially Available Substrates



Compounds **S8**,[15] **S9**,[15] **S10**,[16] **S11**,[17] **S12**,[15] **S13**,[17] **S14**,[15] and **S38**[18] were prepared following literature procedures.

Compound S15



Round-bottom flask equipped with a stir bar was charged with deoxyguanosine (**S39**) (2.50 g, 9.4 mmol, 1.0 equiv.), followed by addition of anhydrous DMF (25 mL). Subsequently, imidazole (1.40 g, 20.6 mmol, 2.2 equiv.) and *tert*-butyldimethylsilyl chloride (1.55 g, 10.3 mmol, 1.1 equiv.) were added and the resulting suspension was stirred at room temperature for 15 min. After that time, the reaction mixture was cooled to -10 °C. Another portion of *tert*-butyldimethylsilyl chloride (1.55 g, 10.3 mmol, 1.1 equiv.) was added and the reaction mixture was stirred at 0 °C for 3 h. The reaction was quenched by addition of water (400 mL), and the resulting suspension was filtered. The solid residue was washed consecutively with water, Et_2O and dried under reduced pressure to provide 2.48 g of a crude compound **S40**, which was used directly in the next step. Crude compound **S40** (2.48, 6.5 mmol, 1.0 equiv.) was suspended in anhydrous MeCN (50 mL), followed by addition of benzoyl anhydride (2.95 g, 13.0 mmol, 2.0 equiv.) and DMAP (0.16 g, 0.13 mmol, 0.2 equiv.). The reaction vessel was equipped with an air condenser and the heterogeneous mixture was refluxed for 3 h. Subsequently, the reaction was cooled to room temperature and quenched by addition of saturated aq.

$NaHCO_3$. The solid residue was filtered off, washed consecutively with water and MeCN, and dried under reduced pressure to provide 2.80 g of a crude compound **S41**, which was used directly in the next step.

Crude compound **S41** (2.80 g, 5.8 mmol, 1.0 equiv.) was suspended in THF (20 mL), followed by addition of H_2O/TFA (1:1; 10 mL). The reaction was stirred at room temperature for 90 min. Subsequently, the solution was neutralized by careful addition of saturated aq. $NaHCO_3$. The crude mixture was concentrated under reduced pressure to ~5 mL and filtered. The solid residue was washed consecutively with water, Et_2O and dried under reduced pressure to provide 1.92 g of pure compound **S15** (**Yield over 3 steps = 55%**).

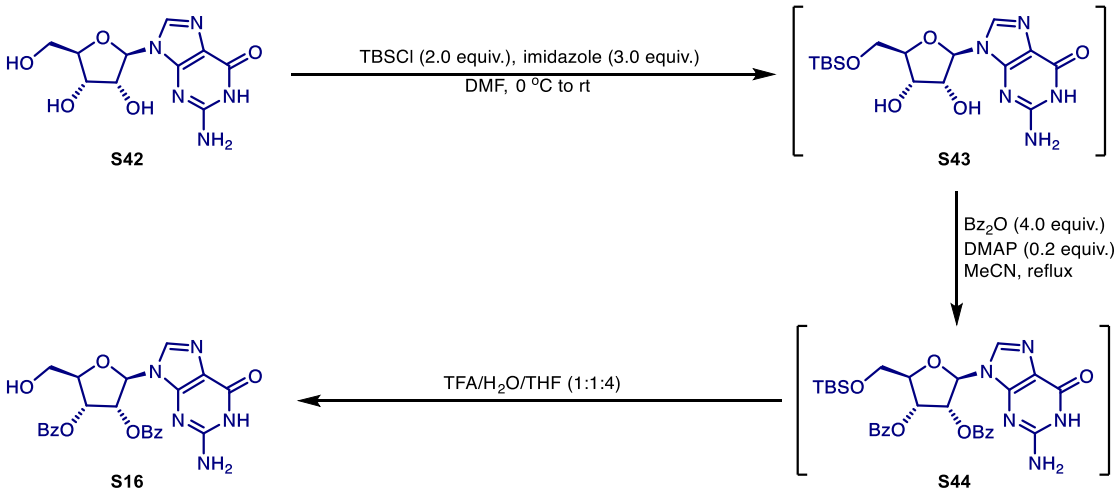
Physical state: white solid

^{1}H NMR (600 MHz, DMSO-*d*$_6$) δ 10.68 (br s, 1H), 8.03 (d, *J* = 7.7 Hz, 2H), 8.00 (s, 1H), 7.72-7.68 (m, 1H), 7.60-7.54 (m, 2H), 6.48 (br s, 2H), 6.23 (dd, *J* = 9.1, 5.7 Hz, 1H), 5.57 (d, *J* = 5.7 Hz, 1H), 5.20 (t, *J* = 5.7 Hz, 1H), 4.23-4.19 (m, 1H), 3.70-3.62 (m, 2H), 2.91 (ddd, *J* = 14.6, 9.1, 5.9 Hz, 1H), 2.58 (dd, *J* = 14.6, 5.7 Hz, 1H).

^{13}C NMR (150 MHz, DMSO-*d*$_6$) δ 165.2, 156.8, 153.8, 151.0, 135.3, 133.7, 129.4, 129.3, 128.8, 116.8, 84.9, 82.8, 76.0, 61.6, 36.7.

HRMS (ESI-TOF) m/z: calculated for $C_{17}H_{18}N_5O_5$ $[M+H]^+$: 372.1303, found: 372.1285.

Compound S16



Round-bottom flask equipped with a stir bar was charged with guanosine (**S42**) (14.2 g, 50 mmol, 1.0 equiv.), followed by addition of anhydrous DMF (200 mL). The heterogeneous mixture was cooled to 0 °C, followed by addition of imidazole (10.2 g, 150 mmol, 3.0 equiv.) and *tert*-butyldimethylsilyl chloride (15.1 g, 100 mmol, 2.0 equiv.). The reaction mixture was warmed to room temperature overnight. Subsequently, the reaction was quenched by addition of water (800 mL), and the resulting suspension was filtered. The solid residue was washed consecutively with water and acetone (*note: solid was repeatedly washed with acetone until the ratio of mono- to di-TBS protected guanosine was > 50:1*) and dried under reduced pressure to provide 12.1 g of a crude compound **S43**, which was used directly in the next step.

Crude compound **S43** (12.1 g, 30.4 mmol, 1.0 equiv.) was suspended in anhydrous MeCN (150 mL), followed by addition of benzoyl anhydride (27.5 g, 121.6 mmol, 4.0 equiv.) and DMAP (0.74 g, 6.2 mmol, 0.2 equiv.).

The reaction vessel was equipped with an air condenser and the heterogeneous mixture was refluxed for 3 h. Subsequently, the reaction was cooled to room temperature and quenched by addition of saturated aq. $NaHCO_3$. The solid residue was filtered off, washed consecutively with water and MeCN, and dried under reduced pressure to provide 14.9 g of a crude compound **S44**, which was used directly in the next step.

Crude compound **S44** (14.9 g, 24.6 mmol, 1.0 equiv.) was suspended in THF (90 mL), followed by addition of H_2O/TFA (1:1; 45 mL). The reaction was stirred at room temperature for 90 min. Subsequently, the solution was neutralized by careful addition of saturated aq. $NaHCO_3$. The crude mixture was concentrated under reduced pressure to ~25 mL and filtered. The solid residue was washed consecutively with water, Et_2O and dried under reduced pressure to provide 11.5 g of pure compound **S16** (**Yield over 3 steps = 47%**).

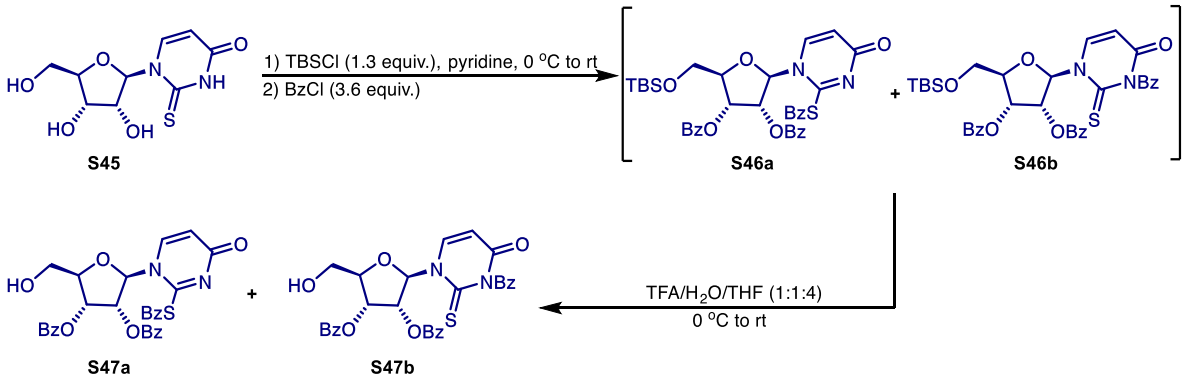
Physical state: white solid

^{1}H NMR (600 MHz, DMSO-*d*$_6$) δ 10.77 (br s, 1H), 8.07 (s, 1H), 7.94 (dd, *J* = 8.3, 1.4 Hz, 2H), 7.79 (dd, *J* = 8.4, 1.4 Hz, 1H), 7.68 (tt, *J* = 7.4, 1.4 Hz, 1H), 7.61 (tt, *J* = 7.4, 1.4 Hz, 1H), 7.54-7.50 (m, 2H), 7.43-7.39 (m, 2H), 6.54 (br s, 2H), 6.26 (d, *J* = 6.7 Hz, 1H), 6.12 (dd, *J* = 6.7, 5.5 Hz, 1H), 5.84 (dd, *J* = 5.5, 2.8 Hz, 1H), 5.51 (t, *J* = 5.5 Hz, 1H), 4.49 (q, *J* = 3.4 Hz, 1H), 3.84-3.75 (m, 2H).

^{13}C NMR (150 MHz, DMSO-*d*$_6$) δ 164.9, 164.4, 157.0, 154.2, 151.2, 135.6, 134.0, 133.9, 129.32, 129.30, 128.91, 128.85, 128.81, 128.2, 116.9, 84.6, 83.6, 73.7, 72.4, 61.2.

HRMS (ESI-TOF) m/z: calculated for $C_{24}H_{22}N_5O_7$ $[M+H]^+$: 492.1514, found: 492.1493.

Compounds S47a and S47b



Round bottom flask equipped with a stir bar was charged with 2-thiouridine (**S45**) (1.87 g, 7.2 mmol, 1.0 equiv.), followed by addition of anhydrous pyridine (40 mL). The solution was cooled to 0 °C, followed by addition of *tert*-butyldimethylsilyl chloride (1.42 g, 9.4 mmol, 1.3 equiv.). The reaction mixture was warmed to room temperature overnight. Subsequently, benzoyl chloride (3.0 mL, 25.9 mmol, 3.6 equiv.) was added and the reaction was stirred for another 8 h. After that time, the reaction mixture was diluted with DCM, washed with 1 M HCl and water. The organic phase was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude was purified by silica gel chromatography (EtOAc/Hexanes/DCM; from 0.5:50:50 to 2:50:50) to provide 3.21 g of a mixture of compounds **S46a** and **S46b** (mixture of *N*- and *S*-benzoylated regioisomers), which was used directly in the next step.

Mixture of regioisomeric compounds **S46a** and **S46b** (3.21 g, 4.7 mmol, 1.0 equiv.) was dissolved in THF (84 mL). The solution was cooled to 0 °C, followed by addition of H_2O/TFA (1:1; 42 mL). The reaction was warmed to room temperature over 3 h. Subsequently, the solution was neutralized by careful addition of saturated aq. $NaHCO_3$. The crude product was extracted with DCM, and the organic phase was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude was purified by silica gel chromatography (EtOAc/Hexanes/DCM; from 10:40:50 to 40:10:50) to provide 1.77 g of regioisomeric mixture of compounds **S47a** and **S47b** (ratio 2:1; *structures of minor and major regioisomers not assigned*) (**Yield over 3 steps = 43%**).

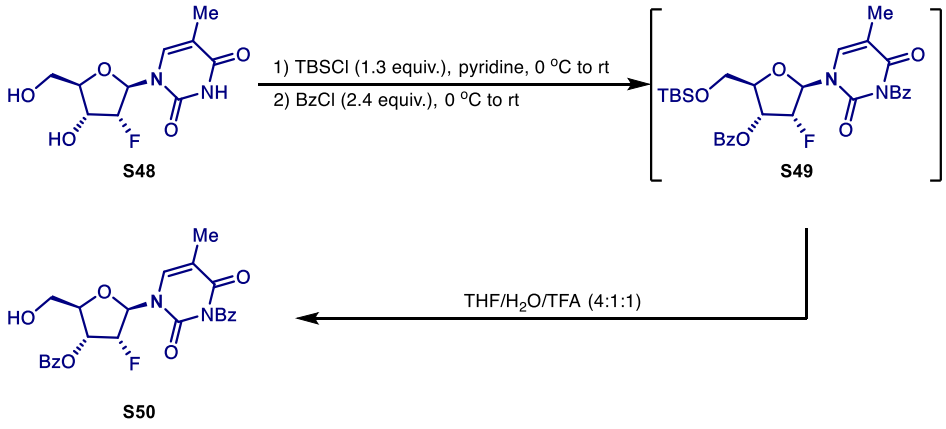
Physical state: white solid

^{1}H NMR (600 MHz, DMSO-d_6) δ 8.57 (d, *J* = 8.2 Hz, 1H; *major*), 8.55 (d, *J* = 8.2 Hz, 1H; *minor*), 8.00-7.79 (m, 6H; *major* + *minor*), 7.75-7.36 (m, 9H; *major* + *minor*), 7.21-7.17 (m, 1H; *major* + *minor*), 6.52 (d, *J* = 8.2 Hz, 1H; *major*), 6.46 (d, *J* = 8.2 Hz, 1H; *minor*), 5.89-5.75 (m, 3H; *major* + *minor*), 4.63 (s, 1H; *major*), 4.62 (s, 1H; *minor*), 3.94-3.83 (m, 2H; *major* + *minor*).

^{13}C NMR (150 MHz, DMSO-d_6) δ 174.4 (*minor*), 174.2 (*major*), 168.1 (*minor*), 167.9 (*major*), 164.9 (*major*), 164.7 (*major*), 164.6 (*minor*), 164.4 (*minor*), 159.0 (*minor*), 158.9 (*major*), 141.5 (*major* + *minor*), 135.0 (*major* + *minor*), 133.91 (*major*), 133.90 (*major* + *minor*), 138.85 (*minor*), 130.8 (*minor*), 130.6 (*major*), 130.20 (*minor*), 130.15 (*major*), 129.5 (*minor*), 129.4 (*major*), 129.34 (*major* + *minor*), 129.28 (*major*), 129.20 (*minor*), 128.9 (*major* + *minor*), 128.82 (*minor*), 128.79 (*major*), 128.71 (*major* + *minor*), 128.4 (*minor*), 129.3 (*major*), 106.9 (*major*), 106.8 (*minor*), 89.5 (*minor*), 89.4 (*major*), 84.0 (*major*), 83.9 (*minor*), 74.9 (*major*), 74.7 (*minor*), 71.6 (*major*), 71.1 (*minor*), 60.2 (*major*), 60.1 (*minor*).

HRMS (ESI-TOF) m/z: calculated for $C_{30}H_{24}N_2O_8SNa$ $[M+Na]^+$: 595.1151, found: 595.1138.

Compound S50



2'-fluorothymidine (**S48**) (0.52 g, 2.0 mmol, 1.0 equiv.), dried by co-evaporation with anhydrous pyridine, was dissolved in anhydrous pyridine (10 mL). The solution was cooled to 0 °C, followed by addition of *tert*-butyldimethylsilyl chloride (0.40 g, 2.6 mmol, 1.3 equiv.). After stirring at 0 °C for 2h, the reaction mixture was warmed to room temperature overnight. Subsequently, the mixture was cooled to 0 °C and benzoyl chloride (0.56 mL, 4.8 mmol, 2.4 equiv.) was added. The ice bath was removed, and the reaction was stirred

for another 8 h at room temperature. After that time, the reaction mixture was diluted with DCM, washed with brine. The aqueous phase was extracted with DCM, and the combined organic layers was dried over Na_2SO_4, filtered and concentrated under reduced pressure to provide crude compound **S49**, which was used directly in the next step.

Crude compound **S49** was redissolved in THF (10 mL). The solution was cooled to 0 °C, followed by addition of H_2O/TFA (1:1; 5 mL). The reaction was warmed to room temperature over 90 min. Subsequently, the solution was neutralized by careful addition of saturated aq. $NaHCO_3$. The crude product was extracted with DCM, and the organic phase was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude was purified by silica gel chromatography (EtOAc/Hexanes; 50:50) to provide 0.68 g of compound **S50** (**Yield over 3 steps = 73%**).

Physical state: white solid

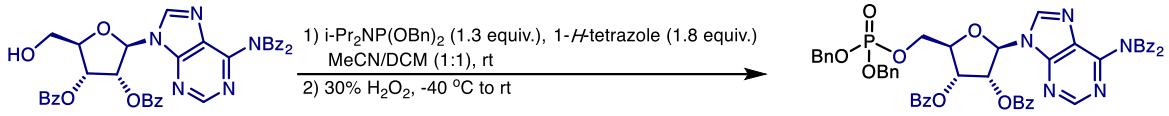
^{1}H NMR (600 MHz, $CDCl_3$) δ 8.06 (dd, *J* = 8.3, 1.2 Hz, 2H), 7.93 (dd, *J* = 8.3, 1.2 Hz, 2H), 7.68-7.64 (m, 2H), 7.63-7.59 (m, 1H), 7.52-7.49 (m, 2H), 7.48-7.45 (m, 2H), 6.02 (dd, *J* = 16.6, 3.0 Hz, 1H), 5.57 (ddd, *J* = 14.5, 6.3, 5.1 Hz, 1H), 5.48 (ddd, *J* = 52.2, 4.8, 3.1 Hz, 1H), 4.40 (d, *J* = 6.3 Hz, 1H), 4.07 (dd, *J* = 12.6, 1.8 Hz, 1H), 3.88 (dd, *J* = 12.6, 2.4 Hz, 1H), 1.98 (d, *J* = 1.0 Hz, 3H).

^{13}C NMR (150 MHz, $CDCl_3$) δ 168.6, 165.9, 162.8, 149.5, 136.7, 135.4, 134.1, 131.5, 130.7, 130.1, 129.4, 128.8, 128.7, 111.8, 91.2 (d, *J* = 194.6 Hz), 90.2 (d, *J* = 34.0 Hz), 82.3, 70.0 (d, *J* = 14.6 Hz), 61.1, 12.8.

^{19}F NMR (376 MHz, $CDCl_3$) δ -205.6.

HRMS (ESI-TOF) m/z: calculated for $C_{24}H_{22}FN_2O_7$ $[M+H]^+$: 469.1406, found: 469.1426.

Compound S51



Flame-dried round bottom flask was charged with protected adenosine **S9** (2.05 g, 3.0 mmol, 1.0 equiv.) and 1-*H*-tetrazole (0.38 g, 5.4 mmol, 1.8 equiv.). Substrates were dissolved in a mixture of anhydrous MeCN (25 mL) and DCM (25 mL). i-$Pr_2NP(OBn)_2$ (1.25 mL, 3.9 mmol, 1.3 equiv.) was added dropwise to the resulting mixture and the reaction was stirred for 1 h under argon atmosphere. Subsequently, the reaction mixture was cooled down to -40 °C, followed by addition of 30% aq. H_2O_2 (10 mL) and the reaction was warmed to room temperature over 1 h. The resulting solution was diluted with DCM and the organic phase was washed with saturated aq. $NaHCO_3$ and brine. The organic layer was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude residue was purified by silica gel chromatography (EtOAc/Hexanes/DCM; from 10:40:50 to 20:30:50) to provide 2.17 g of compound **S51** (**Yield = 77%**).

Physical state: white solid

^{1}H NMR (600 MHz, $CDCl_3$) δ 8.62 (s, 1H), 8.40 (s, 1H), 7.97 (dd, *J* = 8.4, 1.4 Hz, 2H), 7.90 (dd, *J* = 8.4, 1.4 Hz, 2H), 7.88-7.84 (m, 4H), 7.58 (tt, *J* = 7.4, 1.4 Hz, 1H), 7.55 (tt, *J* = 7.4, 1.4 Hz, 1H), 7.49-7.46 (m, 2H),

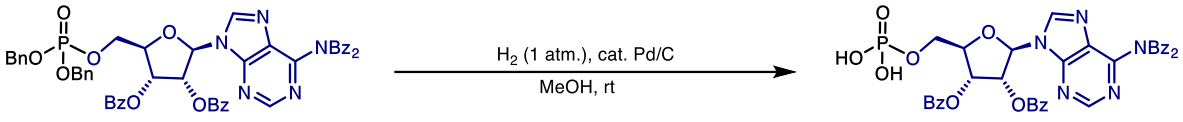
7.42-7.39 (m, 2H), 7.38-7.32 (m, 10H), 7.31-7.24 (m, 6H), 6.50 (d, *J* = 5.7 Hz, 1H), 6.09 (t, *J* = 5.7 Hz, 1H), 5.98 (dd, *J* = 5.7, 4.0 Hz, 1H), 5.11-5.02 (m, 4H), 4.62-4.59 (m, 1H), 4.37 (dd, *J* = 6.2, 3.6 Hz, 2H).

^{13}C NMR (150 MHz, $CDCl_3$) δ 172.4, 165.4, 165.0, 153.1, 152.6, 152.3, 143.4, 135.60 (d, *J* = 6.5 Hz), 135.58 (d, *J* = 6.5 Hz), 134.2, 133.95, 133.93, 133.2, 130.01, 129.96, 129.6, 128.9, 128.81, 128.79, 128.73, 128.66, 128.5, 128.28, 128.27, 127.9, 86.6, 81.9 (d, *J* = 8.0 Hz), 74.3, 71.6, 69.96 (d, *J* = 5.5 Hz), 69.94 (d, *J* = 5.5 Hz), 66.5 (d, *J* = 5.2 Hz). (*Three signals missing due to an overlap in the aromatic region*).

^{31}P NMR (162 MHz, $CDCl_3$) δ -1.0.

HRMS (ESI-TOF) m/z: calculated for $C_{52}H_{43}N_5O_{11}P$ $[M+H]^+$: 944.2697, found: 944.2669.

Compound S52



S51 **S52**

Round bottom flask equipped with a stir bar was charged with protected adenosine phosphate **S51** (2.17 g, 2.3 mmol, 1.0 equiv.), and the atmosphere was exchanged to argon. MeOH (140 mL) was added, followed by Pd/C (10% wt.; 325 mg). The atmosphere in the flask was exchanged for H_2 and the reaction vessel was equipped with a H_2 balloon. The reaction mixture was stirred at room temperature for 1 h, after which TLC indicated full conversion of the starting material. The crude reaction mixture was filtered through a pad of celite, followed by few volumes of MeOH/DCM (1:1). The volatiles were removed under reduced pressure, and the residue was co-evaporated two times with DCM to remove residual MeOH. After being dried under reduced pressure compound **S52** was obtained as a white solid (1.55 g; **Yield = 88%**).

Physical state: white solid

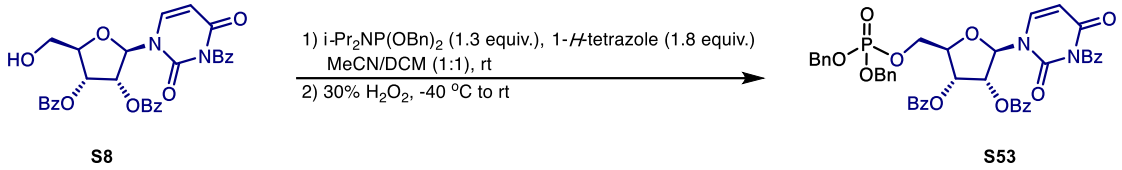
^{1}H NMR (600 MHz, CD_3OD) δ 8.81 (s, 1H), 8.58 (s, 1H), 8.00 (d, *J* = 6.8 Hz, 2H), 7.81 (d, *J* = 7.0 Hz, 4H), 7.76 (d, *J* = 7.4 Hz, 2H), 7.58 (t, *J* = 7.4 Hz, 1H), 7.51-7.45 (m, 3H), 7.41 (t, *J* = 7.8 Hz, 2H), 7.34 (t, *J* = 7.7 Hz, 4H), 7.27 (t, *J* = 7.8 Hz, 2H), 6.67 (d, *J* = 5.8 Hz, 1H), 6.21 (t, *J* = 5.8 Hz, 1H), 6.08 (dd, *J* = 5.8, 3.6 Hz, 1H), 4.77-4.74 (m, 1H), 4.47-4.38 (m, 2H).

^{13}C NMR (150 MHz, CD_3OD) δ 173.7, 166.7, 166.2, 154.4, 153.39, 153.37, 152.9, 146.0, 145.9, 135.3, 134.9, 134.8, 134.3, 130.8, 130.7, 130.5, 130.2, 129.8, 129.71, 129.69, 129.6, 128.9, 87.9, 83.5 (d, *J* = 8.0 Hz), 75.8, 73.2, 66.67 (d, *J* = 3.8 Hz). (*Three signals missing due to an overlap in the aromatic region*).

^{31}P NMR (162 MHz, CD_3OD) δ 0.4.

HRMS (ESI-TOF) m/z: calculated for $C_{38}H_{29}N_5O_{11}P$ $[M-H]^-$: 762.1601, found: 762.1625.

Compound S53



Flame-dried round bottom flask was charged with protected uridine **S8** (3.10 g, 5.6 mmol, 1.0 equiv.) and 1-*H*-tetrazole (0.71 g, 10.1 mmol, 1.8 equiv.). Substrates were dissolved in a mixture of anhydrous MeCN (45 mL) and DCM (45 mL). i-$Pr_2NP(OBn)_2$ (2.30 mL, 7.3 mmol, 1.3 equiv.) was added dropwise to the resulting mixture and the reaction was stirred for 1 h under argon atmosphere. Subsequently, the mixture was cooled down to -40 °C, followed by addition of 30% aq. H_2O_2 (18 mL) and the reaction was warmed to room temperature over 1 h. The resulting solution was diluted with DCM and the organic phase was washed with saturated aq. $NaHCO_3$ and brine. The organic layer was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude residue was purified by silica gel chromatography (EtOAc/Hexanes/DCM; from 10:40:50 to 30:20:50) to provide 3.88 g of compound **S53** (**Yield = 86%**).

Physical state: white amorphous solid

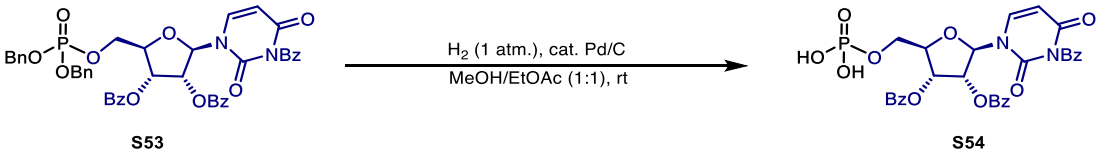
^{1}H NMR (600 MHz, $CDCl_3$) δ 7.98 (d, *J* = 7.2 Hz, 2H), 7.91 (dd, *J* = 8.4, 1.2 Hz, 2H), 7.87 (d, *J* = 7.3 Hz, 2H), 7.68 (d, *J* = 8.3 Hz, 1H), 7.61-7.56 (m, 2H), 7.52 (tt, *J* = 7.3, 1.2 Hz, 1H), 7.43-7.30 (m, 16H), 6.41 (d, *J* = 7.0 Hz, 1H), 5.67-5.64 (m, 2H), 5.46 (t, *J* = 6.5 Hz, 1H), 5.19-5.08 (m, 4H), 4.47-4.44 (m, 1H), 4.37-4.30 (m, 2H).

^{13}C NMR (150 MHz, $CDCl_3$) δ 168.4, 165.42, 165.40, 161.7, 149.7, 139.2, 135.49 (d, *J* = 5.7 Hz), 135.46 (d, *J* = 6.2 Hz), 135.1, 134.0, 133.9, 131.5, 130.6, 130.0, 129.9, 129.2, 129.14, 129.09, 129.0, 128.9, 128.76, 128.75, 128.7, 128.39, 128.36, 103.7, 86.5, 81.7 (d, *J* = 8.1 Hz), 73.7, 71.6, 70.17 (d, *J* = 5.4 Hz), 70.15 (d, *J* = 5.4 Hz), 66.6 (d, *J* = 5.2 Hz). (*Four signals missing due to an overlap in the aromatic region*).

^{31}P NMR (162 MHz, $CDCl_3$) δ -0.6.

HRMS (ESI-TOF) m/z: calculated for $C_{44}H_{38}N_2O_{12}P$ $[M+H]^+$: 817.2157, found: 817.2153.

Compound S54



Round bottom flask equipped with a stir bar was charged with protected uridine phosphate **S53** (3.85 g, 4.8 mmol, 1.0 equiv.), and the atmosphere was exchanged to argon. MeOH (100 mL) and EtOAc (100 mL) were added, followed by Pd/C (10% wt.; 675 mg). The atmosphere in the flask was exchanged for H_2 and the reaction vessel was equipped with a H_2 balloon. The reaction mixture was stirred at room temperature for 1.5 h, after which TLC indicated full conversion of the starting material. The crude reaction mixture was filtered through a pad of celite, followed by few volumes of MeOH/DCM (1:1). The volatiles were removed

under reduced pressure, and the residue was co-evaporated two times with DCM to remove residual MeOH. After being dried under educed pressure compound **S54** was obtained as a white amorphous solid (2.82 g; **Yield = 93%**).

Physical state: white amorphous solid

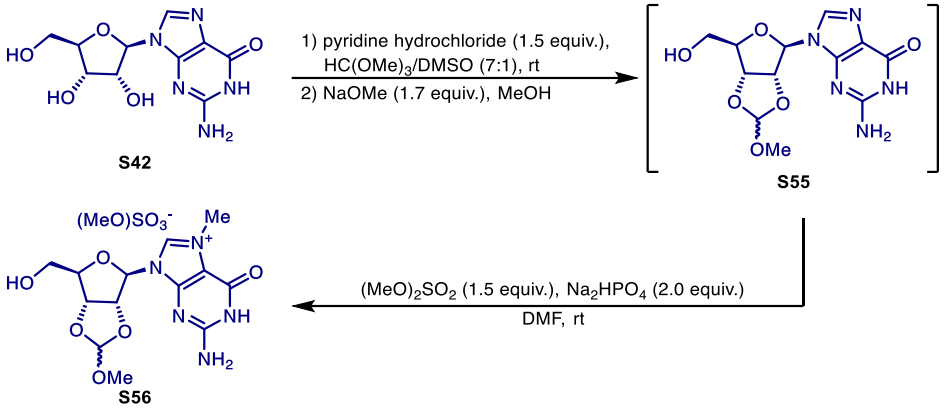
^{1}H NMR (600 MHz, $(CD_3)_2CO$) δ 9.38 (br s, 2H), 8.18 (d, *J* = 7.5 Hz, 1H), 8.03-7.98 (m, 4H), 7.86 (d, *J* = 7.5 Hz, 2H), 7.66 (t, *J* = 7.4 Hz, 1H), 7.61 (t, *J* = 7.4 Hz, 1H), 7.54 (t, *J* = 7.4 Hz, 1H), 7.49 (t, *J* = 7.7 Hz, 2H), 7.43 (t, *J* = 7.7 Hz, 2H), 7.32 (t, *J* = 7.7 Hz, 2H), 6.48 (d, *J* = 6.6 Hz, 1H), 6.08 (d, *J* = 6.9 Hz, 1H), 5.98-5.94 (m, 1H), 5.84-5.79 (m, 1H), 4.82 (br s, 1H), 4.64-4.55 (m, 2H).

^{13}C NMR (150 MHz, $(CD_3)_2CO$) δ 169.8, 165.94, 165.88, 162.6, 150.5, 141.2, 135.8, 134.50, 132.6, 131.2, 130.49, 130.48, 130.1, 130.0, 129.6, 129.49, 129.46, 129.3, 103.7, 87.6, 82.2 (d, *J* = 7.4 Hz), 75.0, 72.7, 66.9.

^{31}P NMR (162 MHz, $(CD_3)_2CO$) δ 0.3.

HRMS (ESI-TOF) m/z: calculated for $C_{30}H_{24}N_2O_{12}P$ $[M-H]^-$: 635.1072, found: 635.1071.

Compound S56



According to the literature procedure[19] guanosine (**S42**) (2.83 g, 10 mmol, 1.0 equiv.) was suspended in anhydrous trimethyl orthoformate (25 mL), followed by addition of pyridine hydrochloride (1.75 g, 15 mmol, 1.5 equiv.). The stirred suspension was treated with DMSO (3.5 mL) and the mixture was stirred at room temperature for 48 h. To a resultant cloudy suspension were added sequentially MeOH (25 mL) and solid NaOMe (0.90 g, 16.5 mmol, 1.7 equiv.) and the reaction was stirred for another 3 h. Subsequently, the mixture was concentrated under reduced pressure and the resulting thick suspension was treated with MeOH/Et_2O (1:1). The obtained pale-yellow solid was filtered off and dried under reduced pressure. Crude compound **S55** was used directly in the next step.

Crude compound **S55** was redissolved in anhydrous DMF (12 mL), followed by addition of Na_2HPO_4 (2.84 g, 20 mmol, 2.0 equiv.). Dimethyl sulfate (1.40 mL, 15 mmol, 1.5 equiv.) was added dropwise and the reaction was stirred at room temperature for 1 h. The heterogeneous mixture was filtered through a pad of celite, and the solid residue was washed with MeOH. The filtrate was concentrated under reduced pressure to ~10 mL and loaded directly on silica gel. The crude was purified by silica gel chromatography (DCM/MeOH; from

100:0 to 80:20) to provide 2.20 g of compound **S56** (2:1 mixture of diastereoisomers). (**Yield over 2 steps = 49%**).

(*Note: compound **S56** is highly hygroscopic and should be stored in a desiccator under argon atmosphere*).

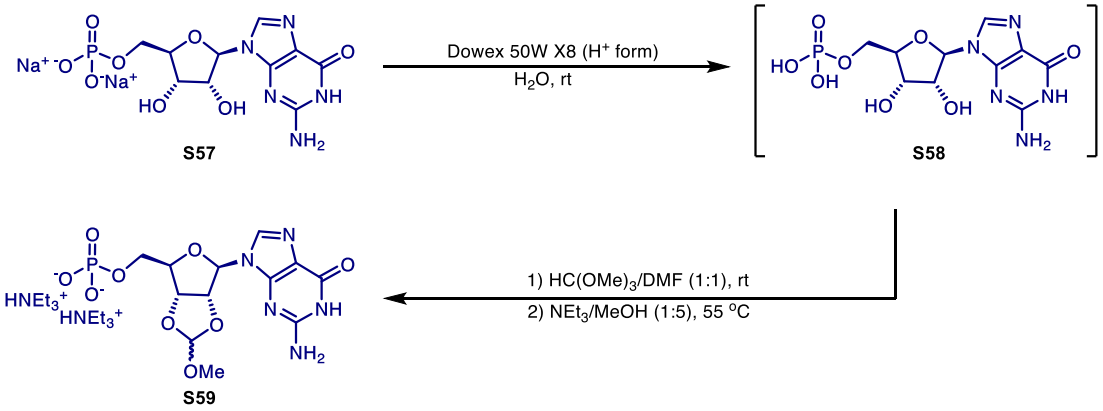
Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 6.31 (d, *J* = 2.3 Hz, 1H; *major*), 6.23 (s, 1H; *minor*), 6.21 (d, *J* = 2.4 Hz, 1H; *minor*), 6.13 (s, 1H; *major*), 5.50 (dd, *J* = 6.2, 2.4 Hz, 1H; *minor*), 4.48 (dd, *J* = 6.8, 2.3 Hz, 1H; *major*), 5.15 (dd, *J* = 6.1, 2.4 Hz, 1H; *minor*), 5.09 (dd, *J* = 6.8, 2.6 Hz, 1H; *major*), 4.72-4.69 (m, 1H; *major*), 4.64-4.61 (m, 1H; *minor*), 4.11 (s, 3H; *major* + *minor*), 3.90-3.86 (m, 1H; *major* + *minor*), 3.86-3.81 (m, 1H; *major* + *minor*), 3.75 (s, 3H; *major* + *minor*), 3.49 (s, 3H; *major*), 3.40 (s, 3H; *minor*).

^{13}C NMR (150 MHz, D_2O) δ 157.5 (*minor*), 157.42 (*minor*), 157.37 (*major*), 157.29 (*major*), 149.4 (*major* + *minor*), 136.1 (t, 1:1:1, $J^1_{C\text{-}D}$ = 35.1 Hz; *major* + *minor*), 118.4 (*major*), 117.3 (*minor*), 108.7 (*major* + *minor*), 92.6 (*major*), 91.9 (*minor*), 89.0 (*major*), 87.6 (*minor*), 84.3 (*major*), 83.6 (*minor*), 81.5 (*major*), 80.7 (*minor*), 61.4 (*major*), 61.2 (*minor*), 55.4 (*major* + *minor*), 52.6 (*major*), 51.6 (*minor*), 35.71 (*minor*), 35.70 (*major*).

HRMS (ESI-TOF) m/z: calculated for $C_{13}H_{18}N_5O_6$ $[M]^+$: 340.1252, found: 340.1264.

Compound S59



Disodium guanosine monophosphate (**S57**) (10 g, 25 mmol, 1.0 equiv.) was converted into dihydrogen guanosine monophosphate (**S58**) according to the literature procedure[20]. **S58** was used in the next step without any further purification.

Dihydrogen guanosine monophosphate (**S58**), obtained as described above, was suspended in a mixture of anhydrous trimethyl orthoformate (50 mL) and anhydrous DMF (50 mL). The resulting mixture was stirred overnight at room temperature. Subsequently, the reaction mixture was concentrated under reduced pressure, followed by addition of Et_2O. The resulting suspension was filtered to provide off-white solid, which was mixed with MeOH (100 mL) and triethylamine (20 mL). The solution was stirred overnight at 55 °C. Volatile components were removed under reduced pressure and the crude residue was purified by reverse phase C18-silica gel chromatography (1 M aq. TEAA/MeCN; from 100:0 to 70:30). Residual water was removed by lyophilization to provide 7.13 g of compound **S59** (9:1 mixture of diastereoisomers). (**Yield over 3 steps = 47%**).

(*Note: compound **S59** obtained after lyophilization usually contain 1-2 equiv. of TEAA buffer as an impurity. We have observed that complete removal of the buffer by multiple lyophilization lead to partial hydrolysis of the orthoester protecting group. Therefore, it is advised to directly use the obtained mixture of compound **S59** and TEAA buffer in the next step*).

Physical state: white amorphous solid

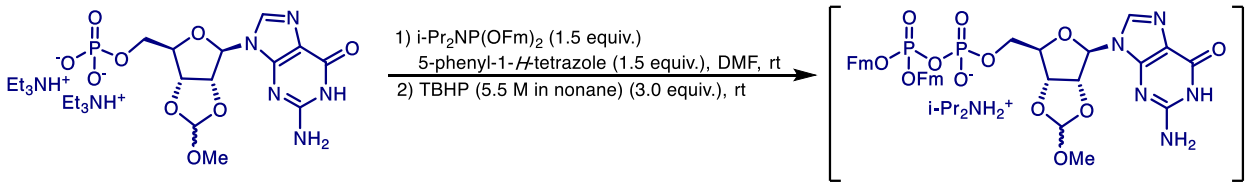
^{1}H NMR (600 MHz, D_2O) δ 7.99 (s, 1H; minor), 7.97 (s, 1H; major), 6.19 (s, 1H; minor), 6.14 (d, J = 2.0 Hz, 1H; major), 6.07 (s, 1H; major), 6.02 (d, J = 2.3 Hz, 1H; minor), 5.40 (dd, J = 5.9, 2.7 Hz, 1H; minor), 5.35 (dd, J = 6.9, 2.3 Hz, 1H; major), 5.22 (dd, J = 5.9, 2.3 Hz, 1H; minor), 5.16 (dd, J = 6.9, 2.7 Hz, 1H; major), 4.61 (q, J = 4.6 Hz, 1H; major), 4.52 (q, J = 4.1 Hz, 1H; minor), 4.08-4.04 (m, 1H; major + minor), 4.04-3.98 (m, 1H; major + minor), 3.46 (s, 3H; major), 3.36 (s, 3H; minor), 3.16 (q, J = 7.3 Hz, 12H; Et_3NH^+), 1.24 (t, J = 7.3 Hz, 18H; Et_3NH^+).

^{13}C NMR (150 MHz, D_2O) δ 158.1 (*major + minor*), 153.3 (*minor*), 153.2 (*major*), 150.6 (*major + minor*), 137.3 (*major*), 137.2 (*minor*), 118.1 (*major*), 116.8 (*minor*), 115.6 (*major + minor*), 89.6 (*major*), 88.6 (*minor*), 85.6 (d, *J* = 8.7 Hz; *major*), 84.1 (d, *J* = 8.5 Hz; *minor*), 83.7 (*major*), 82.8 (*minor*), 81.0 (*major*), 80.3 (*minor*), 64.0 (d, *J* = 4.6 Hz; *major*), 63.9 (d, *J* = 4.3 Hz; *minor*), 52.0 (*major*), 50.9 (*minor*), 46.1 (Et_3NH^+), 7.7 (Et_3NH^+).

^{31}P NMR (162 MHz, D_2O) δ 0.6.

HRMS (ESI-TOF) m/z: calculated for $C_{12}H_{15}N_5O_9P$ $[M-H]^-$: 404.0613, found: 404.0626.

Compound S60



S59 **S60**

Compound **S59** (2.61 g; 4.3 mmol; 1.0 equiv.) was dried by co-evaporation with anhydrous DMF and dissolved in anhydrous DMF (12 mL). i-$Pr_2NP(OFm)_2$ (3.35 g, 6.5 mmol, 1.5 equiv.) was added to the resulting solution, followed by 5-phenyl-1-*H*-tetrazole (0.94 g, 6.5 mmol, 1.5 equiv.) and the reaction was stirred for 1 h at room temperature. Subsequently, *tert*-butyl hydroperoxide (5.5 M in nonane; 2.3 mL, 12.9 mmol, 3.0 equiv.) was added and the mixture was stirred for another 1 h. The reaction was quenched by the addition of Et_2O (100 mL). Solvent was decanted and the remaining oily residue was washed twice with Et_2O. The residue was redissolved in DCM (10 mL), followed by addition of Et_2O (100 mL). The resulting haze mixture was sonicated for 10 min to initiate precipitation. The precipitate was filtered off to provide 4.05 g of crude compound **S60** (75% wt. purity determined by quantitative ^{31}P NMR) (9:1 mixture of diastereoisomers) (**NMR yield = 75%**). Compound **S60** can be used in subsequent steps without any further purification.

(*Note*: *Due to low stability of the compound* ***S60*** *on silica we were not able to obtain analytically pure product on a preparative scale. Small amount (< 50 mg) of compound was purified by silica gel chromatography (DCM/MeOH) and used to gather analytical data.*)

Physical state: white amorphous solid

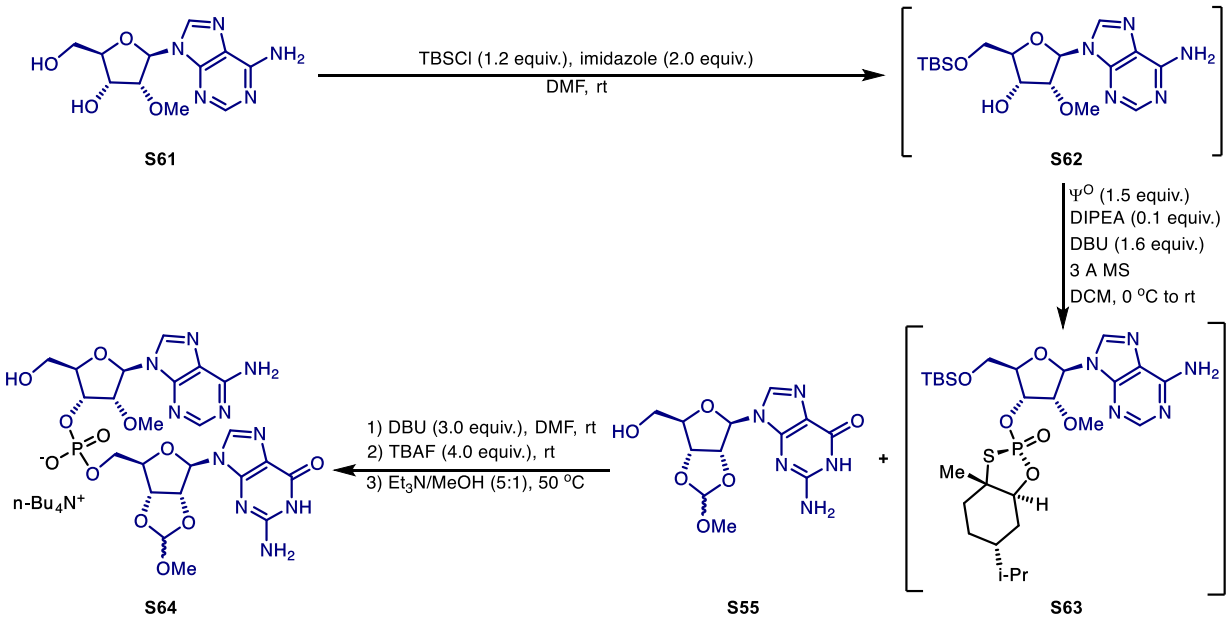
^{1}H NMR (600 MHz, DMSO-*d*$_6$) δ 10.7 (br s, 1H; *major* + *minor*), 8.40 (br s, 2H; i-Pr$_2$NH$_2^+$), 7.94 (s, 1H; *major*), 7.92 (s, 1H; *minor*), 7.86-7.81 (m, 4H; *major* + *minor*), 7.56-7.48 (m, 4H; *major* + *minor*), 7.39-7.32 (m, 4H; *major* + *minor*), 7.26-7.18 (m, 4H; *major* + *minor*), 6.81 (br s, 2H; *major* + *minor*), 6.12 (d, *J* = 1.9 Hz, 1H; *major*), 6.10 (s, 1H; *minor*), 6.02 (d, *J* = 2.4 Hz, 1H; *minor*), 6.01 (s, 1H; *major*), 5.33 (dd, *J* = 7.2, 3.5 Hz, 1H; *minor*), 5.26-5.19 (m; 2H *major* + 1H *minor*), 4.40-4.31 (m, 2H; *major* + *minor*), 4.28-4.14 (m, 6H; *major* + *minor*), 3.97-3.94 (m, 1H; *minor*), 3.83-3.75 (m, 1H; *major*), 3.30 (s, 3H; *major*), 3.27 (hept, *J* = 6.5 Hz, 1H; i-Pr$_2$NH$_2^+$), 3.17 (s, 3H; *minor*), 1.17 (d, *J* = 6.5 Hz, 12H; i-Pr$_2$NH$_2^+$).

^{13}C NMR (150 MHz, DMSO-*d*$_6$) δ 157.5 (*minor*), 156.8 (*major*), 153.9 (*major* + *minor*), 150.5 (*minor*), 150.4 (*major*), 143.4 (*major* + *minor*), 143.2 (*major* + *minor*), 140.79 (*major* + *minor*), 140.77 (*major* + *minor*), 140.76 (*major* + *minor*), 136.3 (*minor*), 136.2 (*major*), 129.4 (*major* + *minor*), 129.0 (*major* + *minor*), 127.6 (*major* + *minor*), 127.0 (*major* + *minor*), 126.8 (*minor*), 126.5 (*major*), 125.14 (*major*), 125.10 (*minor*), 120.0 (*major* + *minor*), 118.2 (*major* + *minor*), 116.91 (*major*), 116.85 (*minor*), 89.6 (*major*), 88.6 (*minor*), 85.9 (d, *J* = 7.5 Hz; *major*), 84.7 (d, *J* = 8.1 Hz; *minor*), 84.2 (*major*), 83.0 (*minor*), 81.4 (*major*), 81.2 (*minor*), 68.3 (*major* + *minor*), 64.9 (*major* + *minor*), 54.9 (*major* + *minor*), 51.9 (*major*), 50.4 (*minor*), 47.3 (d, *J* = 7.1 Hz; *major* + *minor*), 46.1 (i-Pr$_2$NH$_2^+$), 18.7 (i-Pr$_2$NH$_2^+$).

^{31}P NMR (162 MHz, DMSO-*d*$_6$) δ -12.1 (d, *J* = 19.8 Hz), -12.7 (d, *J* = 19.8 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_{40}H_{36}N_5O_{12}P_2$ [M-H]$^-$: 840.1841, found: 840.1861.

Compound S64



Round-bottom flask equipped with a stir bar was charged with 2'-*O*-methyladenosine (**S61**) (1.55 g, 5.5 mmol, 1.0 equiv.), followed by anhydrous DMF (55 mL). Imidazole (0.75 g, 11.0 mmol, 2.0 equiv.) and *tert*-butyldimethylsilyl chloride (1.0 g, 6.6 mmol, 1.2 equiv.) were added consecutively and the reaction mixture was stirred at room temperature overnight. Subsequently, the reaction was quenched by addition of concentrated aq. $NaHCO_3$ and extracted with EtOAc. Organic phase was washed with water, brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure to provide crude compound **S62**, which was used in the next step without any further purification.

The crude compound **S62** was redissolved in anhydrous DCM (30 mL), followed by the addition of Ψ^O reagent[21] (3.47 g, 8.3 mmol, 1.5 equiv.). Freshly dried 3 Å molecular sieves (3.0 g) were added and the mixture was stirred for 5 min. Subsequently, the reaction mixture was cooled to 0 °C, followed by consecutive addition of DIPEA (0.10 mL, 0.6 mmol, 0.1 equiv.) and DBU (1.30 mL, 8.8 mmol, 1.6 equiv.) and the reaction was stirred under argon atmosphere for 30 min. After that time, the mixture was diluted with EtOAc and filtered. The filtrate was washed consecutively with 10% aq. KH_2PO_4 and brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure to provide crude compound **S66**, which was used in the next step without any further purification.

The crude compound **S63** was redissolved in anhydrous DMF (25 mL), followed by addition of protected guanosine **S55**[19] (3.58 g, 11.0 mmol, 2.0 equiv.). DBU (2.46 mL, 16.5 mmol, 3.0 equiv.) was added and the reaction mixture was stirred under argon atmosphere for 30 min. Subsequently, 1.0 M solution of TBAF in THF (22.0 mL, 22.0 mmol, 4.0 equiv.) was added and the reaction was stirred at room temperature for another 4 h. After that time, MeOH (25 mL) and Et_3N (5.0 mL) were added consecutively, and the reaction mixture was stirred at 50 °C overnight. The resulting mixture was concentrated under reduced pressure and the residue was suspended in water (50 mL). The resulting suspension was filtered through a pad of Celite, and the filtrate was concentrated under reduced pressure. The residue was suspended in water and the filtration step was repeated once again. The filtrate was concentrated under reduced pressure and the crude product was purified by reverse phase C18-silica gel chromatography (1 M aq. TEAA/MeCN; from 100:0 to 80:20). Residual water was removed by lyophilization to provide 2.40 g of compound **S64** (2:1 mixture of diastereoisomers). (**Yield over 5 steps = 48%**).

Physical state: white solid

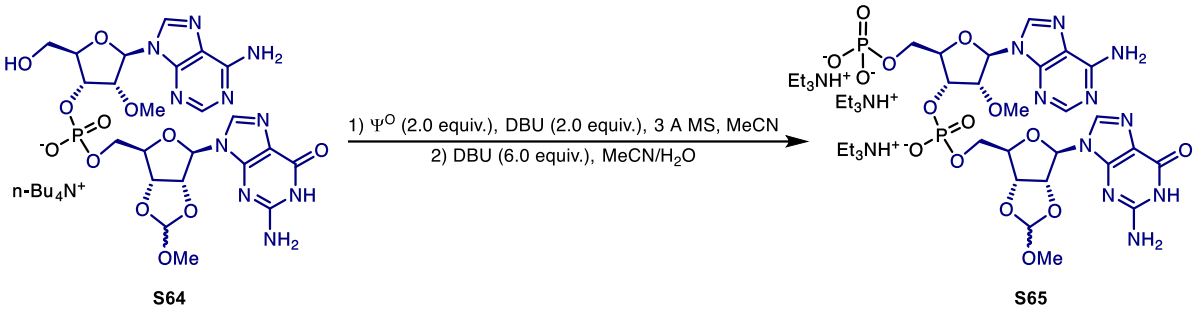
^{1}H NMR (600 MHz, D_2O) δ 8.21 (s, 1H; *major* + *minor*), 8.05 (s, 1H; *minor*), 8.04 (s, 1H; *major*), 7.87 (s, 1H; *minor*); 7.85 (s, 1H; *major*), 6.20 (s, 1H; *minor*), 6.16 (s, 1H; *major*), 6.09 (s, 1H; *major*), 6.06 (s, 1H; *minor*), 5.89 (d, *J* = 6.6 Hz, 1H; *minor*), 5.86 (d, *J* = 6.3 Hz, 1H; *major*), 5.50 (d, *J* = 6.3 Hz, 1H; *minor*), 5.46 (d, *J* = 6.9 Hz, 1H; *major*), 5.29 (dd, *J* = 6.1, 3.1 Hz, 1H; *minor*), 5.19 (dd, *J* = 6.9, 3.1 Hz, 1H; *major*), 4.66-4.62 (m, 1H; *major*), 4.54-4.50 (m, 1H; *minor*), 4.36-4.30 (m, 1H; *major* + *minor*), 4.20-4.11 (m, 2H; *major* + *minor*), 4.06-4.03 (m, 1H; *minor*), 4.00-3.96 (m, 1H; *major*), 3.66-3.59 (m, 1H; *major* + *minor*), 3.57-3.48 (m, 1H; *major* + *minor*), 3.46 (s, 3H; *major*), 3.38 (s, 3H; *minor*), 3.29 (s, 3H; *major* + *minor*), 3.16-3.10 (m, 8H; nBu_4N^+), 1.63-1.54 (m, 8H; nBu_4N^+), 1.36-1.27 (m, 8H; nBu_4N^+), 0.91 (t, *J* = 7.3 Hz, 12H; nBu_4N^+) (*One signal overlapping with D_2O*).

^{13}C NMR (150 MHz, D_2O) δ 159.2 (*minor*), 159.1 (*major*), 154.9 (*major* + *minor*), 154.1 (*minor*), 154.0 (*major*), 151.81 (*minor*), 151.79 (*major*), 150.4 (*minor*), 150.3 (*major*), 147.6 (*major* + *minor*), 140.0 (*major* + *minor*), 137.1 (*major*), 137.0 (*minor*), 118.5 (*major* + *minor*), 118.1 (*major*), 117.0 (*minor*), 116.0 (*major*), 115.9 (*minor*), 89.8 (*major*), 88.5 (*minor*), 86.19 (*major*), 86.15 (*minor*), 85.9 (d, *J* = 9.5 Hz; *major*), 84.7 (*major* + *minor*), 84.4 (d, *J* = 9.4 Hz; *minor*), 83.5 (*major*), 82.4 (*minor*), 81.24 (d, *J* = 5.5 Hz; *major*), 81.21 (d, *J* = 4.7 Hz; *minor*), 80.6 (*major*), 80.1 (*minor*), 72.7 (d, *J* = 5.5 Hz; *major* + *minor*), 65.4 (d, *J* = 5.1 Hz; *major*), 65.1 (d, *J* = 4.9 Hz; *minor*), 60.6 (*major* + *minor*), 57.6 (br; nBu_4N^+), 57.3 (*major* + *minor*), 52.0 (*major*), 51.0 (*minor*), 22.6 (nBu_4N^+), 18.6 (nBu_4N^+), 12.3 (nBu_4N^+).

^{31}P NMR (162 MHz, D_2O) δ -1.2.

HRMS (ESI-TOF) m/z: calculated for $C_{23}H_{28}N_{10}O_{12}P$ $[M-H]^-$: 667.1631, found: 667.1621.

Compound S65



Dinucleoside **S64** (2.40 g, 2.6 mmol, 1.0 equiv.) was dried by co-evaporation with anhydrous MeCN, and the substrate was dissolved in anhydrous MeCN (30 mL). Subsequently Ψ^O reagent[21] (2.22 g, 5.2 mmol, 2.0 equiv.) and 3 Å molecular sieves (3.0 g) were added and the resulting suspension was stirred for 5 min. DBU (0.77 mL, 5.2 mmol, 2.0 equiv.) was added dropwise and the reaction was stirred at room temperature and under argon atmosphere for 45 min. After that time, deionized water (3.0 mL) and DBU (2.32 mL, 15.6 mmol, 6.0 equiv.) were added consecutively and the reaction was stirred for another 15 min. The mixture was diluted with water, filtered through a pad of Celite and the filtrate was concentrated under reduced pressure. The residue was resuspended in water and the filtration step was repeated once again. The filtrate was concentrated under reduced pressure and the crude product was purified by reverse phase C18-silica gel chromatography (1 M aq. TEAA/MeCN; from 100:0 to 85:15). Residual water was removed by lyophilization to provide 1.56 g of compound **S65** (2:1 mixture of diastereoisomers). (**Yield = 63%**).

*(Note: compound **S65** obtained after lyophilization usually contain 1-2 equiv. of TEAA buffer as a sole impurity. We have observed that complete removal of the buffer by multiple lyophilization lead to partial hydrolysis of the orthoester protecting group. Therefore, it is advised to use the obtained mixture of compound **S65** and TEAA buffer directly in the next step).*

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.39 (s, 1H; *minor*), 8.38 (s, 1H; *major*), 8.15 (s, 1H; *major* + *minor*), 7.90 (s, 1H; *major* + *minor*), 6.21 (s, 1H; *minor*), 6.18 (d, *J* = 2.3 Hz, 1H; *major*), 6.10 (s, 1H; *major*), 6.08 (d, *J* = 2.3 Hz, 1H; *minor*), 5.98 (d, *J* = 7.1 Hz, 1H; *minor*), 5.97 (d, *J* = 6.8 Hz; 1H; *major*), 5.47 (dd, *J* = 6.2, 2.3 Hz, 1H;

minor), 5.43 (dd, *J* = 7.0, 2.3 Hz, 1H; *major*), 5.31 (dd, *J* = 6.2, 3.6 Hz, 1H; *minor*), 5.21 (dd, *J* = 7.0, 3.5 Hz, 1H; *major*), 4.91-4.86 (m, 1H; *major* + *minor*), 4.66 (q, *J* = 4.4 Hz, 1H; *major*), 4.54 (q, *J* = 4.2 Hz, 1H; *minor*), 4.37-4.31 (m, 1H; *major* + *minor*), 4.25-4.15 (m, 3H; *major* + *minor*), 4.00-3.92 (m, 1H; *major* + *minor*), 3.86-3.81 (m, 1H; *minor*), 3.80-3.75 (m, 1H; *major*), 3.47 (s, 3H; *major*), 3.37 (s, 3H; *minor*), 3.35 (s, 3H; *minor*), 3.34 (s, 3H; *major*), 3.18 (q, *J* = 7.3 Hz, 12H; Et_3NH^+), 1.26 (t, *J* = 7.3 Hz, 18H; Et_3NH^+).

^{13}C NMR (150 MHz, D_2O) δ 158.1 (*major*), 158.0 (*minor*), 154.5 (*major* + *minor*), 153.1 (*minor*), 153.0 (*major*), 151.8 (*major* + *minor*), 150.5 (*minor*), 150.3 (*major*), 148.3 (*major* + *minor*), 139.2 (*major* + *minor*), 137.6 (*major*), 137.5 (*minor*), 118.1 (*major*), 117.9 (*major* + *minor*), 116.9 (*minor*), 115.9 (*major*), 115.8 (*minor*), 89.9 (*major*), 88.6 (*minor*), 85.8 (d, *J* = 9.5 Hz; *major*), 84.6 (*major* + *minor*), 84.3 (d, *J* = 9.4 Hz; *minor*), 83.6 (*major*), 82.8 (d, *J* = 9.1 Hz; *major* + *minor*), 82.4 (*minor*), 82.1 (d, *J* = 5.5 Hz; *major*), 82.0 (d, *J* = 5.5 Hz; *minor*), 80.5 (*major*), 80.0 (*minor*), 72.71 (d, *J* = 5.7 Hz; *major*), 72.66 (d, *J* = 5.8 Hz; *minor*), 65.4 (d, *J* = 5.1 Hz; *major*), 65.1 (d, *J* = 5.2 Hz; *minor*), 63.7 (d, *J* = 4.7 Hz; *major* + *minor*), 57.2 (*major* + *minor*), 52.0 (*major*), 50.9 (*minor*), 46.2 (Et_3NH^+), 7.7 (Et_3NH^+).

^{31}P NMR (162 MHz, D_2O) δ 0.1, -1.2.

HRMS (ESI-TOF) m/z: calculated for $C_{23}H_{29}N_{10}O_{15}P_2$ $[M-H]^-$: 747.1294, found: 747.1287.

8.3. Experimental Procedures and Characterization Data of Products

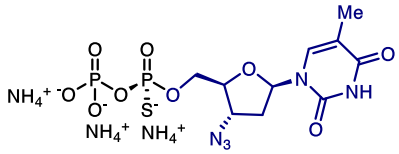
Diastereomeric purities of the products were determined by NMR and LC-MS.

LC traces of pure diastereoisomers were recorded from samples obtained after purification and lyophilization.

LC traces for mixtures of diastereoisomers were obtained from solutions prepared by mixing previously obtained R_P and S_P isomers. In several cases samples of compounds used to prepare such mixtures were partially hydrolysed due to prolonged storage, therefore chromatograms for mixture of isomers does not reflect purity of the obtained compounds.

8.3.1 Nucleoside Thiodiphosphates

Compound (R_P)-4



5'-*O*-azidothymidine triammonium (*R*)-diphosphoro-α-thioate

Following the **General Procedure A** compound **(R_P)-4** was obtained from monophosphate precursor **8g** (62 mg, 0.2 mmol), (+)-Ψ* (128 mg, 0.3 mmol) and azidothymidine (**3**) (133 mg, 0.5 mmol). Deprotection was performed at room temperature. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 20:80) to afford 52 mg of the title compound after lyophilization (**Yield = 53%, d.r. > 20:1**)

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 7.79 (s, 1H), 6.28 (t, *J* = 6.9 Hz, 1H), 4.60 (dt, *J* = 6.7, 3.5 Hz, 1H), 4.29-4.20 (m, 3H), 2.54-2.45 (m, 2H), 1.95 (s, 3H).

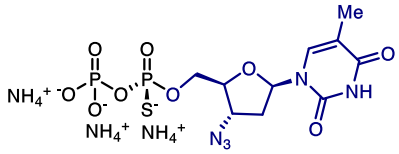
^{13}C NMR (150 MHz, D_2O) δ 166.5, 151.7, 137.3, 111.8, 84.9, 83.0 (d, *J* = 9.8 Hz), 65.7 (d, *J* = 5.8 Hz), 61.0, 36.2, 11.7.

^{31}P NMR (162 MHz, D_2O) δ 41.1 (d, *J* = 29.9 Hz), -6.7 (d, *J* = 29.9 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_{10}H_{14}N_5O_9P_2S$ $[M-H]^-$: 441.9987, found: 441.9979.

Retention time: 11.57 min (*Method 1*)

Compound (S_P)-4



5'-*O*-azidothymidine triammonium (*S*)-diphosphoro-α-thioate

Following the **General Procedure A** compound **(S_P)-4** was obtained from monophosphate precursor **8g** (62 mg, 0.2 mmol), (–)-Ψ* (128 mg, 0.3 mmol) and azidothymidine (**3**) (133 mg, 0.5 mmol). Deprotection was performed at room temperature. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 20:80) to afford 49 mg of the title compound after lyophilization (**Yield = 50%, d.r. > 20:1**)

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 7.78 (s, 1H), 6.28 (t, *J* = 6.8 Hz, 1H), 4.60 (dt, *J* = 6.9, 3.7 Hz, 1H), 4.28-4.21 (m, 3H), 2.56-2.47 (m, 2H), 1.96 (s, 3H).

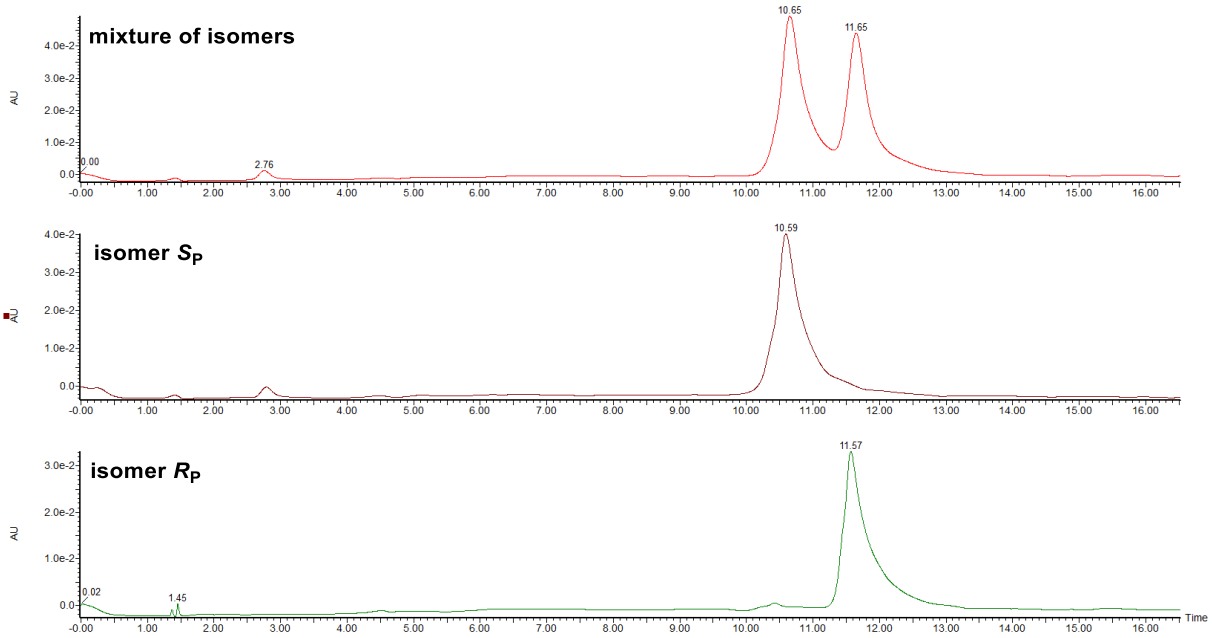
^{13}C NMR (150 MHz, D_2O) δ 166.5, 151.7, 137.3, 111.8, 84.9, 82.9 (d, *J* = 9.7 Hz), 65.4 (d, *J* = 6.1 Hz), 60.7, 36.1, 11.7.

^{31}P NMR (162 MHz, D_2O) δ 42.1 (d, *J* = 28.3 Hz), -9.4 (d, *J* = 28.4 Hz).

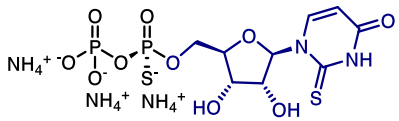
HRMS (ESI-TOF) m/z: calculated for $C_{10}H_{14}N_5O_9P_2S$ $[M-H]^-$: 441.9987, found: 441.9979.

Retention time: 10.59 min (*Method 1*)

LC trace for compound 4:



Compound (R_P)-12



5'-*O*-2-thiouridine triammonium (*R*)-diphosphoro-α-thioate

Following the **General Procedure A** compound **(R_P)-12** was obtained from monophosphate precursor **8g** (62 mg, 0.2 mmol), (+)-Ψ* (128 mg, 0.3 mmol) and regioisomeric mixture of protected 2-thiouridine derivatives **S47a** and **S47b** (286 mg, 0.5 mmol). Deprotection was performed at room temperature. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 35:65) to afford 32 mg of the title compound after lyophilization (**Yield = 33%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.26 (d, *J* = 8.2 Hz, 1H), 6.63 (d, *J* = 2.8 Hz, 1H), 6.27 (d, *J* = 8.2 Hz, 1H), 4.46 (dd, *J* = 4.8, 2.8 Hz, 1H), 4.42-4.37 (m, 2H), 4.35-4.28 (m, 2H).

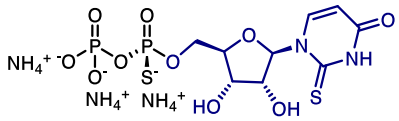
^{13}C NMR (150 MHz, D_2O) δ 175.9, 162.8, 142.3, 106.9, 93.1, 82.7 (d, *J* = 9.7 Hz), 74.6, 68.3, 63.9 (d, *J* = 5.7 Hz).

^{31}P NMR (162 MHz, D_2O) δ 41.9 (d, *J* = 29.0 Hz), -9.6 (d, *J* = 29.0 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_9H_{13}N_2O_{10}P_2S_2$ $[M-H]^-$: 434.9487, found: 434.9483.

Retention time: 4.85 min (*Method 1*)

Compound (S_P)-12



5'-*O*-2-thiouridine triammonium (*S*)-diphosphoro-α-thioate

Following the **General Procedure A** compound **(S_P)-12** was obtained from monophosphate precursor **8g** (62 mg, 0.2 mmol), (–)-Ψ* (128 mg, 0.3 mmol) and regioisomeric mixture of protected 2-thiouridine derivatives **S47a** and **S47b** (286 mg, 0.5 mmol). Deprotection was performed at room temperature. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 35:65) to afford 33 mg of the title compound after lyophilization (**Yield = 34%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.30 (d, *J* = 8.1 Hz, 1H), 6.64 (d, *J* = 2.3 Hz, 1H), 6.28 (d, *J* = 8.1 Hz, 1H), 4.46-4.43 (m, 1H), 4.40-4.36 (m, 2H), 4.35-4.28 (m, 2H).

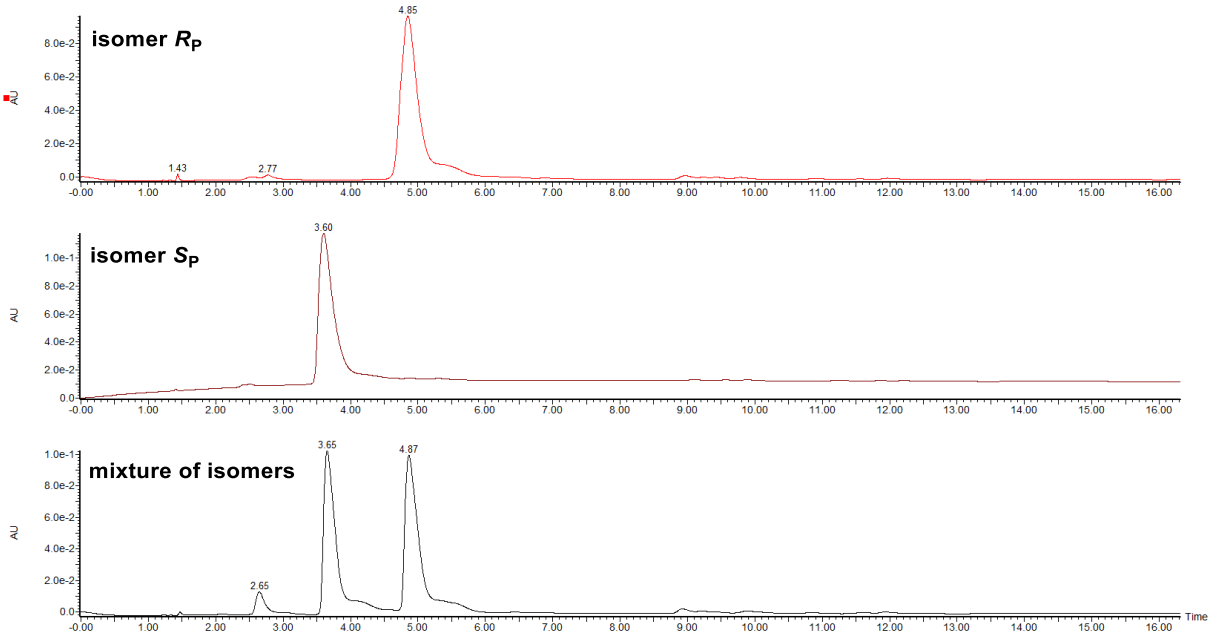
^{13}C NMR (150 MHz, D_2O) δ 175.9, 162.8, 142.4, 107.0, 93.1, 82.7 (d, *J* = 9.4 Hz), 74.6, 68.4, 63.7 (d, *J* = 6.1 Hz).

^{31}P NMR (162 MHz, D_2O) δ 42.5 (d, *J* = 28.9 Hz), -10.3 (d, *J* = 28.9 Hz).

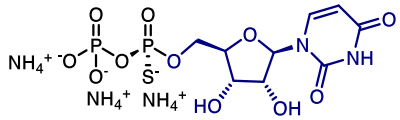
HRMS (ESI-TOF) m/z: calculated for $C_9H_{13}N_2O_{10}P_2S_2$ $[M-H]^-$: 434.9487, found: 434.9483.

Retention time: 3.60 min (*Method 1*)

LC trace for compound 12:



Compound (R_P)-13



5'-*O*-uridine triammonium (*R*)-diphosphoro-α-thioate

Following the **General Procedure A** compound **(R_P)-13** was obtained from monophosphate precursor **8g** (62 mg, 0.2 mmol), (+)-Ψ* (128 mg, 0.3 mmol) and protected uridine **S8** (278 mg, 0.5 mmol). Deprotection was performed at room temperature. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 20:80) to afford 58 mg of the title compound after lyophilization (**Yield = 62%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.05 (d, *J* = 8.1 Hz, 1H), 5.96-5.94 (m, 2H), 4.42 (t, *J* = 5.0 Hz, 1H), 4.39 (t, *J* = 5.0 Hz, 1H), 4.29-4.24 (m, 3H).

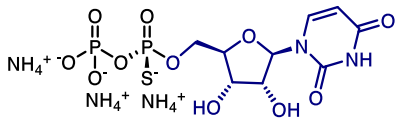
^{13}C NMR (150 MHz, D_2O) δ 166.3, 151.8, 141.9, 102.6, 88.4, 83.2 (d, *J* = 9.7 Hz), 73.8, 69.6, 64.8 (d, *J* = 5.6 Hz).

^{31}P NMR (162 MHz, D_2O) δ 41.1 (d, *J* = 30.9 Hz), -6.8 (d, *J* = 30.9 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_9H_{13}N_2O_{11}P_2S$ $[M-H]^-$: 418.9715, found: 418.9705.

Retention time: 2.87 min (*Method 1*)

Compound (S_P)-13



5'-*O*-uridine triammonium (*S*)-diphosphoro-*α*-thioate

Following the **General Procedure A** compound **(S_P)-13** was obtained from monophosphate precursor **8g** (62 mg, 0.2 mmol), (–)-Ψ* (128 mg, 0.3 mmol) and protected uridine **S8** (278 mg, 0.5 mmol). Deprotection was performed at room temperature. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 20:80) to afford 60 mg of the title compound after lyophilization (**Yield = 64%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.09 (d, *J* = 8.1 Hz, 1H), 5.99-5.97 (m, 2H), 4.42 (t, *J* = 4.8 Hz, 1H), 4.40 (t, *J* = 4.8 Hz, 1H), 4.31-4.29 (m, 1H), 4.29-4.25 (m, 2H).

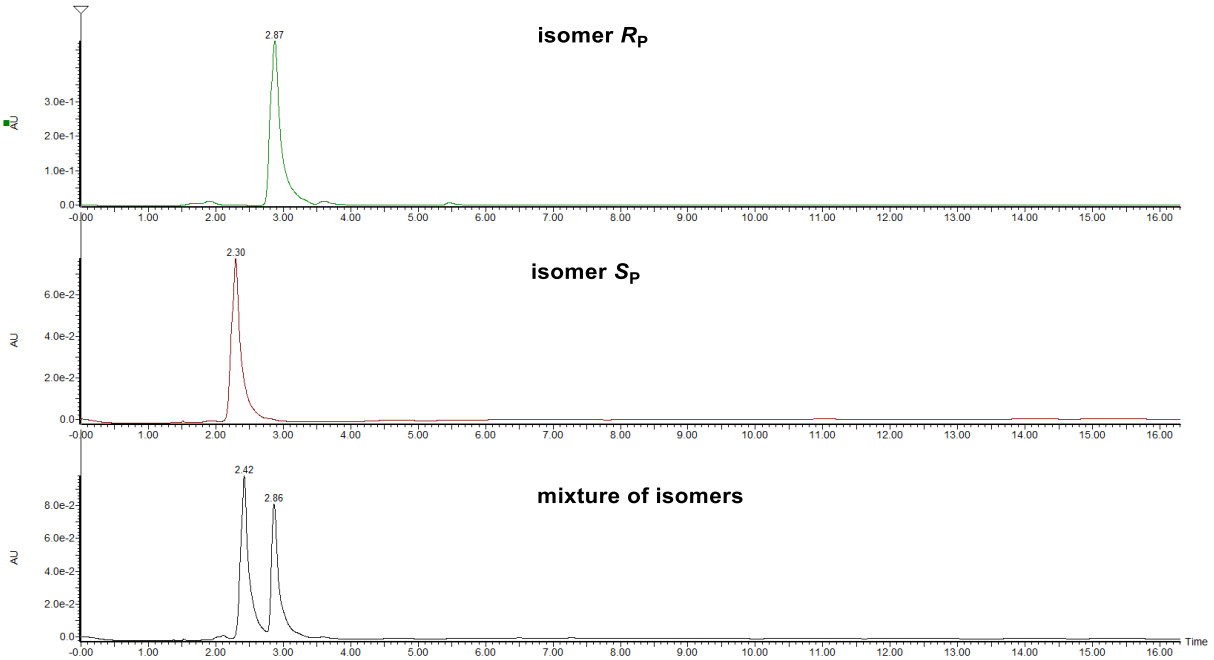
^{13}C NMR (150 MHz, D_2O) δ 166.3, 151.8, 142.0, 102.6, 88.5, 83.1 (d, *J* = 9.5 Hz), 73.8, 69.6, 64.5 (d, *J* = 6.5 Hz).

^{31}P NMR (162 MHz, D_2O) δ 41.7 (d, *J* = 28.5 Hz), -9.7 (d, *J* = 28.5 Hz).

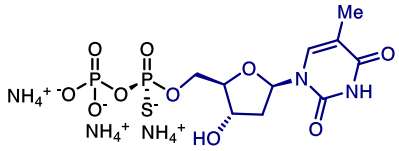
HRMS (ESI-TOF) m/z: calculated for $C_9H_{13}N_2O_{11}P_2S$ $[M-3NH_3-H]^-$: 418.9715, found: 418.9705.

Retention time: 2.30 min (*Method 1*)

LC trace for compound 13:



Compound (R_P)-14



5'-*O*-thymidine triammonium (*R*)-diphosphoro-α-thioate

Following the **General Procedure A** compound **(R_P)-14** was obtained from monophosphate precursor **8g** (62 mg, 0.2 mmol), (+)-Ψ* (128 mg, 0.3 mmol) and protected thymidine **S11** (225 mg, 0.5 mmol). Deprotection was performed at room temperature. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 20:80) to afford 44 mg of the title compound after lyophilization (**Yield = 47%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 7.79 (s, 1H), 6.35 (t, *J* = 6.9 Hz, 1H), 4.67 (dt, *J* = 6.5, 3.7 Hz, 1H), 4.25-4.18 (m, 3H), 2.41 (dt, *J* = 13.8, 6.9 Hz, 1H), 2.35 (ddd, *J* = 13.8, 6.9, 3.0 Hz, 1H), 1.96 (s, 3H).

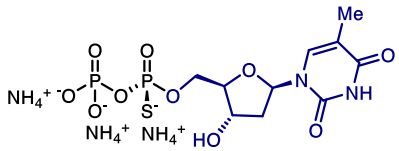
^{13}C NMR (150 MHz, D_2O) δ 166.6, 151.8, 137.4, 111.8, 85.3 (d, *J* = 9.6 Hz), 84.9, 70.9, 65.4 (d, *J* = 5.9 Hz), 38.5, 11.7.

^{31}P NMR (162 MHz, D_2O) δ 41.3 (d, *J* = 27.3 Hz), -7.1 (d, *J* = 27.3 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_{10}H_{15}N_2O_{10}P_2S$ $[M-H]^-$: 416.9923, found: 416.9934.

Retention time: 4.99 min (*Method 1*)

Compound (S_P)-14



5'-*O*-thymidine triammonium (*S*)-diphosphoro-α-thioate

Following the **General Procedure A** compound **(S_P)-14** was obtained from monophosphate precursor **8g** (62 mg, 0.2 mmol), (–)-Ψ* (128 mg, 0.3 mmol) and protected thymidine **S11** (225 mg, 0.5 mmol). Deprotection was performed at room temperature. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 20:80) to afford 49 mg of the title compound after lyophilization (**Yield = 52%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 7.77 (s, 1H), 6.35 (t, *J* = 6.9 Hz, 1H), 4.66 (dt, *J* = 6.2, 3.2 Hz, 1H), 4.26-4.17 (m, 3H), 2.41 (dt, *J* = 13.8, 6.9 Hz, 1H), 2.36 (ddd, *J* = 13.8, 6.9, 3.6 Hz, 1H), 1.96 (s, 3H).

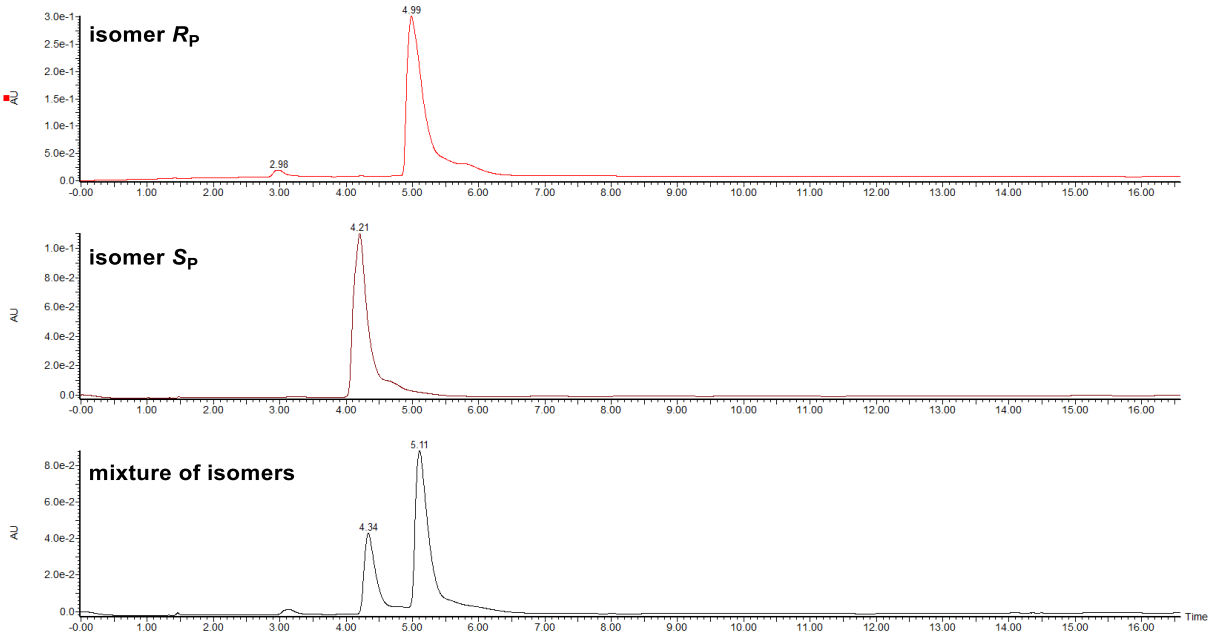
^{13}C NMR (150 MHz, D_2O) δ 166.6, 151.8, 137.4, 111.8, 85.2 (d, *J* = 9.5 Hz), 84.9, 70.9, 65.2 (d, *J* = 6.2 Hz), 38.3, 11.7.

^{31}P NMR (162 MHz, D_2O) δ 42.1 (d, *J* = 29.4 Hz), -9.3 (d, *J* = 29.4 Hz).

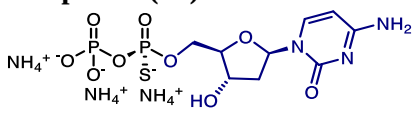
HRMS (ESI-TOF) m/z: calculated for $C_{10}H_{15}N_2O_{10}P_2S$ $[M-H]^-$: 416.9923, found: 416.9934.

Retention time: 4.21 min (*Method 1*)

LC trace for compound 14:



Compound (R_P)-15



5'-*O*-deoxycytidine triammonium (*R*)-diphosphoro-α-thioate

Following the **General Procedure A** with slight modifications compound **(R_P)-15** was obtained from monophosphate precursor **8g** (62 mg, 0.2 mmol), (+)-Ψ* (128 mg, 0.3 mmol) and protected deoxycytidine **S13** (217 mg, 0.5 mmol). Phosphate transfer step was performed using 5.0 equiv. of DBU. Deprotection was performed at 40 °C. The crude product after work-up was neutralized using 10% aq. AcOH and purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 30:70) to afford 38 mg of the title compound after lyophilization (**Yield = 42%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.04 (d, *J* = 7.6 Hz, 1H), 6.33 (t, *J* = 6.6 Hz, 1H), 6.13 (d, *J* = 7.6 Hz, 1H), 4.63 (dt, *J* = 6.6, 3.5 Hz, 1H), 4.25-4.20 (m, 3H), 2.42 (ddd, *J* = 13.9, 6.6, 4.1 Hz, 1H), 2.33 (dt, *J* = 13.9, 6.6 Hz, 1H).

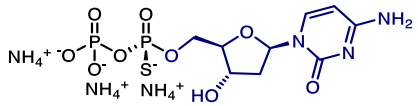
^{13}C NMR (150 MHz, D_2O) δ 165.8, 157.2, 142.0, 96.5, 85.9, 85.3 (d, *J* = 9.6 Hz), 70.6, 65.1 (d, *J* = 5.8 Hz), 39.3.

^{31}P NMR (162 MHz, D_2O) δ 42.2 (d, *J* = 28.5 Hz), -10.0 (d, *J* = 28.5 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_9H_{14}N_3O_9P_2S$ $[M-H]^-$: 401.9926, found: 401.9918.

Retention time: 2.83 min (*Method 1*)

Compound (S_P)-15



5'-*O*-deoxycytidine triammonium (*S*)-diphosphoro-*α*-thioate

Following the **General Procedure A** with slight modifications compound **(S_P)-15** was obtained from monophosphate precursor **8g** (62 mg, 0.2 mmol), (–)-Ψ* (128 mg, 0.3 mmol) and protected deoxycytidine **S13** (217 mg, 0.5 mmol). Phosphate transfer step was performed using 5.0 equiv. of DBU. Deprotection was performed at 40 °C. The crude product after work-up was neutralized using 10% aq. AcOH and purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 30:70) to afford 41 mg of the title compound after lyophilization (**Yield = 45%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.04 (d, *J* = 7.6 Hz, 1H), 6.32 (t, *J* = 6.6 Hz, 1H), 6.13 (d, *J* = 7.6 Hz, 1H), 4.62 (dt, *J* = 6.7, 3.5 Hz, 1H), 4.24-4.18 (m, 3H), 2.42 (ddd, *J* = 13.9, 6.6, 4.1 Hz, 1H), 2.32 (dt, *J* = 13.9, 6.6 Hz, 1H).

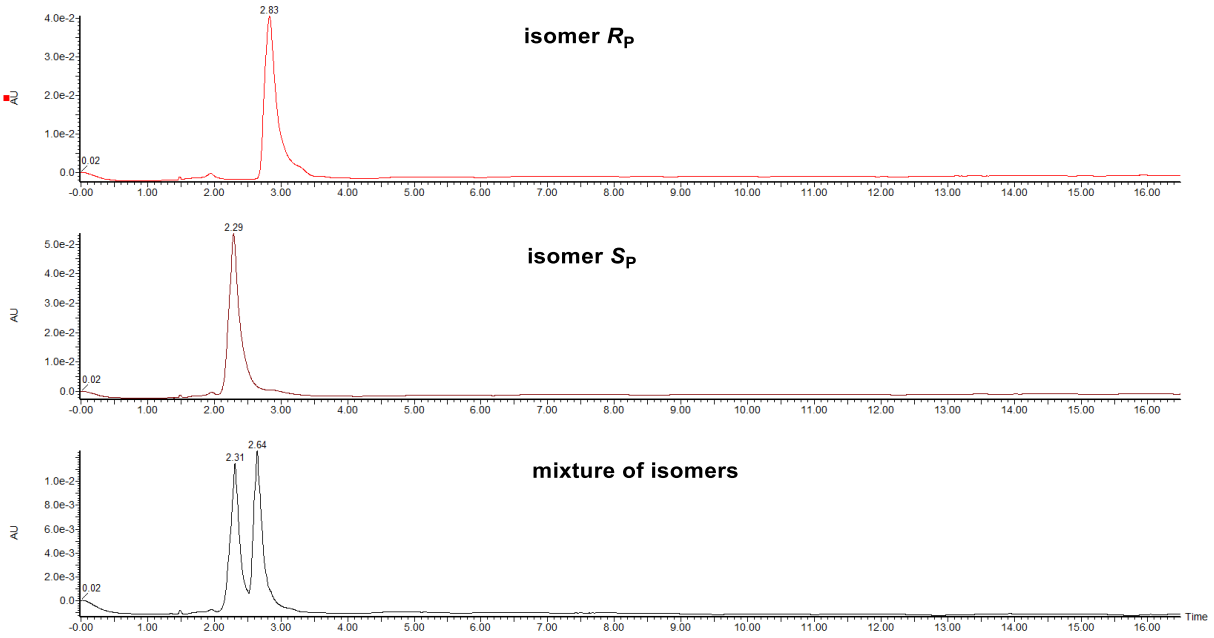
^{13}C NMR (150 MHz, D_2O) δ 165.8, 157.1, 142.0, 96.5, 85.9, 85.3 (d, *J* = 9.7 Hz), 70.7, 65.1 (d, *J* = 6.2 Hz), 39.3.

^{31}P NMR (162 MHz, D_2O) δ 42.2 (d, *J* = 29.1 Hz), -9.7 (d, *J* = 29.1 Hz).

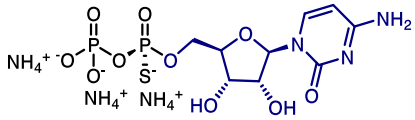
HRMS (ESI-TOF) m/z: calculated for $C_9H_{14}N_3O_9P_2S$ $[M-H]^-$: 401.9926, found: 401.9918.

Retention time: 2.29 min (*Method 1*)

LC trace for compound 15:



Compound (R_P)-16



5'-*O*-cytidine triammonium (*R*)-diphosphoro-α-thioate

Following the **General Procedure A** with slight modifications compound **(R_P)-16** was obtained from monophosphate precursor **8g** (62 mg, 0.2 mmol), (+)-Ψ* (128 mg, 0.3 mmol) and protected cytidine **S14** (277 mg, 0.5 mmol). Phosphate transfer step was performed using 5.0 equiv. of DBU. Deprotection was performed at 40 °C. The crude product after work-up was neutralized using 10% aq. AcOH and purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 30:70) to afford 35 mg of the title compound after lyophilization (**Yield = 37%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.06 (d, *J* = 7.6 Hz, 1H), 6.15 (d, *J* = 7.6 Hz, 1H), 6.00 (d, *J* = 3.8 Hz, 1H), 4.40 (t, *J* = 5.1 Hz, 1H), 4.35-4.25 (m, 4H).

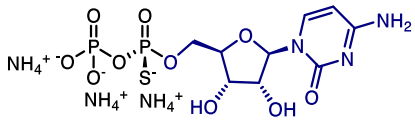
^{13}C NMR (150 MHz, D_2O) δ 165.9, 157.4, 141.8, 96.5, 89.3, 82.6 (d, *J* = 9.9 Hz), 74.2, 69.2, 64.6 (d, *J* = 5.9 Hz).

^{31}P NMR (162 MHz, D_2O) δ 41.3 (d, *J* = 29.1 Hz), -6.8 (d, *J* = 29.1 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_9H_{14}N_3O_{10}P_2S$ $[M-H]^-$: 417.9875, found: 417.9983.

Retention time: 5.75 min (*Method 2*)

Compound (S_P)-16



5'-*O*-cytidine triammonium (*S*)-diphosphoro-α-thioate

Following the **General Procedure A** with slight modifications compound **(S_P)-16** was obtained from monophosphate precursor **8g** (62 mg, 0.2 mmol), (–)-Ψ* (128 mg, 0.3 mmol) and protected cytidine **S14** (277 mg, 0.5 mmol). Phosphate transfer step was performed using 5.0 equiv. of DBU. Deprotection was performed at 40 °C. The crude product after work-up was neutralized using 10% aq. AcOH and purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 30:70) to afford 32 mg of the title compound after lyophilization (**Yield = 34%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.10 (d, *J* = 7.6 Hz, 1H), 6.14 (d, *J* = 7.6 Hz, 1H), 6.00 (d, *J* = 4.1 Hz, 1H), 4.41 (t, *J* = 5.0 Hz, 1H), 4.33 (t, *J* = 4.6 Hz, 1H), 4.31-4.27 (m, 3H).

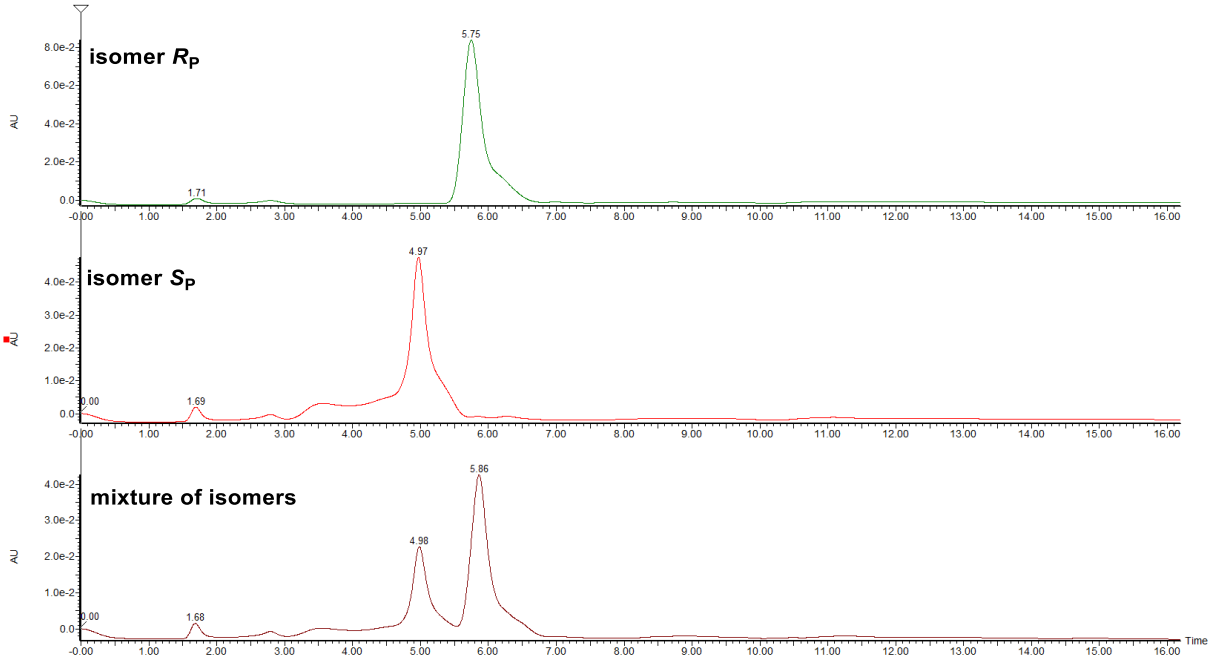
^{13}C NMR (150 MHz, D_2O) δ 166.1, 157.6, 141.9, 96.6, 89.3, 82.6 (d, *J* = 9.7 Hz), 74.3, 69.2, 64.2 (d, *J* = 6.6 Hz).

^{31}P NMR (162 MHz, D_2O) δ 41.2 (d, *J* = 29.3 Hz), -6.7 (d, *J* = 29.3 Hz).

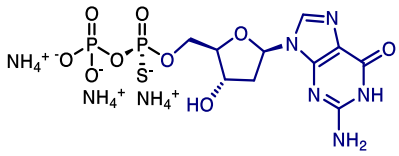
HRMS (ESI-TOF) m/z: calculated for $C_9H_{14}N_3O_{10}P_2S$ $[M-H]^-$: 417.9875, found: 417.9983.

Retention time: 4.97 min (*Method 2*)

LC trace for compound 16:



Compound (R_P)-17



5'-*O*-deoxyguanosine triammonium (*R*)-diphosphoro-α-thioate

Following the **General Procedure A** with slight modifications compound **(R_P)-17** was obtained from monophosphate precursor **8g** (92 mg, 0.3 mmol), (+)-Ψ* (85 mg, 0.2 mmol) and protected deoxyguanosine **S15** (185 mg, 0.5 mmol). Phosphate transfer step was performed using 5.0 equiv. of DBU. Deprotection was performed at 40 °C. After extraction, the aqueous phase was concentrated to ~3 mL, neutralized using 10% aq. AcOH and directly purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 30:70) to afford 32 mg of the title compound after lyophilization (**Yield = 33%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.14 (s, 1H), 6.29 (t, *J* = 6.9 Hz, 1H), 4.29-4.26 (m, 1H), 4.21-4.18 (m, 2H), 2.80 (dt, *J* = 13.8, 6.9 Hz, 1H), 2.51 (ddd, *J* = 13.8, 6.9, 3.5 Hz, 1H).

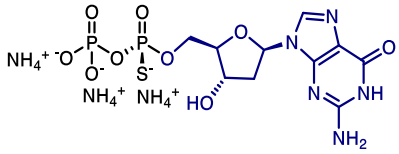
^{13}C NMR (150 MHz, D_2O) δ 158.8, 153.8, 151.3, 137.7, 116.1, 85.5 (d, *J* = 9.5 Hz), 83.5, 71.3, 65.4 (d, *J* = 6.0 Hz), 38.6.

^{31}P NMR (162 MHz, D_2O) δ 42.3 (d, *J* = 28.5 Hz), -10.2 (d, *J* = 28.5 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_{10}H_{14}N_5O_9P_2S$ $[M-H]^-$: 441.9993, found: 441.9999.

Retention time: 9.03 min (*Method 2*)

Compound (S_P)-17



5'-*O*-deoxyguanosine triammonium (*S*)-diphosphoro-α-thioate

Following the **General Procedure A** with slight modifications compound **(S_P)-17** was obtained from monophosphate precursor **8g** (92 mg, 0.3 mmol), (–)-Ψ* (85 mg, 0.2 mmol) and protected deoxyguanosine **S15** (185 mg, 0.5 mmol). Phosphate transfer step was performed using 5.0 equiv. of DBU. Deprotection was performed at 40 °C. After extraction, the aqueous phase was concentrated to ~3 mL, neutralized using 10% aq. AcOH and directly purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 30:70) to afford 31 mg of the title compound after lyophilization (**Yield = 31%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.16 (s, 1H), 6.29 (t, *J* = 6.9 Hz, 1H), 4.29-4.26 (m, 1H), 4.23-4.16 (m, 2H), 2.79 (dt, *J* = 13.7, 6.9 Hz, 1H), 2.52 (ddd, *J* = 13.7, 6.9, 3.5 Hz, 1H).

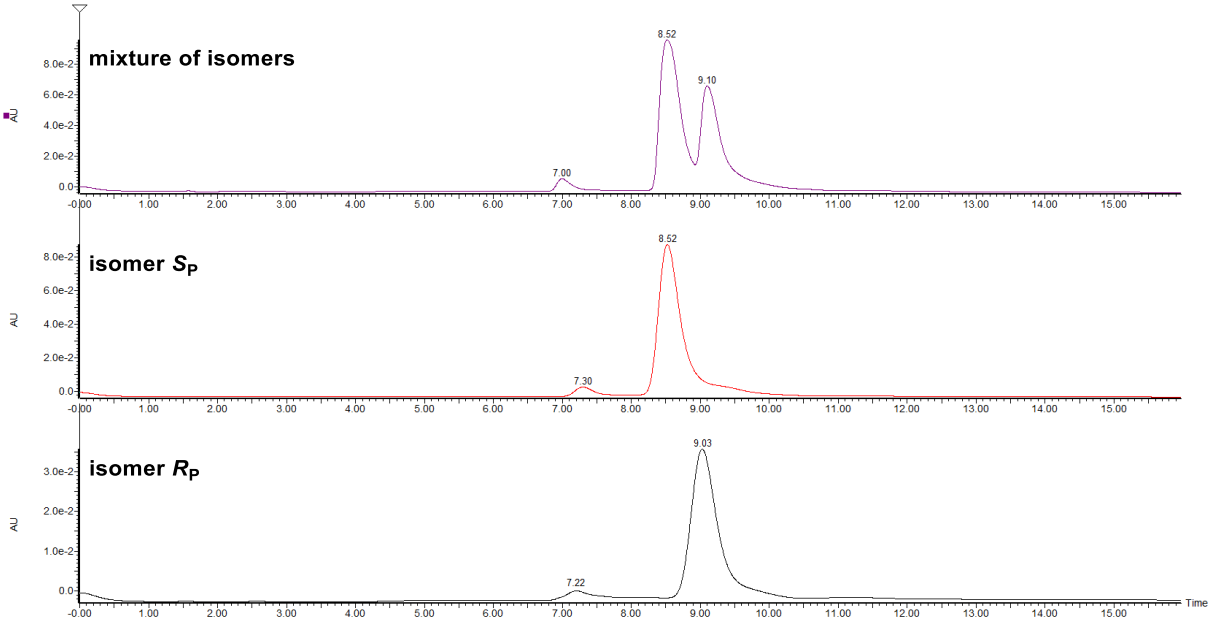
^{13}C NMR (150 MHz, D_2O) δ 158.8, 153.7, 151.2, 137.7, 116.1, 85.5 (d, *J* = 9.4 Hz), 83.6, 71.4, 65.4 (d, *J* = 6.2 Hz), 38.6.

^{31}P NMR (162 MHz, D_2O) δ 42.6 (d, *J* = 29.4 Hz), -10.1 (d, *J* = 29.4 Hz).

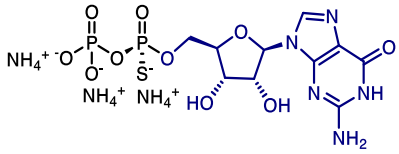
HRMS (ESI-TOF) m/z: calculated for $C_{10}H_{14}N_5O_9P_2S$ $[M-H]^-$: 441.9993, found: 441.9999.

Retention time: 8.52 min (*Method 2*)

LC trace for compound 17:



Compound (R_P)-18



5'-*O*-guanosine triammonium (*R*)-diphosphoro-α-thioate

Following the **General Procedure A** with slight modifications compound **(R_P)-18** was obtained from monophosphate precursor **8g** (92 mg, 0.3 mmol), (+)-Ψ* (85 mg, 0.2 mmol) and protected guanosine **S16** (245 mg, 0.5 mmol). Phosphate transfer step was performed using 5.0 equiv. of DBU. Deprotection was performed at 40 °C. After extraction, the aqueous phase was concentrated to ~3 mL, neutralized using 10% aq. AcOH and directly purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 30:70) to afford 34 mg of the title compound after lyophilization (**Yield = 33%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.18 (s, 1H), 5.92 (d, *J* = 5.6 Hz, 1H), 4.58-4.54 (m, 1H), 4.40-4.34 (m, 1H), 4.29-4.24 (m, 2H) (*One signal overlapping with D_2O*).

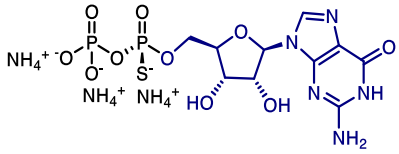
^{13}C NMR (150 MHz, D_2O) δ 158.9, 153.9, 151.7, 137.7, 116.1, 86.9, 83.7 (d, *J* = 9.7 Hz), 73.9, 70.4, 65.2 (d, *J* = 5.8 Hz).

^{31}P NMR (162 MHz, D_2O) δ 42.3 (d, *J* = 28.2 Hz), -10.2 (d, *J* = 28.2 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_{10}H_{14}N_5O_{10}P_2S$ $[M-H]^-$: 457.9942, found: 457.9951.

Retention time: 7.60 min (*Method 2*)

Compound (S_P)-18



5'-*O*-guanosine triammonium (*S*)-diphosphoro-α-thioate

Following the **General Procedure A** with slight modifications compound **(S_P)-18** was obtained from monophosphate precursor **8g** (92 mg, 0.3 mmol), (–)-Ψ* (85 mg, 0.2 mmol) and protected guanosine **S16** (245 mg, 0.5 mmol). Phosphate transfer step was performed using 5.0 equiv. of DBU. Deprotection was performed at 40 °C. After extraction, the aqueous phase was concentrated to ~3 mL, neutralized using 10% aq. AcOH and directly purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 30:70) to afford 38 mg of the title compound after lyophilization (**Yield = 37%, d.r. > 20:1**).

Physical state: white amorphous solid

^{1}H NMR (600 MHz, D_2O) δ 8.21 (s, 1H), 5.92 (d, *J* = 5.8 Hz, 1H), 4.78-4.75 (m, 1H), 4.57-4.55 (m, 1H), 4.39-4.36 (m, 1H), 4.30-4.23 (m, 2H).

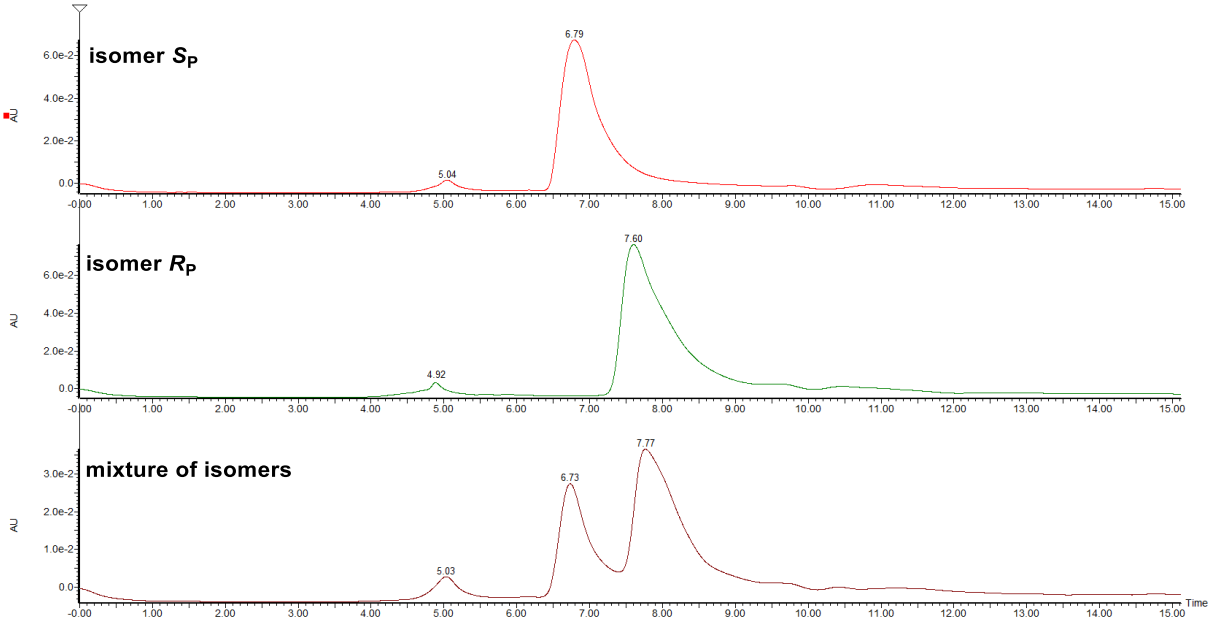
^{13}C NMR (150 MHz, D_2O) δ 158.8, 153.8, 151.6, 137.7, 116.1, 86.9, 83.6 (d, *J* = 9.7 Hz), 73.9, 70.4, 65.1 (d, *J* = 6.1 Hz).

^{31}P NMR (162 MHz, D_2O) δ 42.6 (d, *J* = 27.0 Hz), -10.2 (d, *J* = 27.0 Hz).

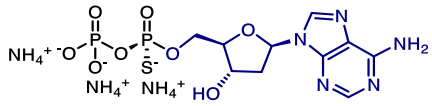
HRMS (ESI-TOF) m/z: calculated for $C_{10}H_{14}N_5O_{10}P_2S$ $[M-H]^-$: 457.9942, found: 457.9951.

Retention time: 6.79 min (*Method 2*)

LC trace for compound 18:



Compound (R_P)-19



5'-*O*-deoxyadenosine triammonium (*R*)-diphosphoro-α-thioate

Following the **General Procedure A** compound **(R_P)-19** was obtained from monophosphate precursor **8g** (62 mg, 0.2 mmol), (+)-Ψ* (128 mg, 0.3 mmol) and protected deoxyadenosine **S12** (281 mg, 0.5 mmol). Deprotection was performed at 40 °C. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 30:70) to afford 60 mg of the title compound after lyophilization (**Yield = 63%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.47 (s, 1H), 8.11 (s, 1H), 6.41 (t, *J* = 6.7 Hz, 1H), 4.30-4.28 (m, 1H), 4.22 (ddd, *J* = 11.2, 7.4, 3.7 Hz, 1H), 4.16 (ddd, *J* = 11.2, 6.6, 3.8 Hz, 1H), 2.79 (dt, *J* = 13.6, 6.7 Hz, 1H), 2.59 (ddd, *J* = 13.6, 6.7, 4.0 Hz, 1H) (*One signal overlapping with D_2O*).

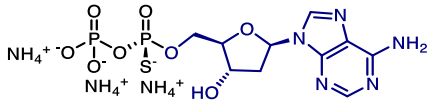
^{13}C NMR (150 MHz, D_2O) δ 155.2, 152.4, 148.3, 139.9, 118.3, 85.6 (d, *J* = 9.5 Hz), 83.5, 71.0, 65.1 (d, *J* = 6.0 Hz), 39.0.

^{31}P NMR (162 MHz, D_2O) δ 41.1 (d, *J* = 29.4 Hz), -8.9 (d, *J* = 29.4 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_{10}H_{14}N_5O_8P_2S$ $[M-H]^-$: 426.0038, found: 426.0033.

Retention time: 6.17 min (*Method 1*)

Compound (S_P)-19



5'-*O*-deoxyadenosine triammonium (*S*)-diphosphoro-α-thioate

Following the **General Procedure A** compound **(S_P)-19** was obtained from monophosphate precursor **8g** (62 mg, 0.2 mmol), (–)-Ψ* (128 mg, 0.3 mmol) and protected deoxyadenosine **S12** (281 mg, 0.5 mmol). Deprotection was performed at 40 °C. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 30:70) to afford 55 mg of the title compound after lyophilization (**Yield = 57%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.47 (s, 1H), 8.08 (s, 1H), 6.39 (t, *J* = 6.7 Hz, 1H), 4.79-4.75 (m, 1H), 4.31-4.29 (m, 1H), 4.22 (ddd, *J* = 11.5, 7.7, 3.9 Hz, 1H), 4.16 (ddd, *J* = 11.5, 6.6, 3.7 Hz, 1H), 2.77 (dt, *J* = 13.6, 6.7 Hz, 1H), 2.59 (ddd, *J* = 13.6, 6.7, 3.6 Hz, 1H).

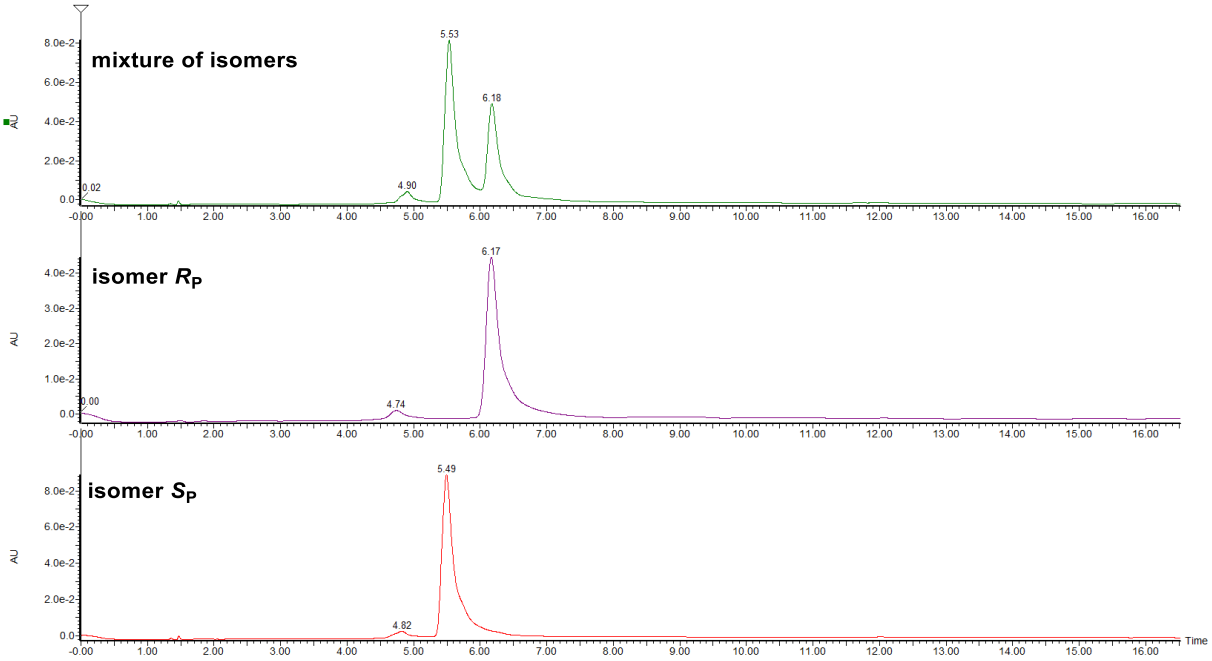
^{13}C NMR (150 MHz, D_2O) δ 154.9, 152.0, 148.2, 140.0, 118.2, 85.6 (d, *J* = 9.4 Hz), 83.7, 71.2, 65.4 (d, *J* = 6.3 Hz), 39.2.

^{31}P NMR (162 MHz, D_2O) δ 42.0 (d, *J* = 28.0 Hz), -10.8 (d, *J* = 28.0 Hz).

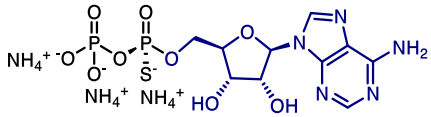
HRMS (ESI-TOF) m/z: calculated for $C_{10}H_{14}N_5O_8P_2S$ $[M-H]^-$: 426.0038, found: 426.0033.

Retention time: 5.49 min (*Method 1*)

LC trace for compound 19:



Compound (R_P)-20



5'-*O*-adenosine triammonium (*R*)-diphosphoro-α-thioate

Following the **General Procedure A** compound **(R_P)-20** was obtained from monophosphate precursor **8g** (62 mg, 0.2 mmol), (+)-Ψ* (128 mg, 0.3 mmol) and protected adenosine **S9** (341 mg, 0.5 mmol). Deprotection was performed at 40 °C. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 30:70) to afford 55 mg of the title compound after lyophilization (**Yield = 56%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.54 (s, 1H), 8.13 (s, 1H), 6.08 (d, *J* = 5.4 Hz, 1H), 4.79-4.74 (m, 1H), 4.59 (t, *J* = 4.6 Hz, 1H), 4.41-4.39 (m, 1H), 4.31-4.25 (m, 2H).

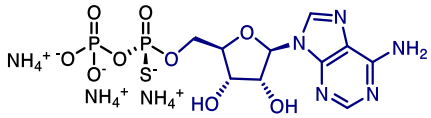
^{13}C NMR (150 MHz, D_2O) δ 155.4, 152.6, 148.9, 140.0, 118.4, 87.0, 83.8 (d, *J* = 9.6 Hz), 74.4, 70.4, 65.1 (d, *J* = 5.6 Hz).

^{31}P NMR (162 MHz, D_2O) δ 42.0 (d, *J* = 29.2 Hz), -8.3 (d, *J* = 29.2 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_{10}H_{14}N_5O_9P_2S$ $[M-H]^-$: 441.9987, found: 441.9988.

Retention time: 5.06 min (*Method 1*)

Compound (S_P)-20



5'-*O*-adenosine triammonium (*S*)-diphosphoro-α-thioate

Following the **General Procedure A** compound **(S_P)-20** was obtained from monophosphate precursor **8g** (62 mg, 0.2 mmol), (–)-Ψ* (128 mg, 0.3 mmol) and protected adenosine **S9** (342 mg, 0.5 mmol). Deprotection was performed at 40 °C. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 30:70) to afford 52 mg of the title compound after lyophilization (**Yield = 53%, d.r. > 20:1**).

Preparative scale: Following the **General Procedure A** compound **(S_P)-20** was obtained from monophosphate precursor **8g** (0.62 g, 2.0 mmol), (–)-Ψ* (1.28 g, 3.0 mmol) and protected adenosine **S9** (3.42 g, 5.0 mmol). The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 30:70) to afford 620 mg of the title compound after lyophilization (**Yield = 63%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.55 (s, 1H), 8.09 (s, 1H), 6.06 (d, *J* = 5.3 Hz, 1H), 4.74-4.72 (m, 1H), 4.57 (t, *J* = 4.6 Hz, 1H), 4.40-4.37 (m, 1H), 4.29 (ddd, *J* = 10.7, 7.5, 3.0 Hz, 1H), 4.26-4.23 (m, 1H).

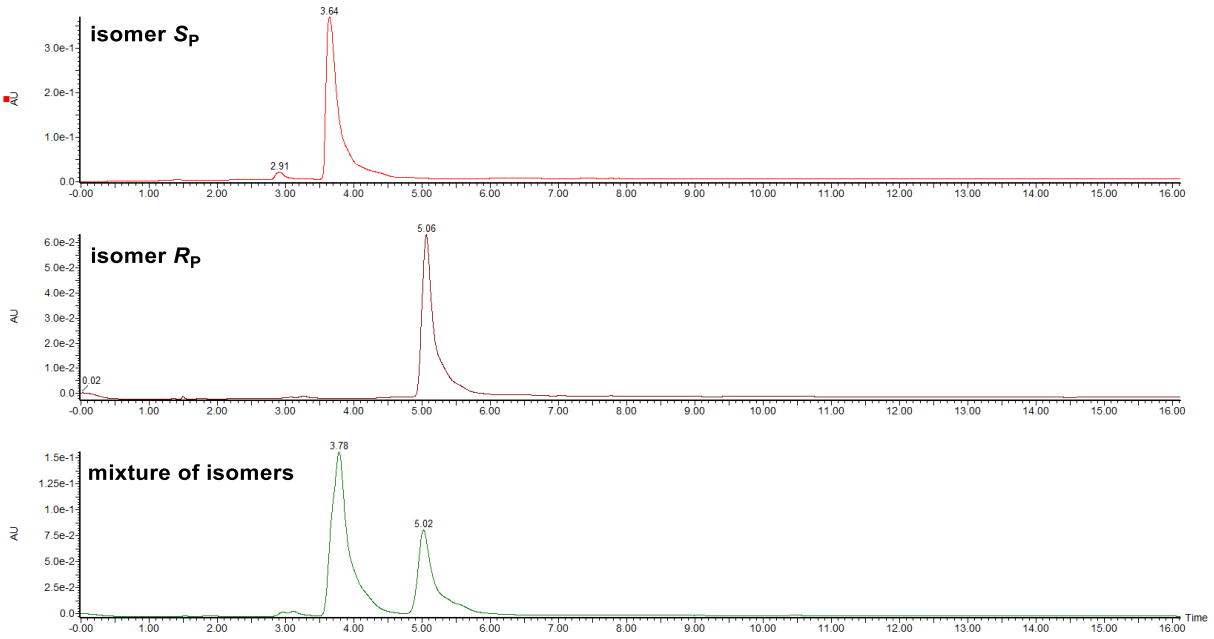
^{13}C NMR (150 MHz, D_2O) δ 155.1, 152.4, 148.6, 140.0, 118.2, 87.0, 83.6 (d, *J* = 9.4 Hz), 74.4, 70.2, 64.7 (d, *J* = 6.1 Hz).

^{31}P NMR (162 MHz, D_2O) δ 42.0 (d, *J* = 29.1 Hz), -8.1 (d, *J* = 29.1 Hz).

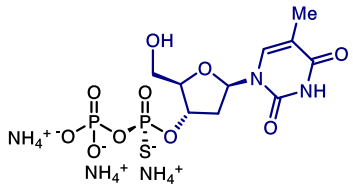
HRMS (ESI-TOF) m/z: calculated for $C_{10}H_{14}N_5O_9P_2S$ [M-H]$^-$: 441.9987, found: 441.9988.

Retention time: 3.64 min (*Method 1*)

LC trace for compound 20:



Compound (R_P)-21



3'-*O*-thymidine triammonium (*R*)-diphosphoro-α-thioate

Following the **General Procedure A** compound **(R_P)-21** was obtained from monophosphate precursor **8g** (62 mg, 0.2 mmol), (+)-Ψ* (128 mg, 0.3 mmol) and protected thymidine **S10** (173 mg, 0.5 mmol). Deprotection was performed at 40 °C. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 20:80) to afford 47 mg of the title compound after lyophilization (**Yield = 50%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 7.70 (s, 1H), 6.34 (t, *J* = 6.8 Hz, 1H), 5.05 (ddt, *J* = 10.7, 7.0, 3.8 Hz, 1H), 4.27 (q, *J* = 3.8 Hz, 1H), 3.88 (d, *J* = 3.8 Hz, 2H), 2.63 (ddd, *J* = 14.3, 6.8, 3.8 Hz, 1H), 2.49 (ddd, *J* = 14.3, 7.0, 6.8 Hz, 1H), 1.90 (s, 3H).

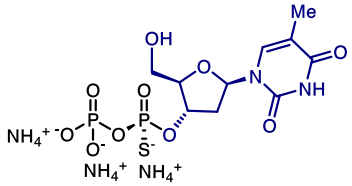
^{13}C NMR (150 MHz, D_2O) δ 166.5, 151.7, 137.6, 111.5, 85.4 (d, *J* = 6.2 Hz), 84.8, 74.6 (d, *J* = 5.7 Hz), 60.7, 37.5 (d, *J* = 3.7 Hz), 11.5.

^{31}P NMR (162 MHz, D_2O) δ 41.0 (d, *J* = 28.5 Hz), -8.3 (d, *J* = 28.5 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_{10}H_{15}N_2O_{10}P_2S$ [M-H]$^-$: 416.9928, found: 416.9926.

Retention time: 7.96 min (*Method 3*)

Compound (S_P)-21



3'-*O*-thymidine triammonium (*S*)-diphosphoro-α-thioate

Following the **General Procedure A** compound **(S_P)-21** was obtained from monophosphate precursor **8g** (62 mg, 0.2 mmol), (–)-Ψ* (128 mg, 0.3 mmol) and protected thymidine **S10** (173 mg, 0.5 mmol). Deprotection was performed at 40 °C. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 20:80) to afford 50 mg of the title compound after lyophilization (**Yield = 53%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 7.69 (d, *J* = 1.2 Hz, 1H), 6.35 (t, *J* = 7.0 Hz, 1H), 5.05 (ddt, *J* = 10.4, 7.0, 3.6 Hz, 1H), 4.26 (dt, *J* = 4.6, 3.6 Hz, 1H), 3.90 (dd, *J* = 12.7, 3.6 Hz, 1H), 3.87 (dd, *J* = 12.7, 4.6 Hz, 1H), 2.65 (ddd, *J* = 14.3, 7.0, 3.6 Hz, 1H), 2.46 (dt, *J* = 14.3, 7.0 Hz, 1H), 1.90 (d, *J* = 1.2 Hz, 3H).

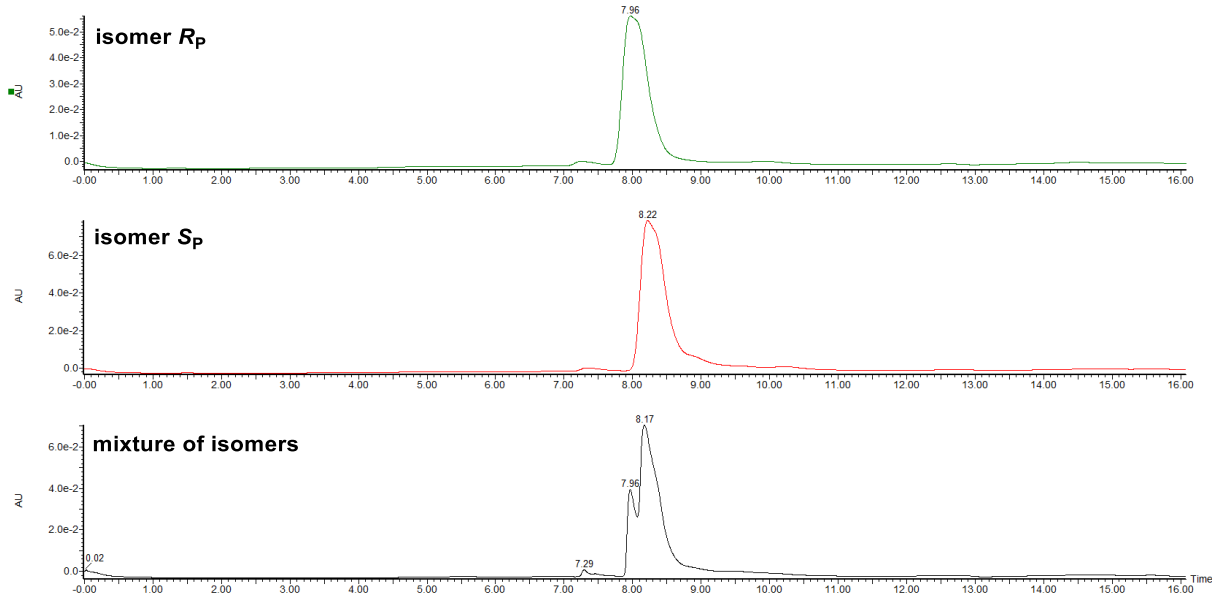
^{13}C NMR (150 MHz, D_2O) δ 166.5, 151.7, 137.6, 111.5, 85.6 (d, *J* = 6.5 Hz), 85.0, 75.1 (d, *J* = 6.1 Hz), 60.9, 37.4 (d, *J* = 3.5 Hz), 11.5.

^{31}P NMR (162 MHz, D_2O) δ 41.7 (d, *J* = 27.8 Hz), -10.2 (d, *J* = 27.8 Hz).

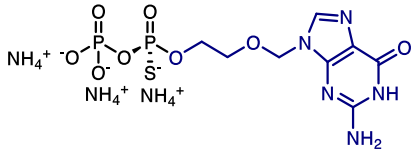
HRMS (ESI-TOF) m/z: calculated for $C_{10}H_{15}N_2O_{10}P_2S$ $[M-H]^-$: 416.9928, found: 416.9926.

Retention time: 8.22 min (*Method 3*)

LC trace for compound 21:



Compound (R_P)-22



acycloguanosine triammonium (*R*)-diphosphoro-α-thioate

Following the **General Procedure A** with slight modifications compound **(R_P)-22** was obtained from monophosphate precursor **8g** (92 mg, 0.3 mmol), (+)-Ψ* (85 mg, 0.2 mmol) and acyclovir (112 mg, 0.5 mmol), using anhydrous DMF (2.0 mL) as a solvent. Phosphate transfer step was performed using 5.0 equiv. of DBU. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 25:75) to afford 42 mg of the title compound after lyophilization (**Yield = 47%, *e.r.* > 20:1**).

Note: Due to difficulties in separation of the enantiomers by HPLC, e.e. was confirmed via derivatization strategy (see section 8.4).

Physical state: white amorphous solid

$[\alpha]_D^{20}$ = +10.6 (*c* 1.01, DMSO) (*measured as triethylammonium salt*)

^{1}H NMR (600 MHz, D_2O) δ 7.91 (s, 1H), 5.50 (s, 2H), 4.11-4.04 (m, 2H), 3.77 (t, *J* = 4.5 Hz, 2H).

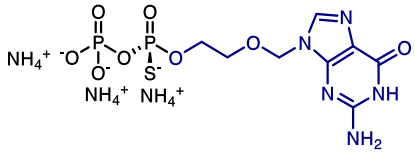
^{13}C NMR (150 MHz, D_2O) δ 158.4, 153.5, 151.1, 139.5, 115.5, 72.2, 67.7 (d, *J* = 8.8 Hz), 64.4 (d, *J* = 5.9 Hz).

^{31}P NMR (162 MHz, D_2O) δ 41.4 (d, *J* = 29.3 Hz), -6.8 (d, *J* = 29.3 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_8H_{12}N_5O_8P_2S$ $[M-H]^-$: 399.9887, found: 399.9879.

Retention time: 7.75 min (*Method 2*)

Compound (S_P)-22



acycloguanosine triammonium (*S*)-diphosphoro-α-thioate

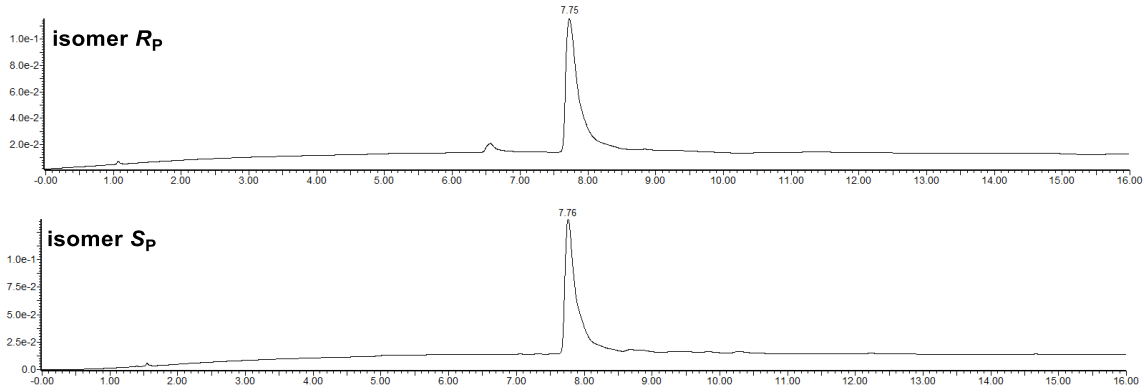
Following the **General Procedure A** with slight modifications compound **(S_P)-22** was obtained from monophosphate precursor **8g** (92 mg, 0.3 mmol), (–)-Ψ* (85 mg, 0.2 mmol) and acyclovir (112 mg, 0.5 mmol), using anhydrous DMF (2.0 mL) as a solvent. Phosphate transfer step was performed using 5.0 equiv. of DBU. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 25:75) to afford 46 mg of the title compound after lyophilization (**Yield = 51%, *e.r.* > 20:1**).

Note: Due to difficulties in separation of the enantiomers by HPLC, e.e. was confirmed via derivatization strategy (see section 8.4).

All characterization data were identical with **(R_P)-22**, except of the optical rotation.

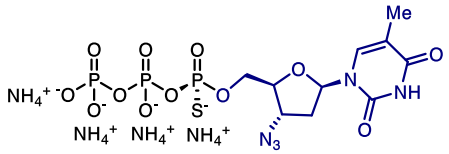
$[\alpha]_D^{20}$ = -11.0 (*c* 0.98, DMSO) (*measured as triethylammonium salt*)

LC trace for compound 22:



8.3.2 Nucleoside Thiotriphosphates

Compound (R_P)-23



5'-*O*-azidothymidine tetraammonium (*R*)-triphosphoro-α-thioate

Following the **General Procedure B** compound **(R_P)-23** was obtained from diphosphate precursor **9c** (169 mg, 0.2 mmol), (+)-Ψ* (112 mg, 0.26 mmol) and azidothymidine (**3**) (107 mg, 0.4 mmol). Deprotection was performed at room temperature. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 30:70) to afford 67 mg of the title compound after lyophilization (**Yield = 57%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 7.80 (s, 1H), 6.29 (t, *J* = 6.9 Hz, 1H), 4.64 (dt, *J* = 6.7, 3.4 Hz, 1H), 4.34-4.25 (m, 3H), 2.54-2.45 (m, 2H), 1.96 (s, 3H).

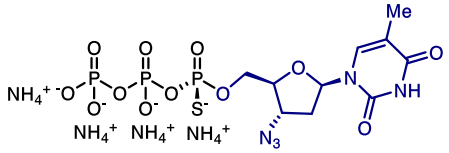
^{13}C NMR (150 MHz, D_2O) δ 166.6, 151.7, 137.3, 111.9, 84.8, 82.9 (d, *J* = 9.8 Hz), 66.1 (d, *J* = 6.1 Hz), 61.1, 36.3, 11.7.

^{31}P NMR (162 MHz, D_2O) δ 42.3 (d, *J* = 27.7 Hz), -9.1 (d, *J* = 19.7 Hz), -24.2 (dd, *J* = 27.7, 19.7 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_{10}H_{15}N_5O_{12}P_3S$ $[M-H]^-$: 521.9651, found: 521.9650.

Retention time: 11.27 min (*Method 1*)

Compound (S_P)-23



5'-*O*-azidothymidine tetraammonium (*S*)-triphosphoro-α-thioate

Following the **General Procedure B** compound **(S_P)-23** was obtained from diphosphate precursor **9c** (169 mg, 0.2 mmol), (–)-Ψ* (112 mg, 0.26 mmol) and azidothymidine (**3**) (107 mg, 0.4 mmol). Deprotection was performed at room temperature. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 30:70) to afford 70 mg of the title compound after lyophilization (**Yield = 59%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 7.78 (s, 1H), 6.28 (t, *J* = 6.9 Hz, 1H), 4.61 (dt, *J* = 7.1, 3.7 Hz, 1H), 4.34-4.23 (m, 3H), 2.55-2.45 (m, 2H), 1.96 (s, 3H).

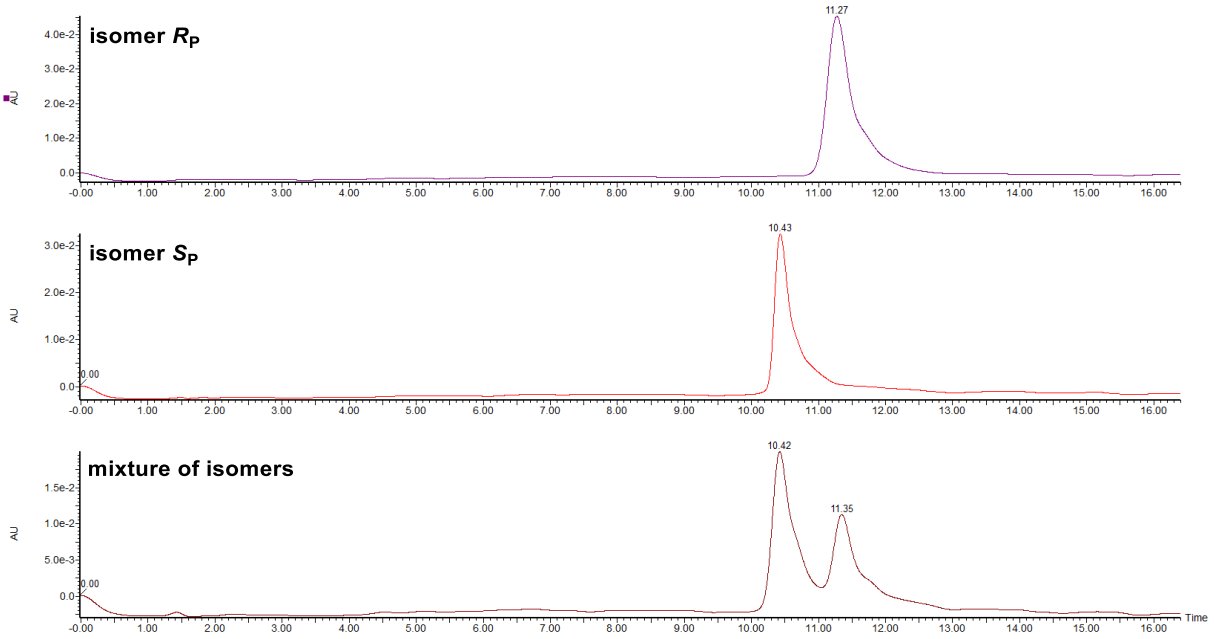
^{13}C NMR (150 MHz, D_2O) δ 166.6, 151.7, 137.3, 111.8, 84.9, 82.8 (d, *J* = 9.8 Hz), 65.7 (d, *J* = 6.4 Hz), 60.8, 36.2, 11.7.

^{31}P NMR (162 MHz, D_2O) δ 43.3 (d, *J* = 26.8 Hz), -9.1 (d, *J* = 20.0 Hz), -23.6 (dd, *J* = 26.8, 20.0 Hz).

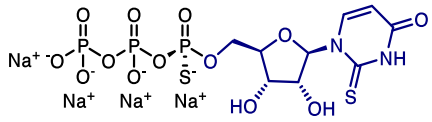
HRMS (ESI-TOF) m/z: calculated for $C_{10}H_{15}N_5O_{12}P_3S$ $[M-H]^-$: 521.9651, found: 521.9650.

Retention time: 10.43 min (*program: Method 1*)

LC trace for compound 23:



Compound (R_P)-24



5'-*O*-2-thiouridine tetrasodium (*R*)-triphosphoro-*α*-thioate

Following the **General Procedure B** compound **(R_P)-24** was obtained from diphosphate precursor **9c** (169 mg, 0.2 mmol), (+)-Ψ* (112 mg, 0.26 mmol) and regioisomeric mixture of protected 2-thiouridine derivatives **S47a** and **S47b** (229 mg, 0.4 mmol). Deprotection was performed at room temperature. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 40:60), followed by reverse-phase chromatography on C18-silica gel (1 M aq. TEAA/MeCN, from 100:0 to 95:5). Fractions containing product were pooled and lyophilized. Obtained solid was redissolved in minimal amount of water and the product was precipitated as sodium salt from 0.2 M $NaClO_4$ in acetone to afford 35 mg of the title compound after drying under high vacuum (**Yield = 29%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.23 (d, *J* = 8.0 Hz, 1H), 6.69 (s, 1H), 6.25 (d, *J* = 8.0 Hz, 1H), 4.48-4.40 (m, 3H), 4.39-4.31 (m, 2H).

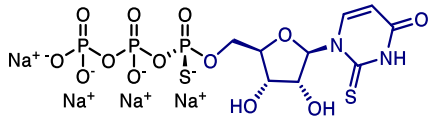
^{13}C NMR (150 MHz, D_2O) δ 176.5, 164.5, 142.2, 106.8, 93.1, 82.7 (d, *J* = 9.7 Hz), 74.7, 68.3, 64.4 (d, *J* = 5.7 Hz).

^{31}P NMR (162 MHz, D_2O) δ 42.6 (d, *J* = 28.3 Hz), -6.0 (d, *J* = 20.3 Hz), -22.7 (dd, *J* = 28.3, 20.3 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_9H_{14}N_2O_{13}P_3S_2$ $[M-H]^-$: 514.9150, found: 514.9168.

Retention time: 5.44 min (*Method 1*)

Compound (S_P)-24



5'-*O*-2-thiouridine tetrasodium (*S*)-triphosphoro-α-thioate

Following the **General Procedure B** compound **(S_P)-24** was obtained from diphosphate precursor **9c** (169 mg, 0.2 mmol), (–)-Ψ* (112 mg, 0.26 mmol) and regioisomeric mixture of protected 2-thiouridine derivatives **S47a** and **S47b** (229 mg, 0.4 mmol). Deprotection was performed at room temperature. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 40:60), followed by reverse-phase chromatography on C18-silica gel (1 M aq. TEAA/MeCN, from 100:0 to 95:5). Fractions containing product were pooled and lyophilized. Obtained solid was redissolved in minimal amount of water and the product was precipitated as sodium salt from 0.2 M $NaClO_4$ in acetone to afford 33 mg of the title compound after drying under high vacuum (**Yield = 27%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.34 (d, *J* = 8.1 Hz, 1H), 6.67 (s, 1H), 6.28 (d, *J* = 8.1 Hz, 1H), 4.48-4.43 (m, 2H), 4.42-4.38 (m, 2H), 4.35-4.31 (m, 1H).

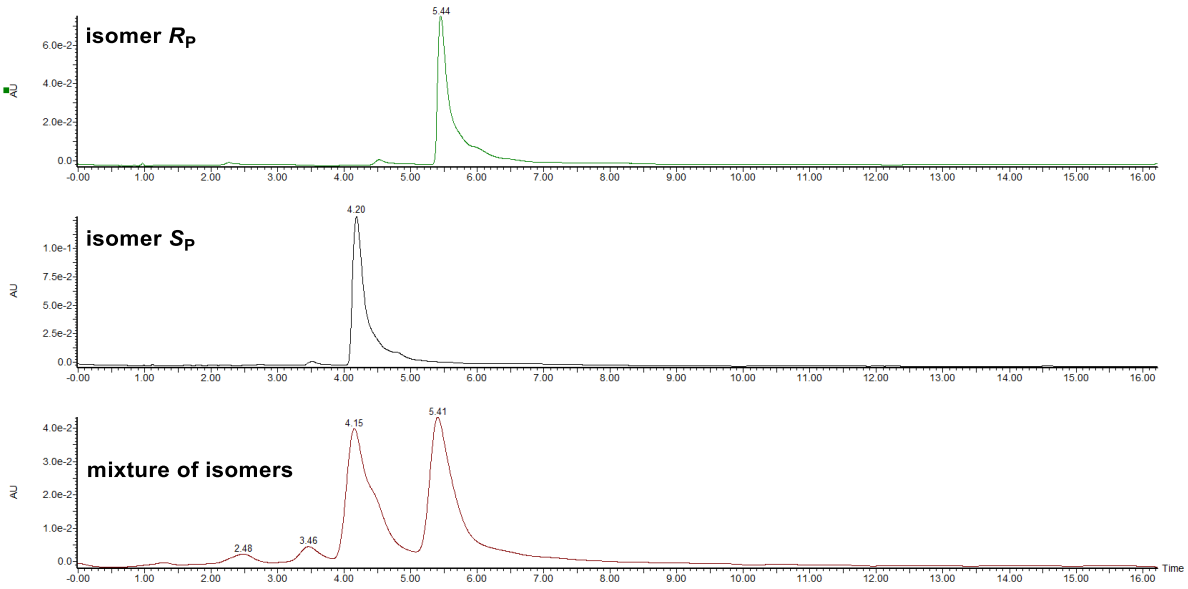
^{13}C NMR (150 MHz, D_2O) δ 176.2, 163.6, 142.5, 107.0, 93.2, 82.8 (d, *J* = 9.4 Hz), 74.7, 68.2, 63.7 (d, *J* = 6.8 Hz).

^{31}P NMR (162 MHz, D_2O) δ 42.9 (d, *J* = 27.1 Hz), -6.2 (d, *J* = 20.0 Hz), -22.6 (dd, *J* = 27.1, 20.0 Hz).

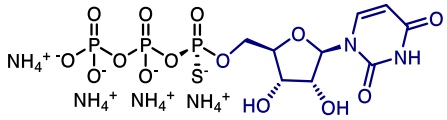
HRMS (ESI-TOF) m/z: calculated for $C_9H_{14}N_2O_{13}P_3S_2$ $[M-H]^-$: 514.9150, found: 514.9168.

Retention time: 4.20 min (*Method 1*)

LC trace for compound 24:



Compound (R_P)-25



5'-*O*-uridine tetraammonium (*R*)-triphosphoro-α-thioate

Following the **General Procedure B** compound **(R_P)-25** was obtained from diphosphate precursor **9c** (169 mg, 0.2 mmol), (+)-Ψ* (112 mg, 0.26 mmol) and protected uridine **S8** (222 mg, 0.4 mmol). Deprotection was performed at room temperature. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 30:70) to afford 53 mg of the title compound after lyophilization (**Yield = 47%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.04 (d, *J* = 8.1 Hz, 1H), 5.99 (d, *J* = 5.1 Hz, 1H), 5.97 (d, *J* = 8.1 Hz, 1H), 4.46-4.43 (m, 1H), 4.41 (t, *J* = 5.1 Hz, 1H), 4.34-4.27 (m, 3H).

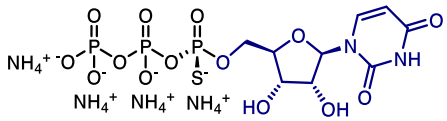
^{13}C NMR (150 MHz, D_2O) δ 166.2, 151.8, 141.9, 102.6, 88.2, 83.2 (d, *J* = 9.7 Hz), 73.8, 69.6, 65.3 (d, *J* = 5.9 Hz).

^{31}P NMR (162 MHz, D_2O) δ 43.1 (d, *J* = 27.7 Hz), -8.9 (d, *J* = 20.0 Hz), -23.6 (dd, *J* = 27.7, 20.0 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_9H_{14}N_2O_{14}P_3S$ $[M-H]^-$: 498.9379, found: 498.9383.

Retention time: 3.67 min (*Method 1*)

Compound (S_P)-25



5'-*O*-uridine tetraammonium (*S*)-triphosphoro-α-thioate

Following the **General Procedure B** compound **(S_P)-25** was obtained from diphosphate precursor **9c** (169 mg, 0.2 mmol), (+)-Ψ* (112 mg, 0.26 mmol) and protected uridine **S8** (222 mg, 0.4 mmol). Deprotection was performed at room temperature. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 30:70) to afford 52 mg of the title compound after lyophilization (**Yield = 46%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.10 (d, *J* = 8.1 Hz, 1H), 6.00 (d, *J* = 5.1 Hz, 1H), 5.98 (d, *J* = 8.1 Hz, 1H), 4.45-4.43 (m, 1H), 4.44 (t, *J* = 5.1 Hz, 1H), 4.33-4.27 (m, 3H).

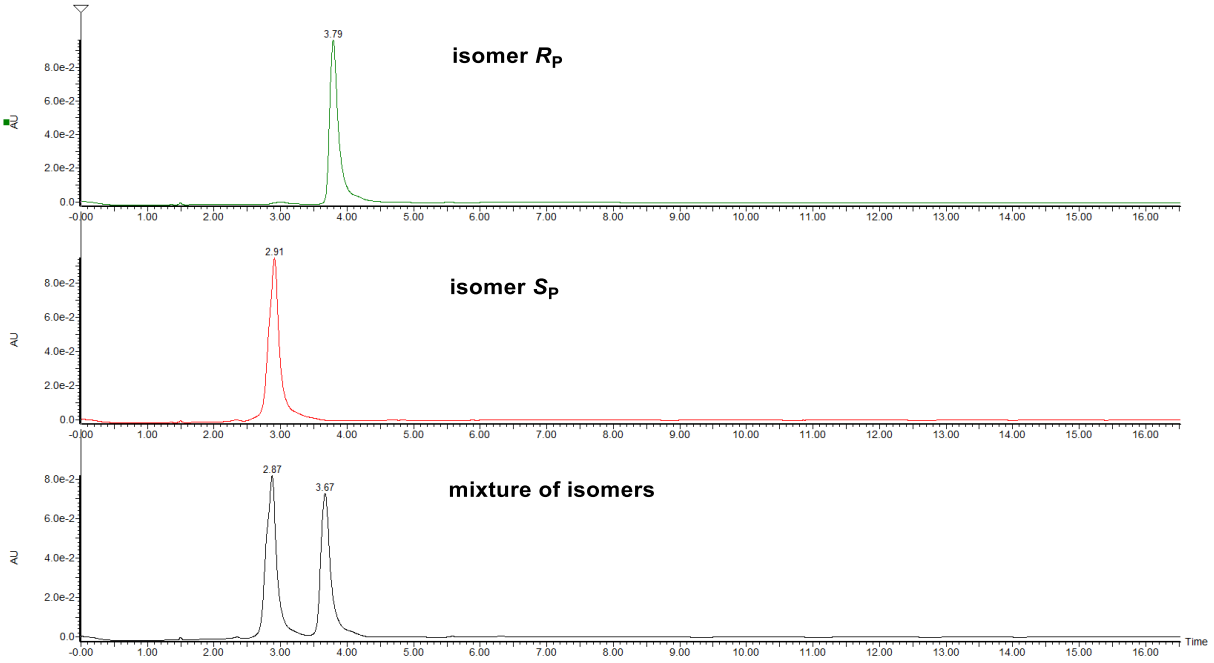
^{13}C NMR (150 MHz, D_2O) δ 166.3, 151.8, 142.1, 102.6, 88.3, 83.2 (d, *J* = 9.5 Hz), 73.8, 69.6, 64.8 (d, *J* = 6.6 Hz).

^{31}P NMR (162 MHz, D_2O) δ 43.4 (d, *J* = 27.1 Hz), -8.7 (d, *J* = 19.9 Hz), -23.5 (dd, *J* = 27.1, 19.9 Hz).

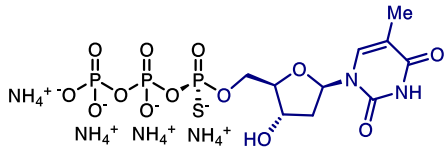
HRMS (ESI-TOF) m/z: calculated for $C_9H_{14}N_2O_{14}P_3S$ $[M-H]^-$: 498.9379, found: 498.9383.

Retention time: 2.91 min (*Method 1*)

LC trace for compound 25:



Compound (R_P)-26



5'-*O*-thymidine tetraammonium (*R*)-triphosphoro-α-thioate

Following the **General Procedure B** compound **(R_P)-26** was obtained from diphosphate precursor **9c** (169 mg, 0.2 mmol), (+)-Ψ* (112 mg, 0.26 mmol) and protected thymidine **S11** (180 mg, 0.4 mmol). Deprotection was performed at room temperature. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 30:70) to afford 54 mg of the title compound after lyophilization (**Yield = 48%, d.r. > 20:1**).

Preparative scale: Following the **General Procedure B** compound **(R_P)-26** was obtained from diphosphate precursor **9c** (1.69 g, 2.0 mmol), (+)-Ψ* (1.12 g, 2.6 mmol) and protected thymidine **S11** (1.80 g, 4.0 mmol). The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 30:70) to afford 566 mg of the title compound after lyophilization (**Yield = 50%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 7.80 (s, 1H), 6.35 (t, *J* = 6.9 Hz, 1H), 4.70 (dt, *J* = 6.5, 3.4 Hz, 1H), 4.30-4.26 (m, 2H), 4.23-4.20 (m, 1H), 2.41 (ddd, *J* = 13.9, 7.5, 6.9 Hz, 1H), 2.35 (ddd, *J* = 13.9, 6.9, 3.6 Hz, 1H), 1.96 (s, 3H).

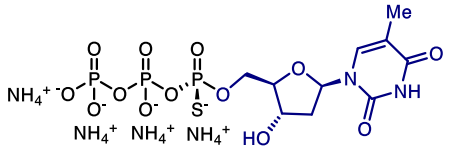
^{13}C NMR (150 MHz, D_2O) δ 166.6, 151.8, 137.4, 111.8, 85.3 (d, *J* = 9.6 Hz), 84.9, 70.8, 65.7 (d, *J* = 6.2 Hz), 38.5, 11.7.

^{31}P NMR (162 MHz, D_2O) δ 42.9 (d, *J* = 28.2 Hz), -8.2 (d, *J* = 20.7 Hz), -23.5 (dd, *J* = 28.2, 20.7 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_{10}H_{16}N_2O_{13}P_3S$ $[M-H]^-$: 496.9586, found: 496.9590.

Retention time: 5.87 min (*Method 1*)

Compound (S_P)-26



5'-*O*-thymidine tetraammonium (*S*)-triphosphoro-α-thioate

Following the **General Procedure B** compound **(S_P)-26** was obtained from diphosphate precursor **9c** (169 mg, 0.2 mmol), (–)-Ψ* (112 mg, 0.26 mmol) and protected thymidine **S11** (180 mg, 0.4 mmol). Deprotection was performed at room temperature. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 30:70) to afford 52 mg of the title compound after lyophilization (**Yield = 46%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 7.77 (s, 1H), 6.36 (t, *J* = 6.9 Hz, 1H), 4.67 (dt, *J* = 6.2, 3.2 Hz, 1H), 4.29 (ddd, *J* = 11.9, 7.6, 4.0 Hz, 1H), 4.25-4.19 (m, 2H), 2.41 (dt, *J* = 13.9, 6.9 Hz, 1H), 2.35 (ddd, *J* = 13.9, 6.9, 3.2 Hz, 1H), 1.96 (s, 3H).

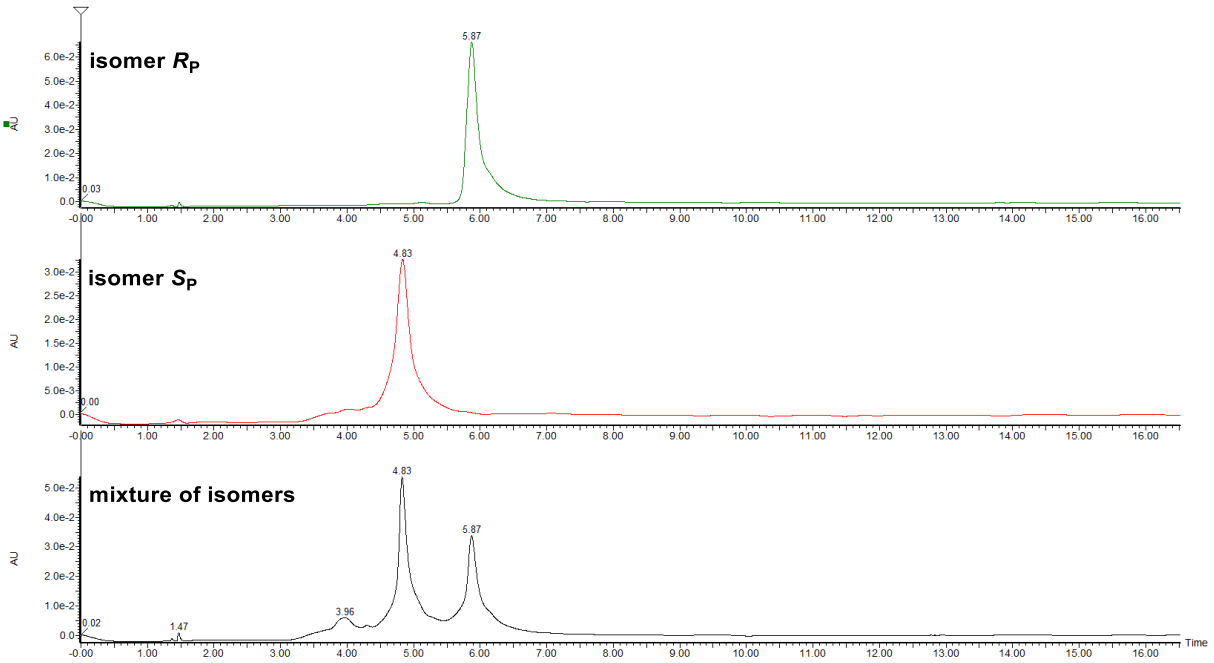
^{13}C NMR (150 MHz, D_2O) δ 166.6, 151.8, 137.4, 111.8, 85.3 (d, *J* = 9.6 Hz), 85.0, 70.9, 65.6 (d, *J* = 6.7 Hz), 38.4, 11.7.

^{31}P NMR (162 MHz, D_2O) δ 42.6 (d, *J* = 26.7 Hz), -7.1 (d, *J* = 20.0 Hz), -23.3 (dd, *J* = 26.7, 20.0 Hz).

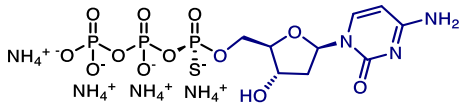
HRMS (ESI-TOF) m/z: calculated for $C_{10}H_{16}N_2O_{13}P_3S$ $[M-H]^-$: 496.9586, found: 496.9590.

Retention time: 4.83 min (*Method 1*)

LC trace for compound 26:



Compound (R_P)-27



5'-*O*-deoxycytidine tetraammonium (*R*)-triphosphoro-*α*-thioate

Following the **General Procedure B** with slight modifications compound **(R_P)-27** was obtained from diphosphate precursor **9c** (169 mg, 0.2 mmol), (+)-Ψ* (112 mg, 0.26 mmol) and protected deoxycytidine **S13** (174 mg, 0.4 mmol). Phosphate transfer step was performed using 8.0 equiv. of DBU. Deprotection was performed at 40 °C. The crude product after work-up was neutralized using 10% aq. AcOH and purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 40:60) to afford 40 mg of the title compound after lyophilization (**Yield = 36%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.02 (d, *J* = 7.6 Hz, 1H), 6.33 (t, *J* = 6.7 Hz, 1H), 6.14 (d, *J* = 7.6 Hz, 1H), 4.66-4.62 (m, 1H), 4.30-4.24 (m, 2H), 4.24-4.19 (m, 1H), 2.44-2.38 (m, 1H), 2.33 (dt, *J* = 13.9, 6.7, 1H).

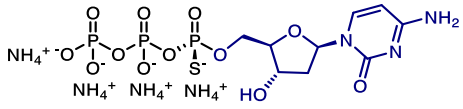
^{13}C NMR (150 MHz, D_2O) δ 166.0, 157.4, 141.8, 96.5, 85.8, 85.3 (d, *J* = 9.6 Hz), 70.4, 65.4 (d, *J* = 6.0 Hz), 39.3.

^{31}P NMR (162 MHz, D_2O) δ 43.1 (d, *J* = 27.4 Hz), -8.1 (d, *J* = 20.4 Hz), -23.4 (dd, *J* = 27.4, 20.4 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_9H_{15}N_3O_{12}P_3S$ $[M-H]^-$: 481.9589, found: 481.9575.

Retention time: 7.58 min (*Method 2*)

Compound (S_P)-27



5'-*O*-deoxycytidine tetraammonium (*S*)-triphosphoro-*α*-thioate

Following the **General Procedure B** with slight modifications compound **(S_P)-27** was obtained from diphosphate precursor **9c** (169 mg, 0.2 mmol), (–)-Ψ* (112 mg, 0.26 mmol) and protected deoxycytidine **S13** (174 mg, 0.4 mmol). Phosphate transfer step was performed using 8.0 equiv. of DBU. Deprotection was performed at 40 °C. The crude product after work-up was neutralized using 10% aq. AcOH and purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 40:60) to afford 41 mg of the title compound after lyophilization (**Yield = 37%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.07 (d, *J* = 7.6 Hz, 1H), 6.34 (t, *J* = 6.6 Hz, 1H), 6.16 (d, *J* = 7.6 Hz, 1H), 4.64 (dt, *J* = 6.8, 3.6 Hz, 1H), 4.31-4.21 (m, 3H), 2.42 (ddd, *J* = 14.1, 6.6, 4.0 Hz, 1H), 2.33 (dt, *J* = 14.1, 6.6, 1H).

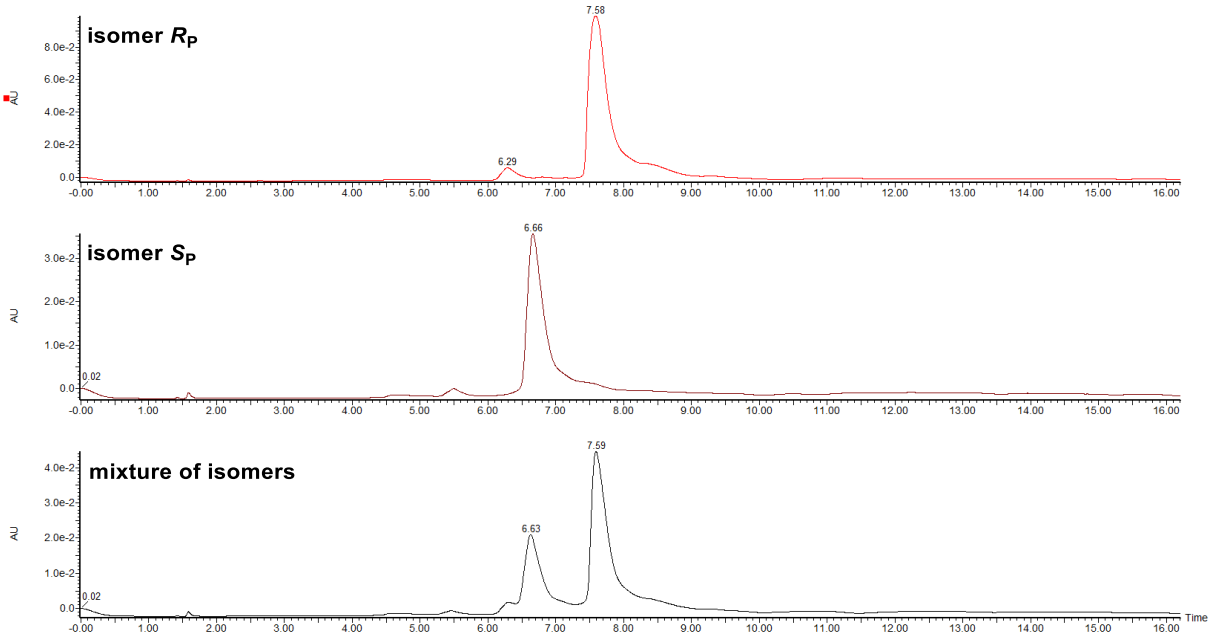
^{13}C NMR (150 MHz, D_2O) δ 165.7, 156.9, 142.1, 96.5, 86.0, 85.4 (d, *J* = 9.7 Hz), 70.7, 65.4 (d, *J* = 6.3 Hz), 39.4.

^{31}P NMR (162 MHz, D_2O) δ 43.4 (d, *J* = 27.1 Hz), -9.9 (d, *J* = 19.9 Hz), -23.9 (dd, *J* = 27.1, 19.9 Hz).

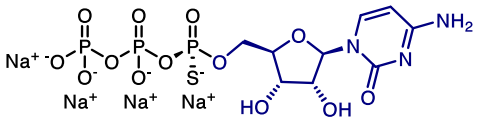
HRMS (ESI-TOF) m/z: calculated for $C_9H_{15}N_3O_{12}P_3S$ $[M-H]^-$: 481.9589, found: 481.9575.

Retention time: 6.66 min (*Method 2*)

LC trace for compound 27:



Compound (R_P)-28



5'-*O*-cytidine tetrasodium (*R*)-triphosphoro-*α*-thioate

Following the **General Procedure B** with slight modifications compound **(R_P)-28** was obtained from diphosphate precursor **9c** (169 mg, 0.2 mmol), (+)-Ψ* (112 mg, 0.26 mmol) and protected cytidine **S14** (222 mg, 0.4 mmol). Phosphate transfer step was performed using 8.0 equiv. of DBU. Deprotection was performed at 40 °C. The crude product after work-up was neutralized using 10% aq. AcOH and purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 40:60), followed by reverse-phase chromatography on C18-silica gel (1 M aq. TEAA/MeCN, from 100:0 to 95:5). Fractions containing product were pooled and lyophilized. Obtained solid was redissolved in minimal amount of water and the product was precipitated as sodium salt from 0.2 M $NaClO_4$ in acetone to afford 45 mg of the title compound, after drying under high vacuum. (**Yield = 38%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.06 (d, *J* = 7.6 Hz, 1H), 6.15 (d, *J* = 7.6 Hz, 1H), 6.01 (d, *J* = 4.3 Hz, 1H), 4.47 (t, *J* = 5.2 Hz, 1H), 4.36 (t, *J* = 4.7 Hz, 1H), 4.36-4.32 (m, 2H), 4.31-4.28 (m, 1H).

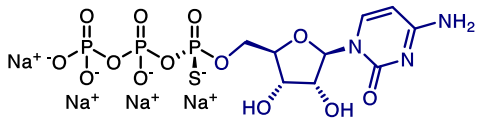
^{13}C NMR (150 MHz, D_2O) δ 166.2, 157.8, 141.7, 96.6, 89.0, 82.6 (d, *J* = 9.7 Hz), 74.2, 69.1, 64.9 (d, *J* = 5.6 Hz).

^{31}P NMR (162 MHz, D_2O) δ 42.8 (d, *J* = 27.9 Hz), -5.8 (d, *J* = 20.2 Hz), -22.5 (dd, *J* = 27.9, 20.2 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_9H_{15}N_3O_{13}P_3S$ $[M-H]^-$: 497.9538, found: 497.9533.

Retention time: 6.81 min (*Method 2*)

Compound (S_P)-28



5'-*O*-cytidine tetrasodium (*S*)-triphosphoro-α-thioate

Following the **General Procedure B** with slight modifications compound **(S_P)-28** was obtained from diphosphate precursor **9c** (169 mg, 0.2 mmol), (–)-Ψ* (112 mg, 0.26 mmol) and protected cytidine **S14** (222 mg, 0.4 mmol). Phosphate transfer step was performed using 8.0 equiv. of DBU. Deprotection was performed at 40 °C. The crude product after work-up was neutralized using 10% aq. AcOH and purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 40:60) followed by reverse-phase chromatography on C18-silica gel (1 M aq. TEAA/MeCN, from 100:0 to 95:5). Fractions containing product were pooled and lyophilized. Obtained solid was redissolved in minimal amount of water and the product was precipitated as sodium salt from 0.2 M $NaClO_4$ in acetone to afford 47 mg of the title compound after drying under high vacuum (**Yield = 40%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.13 (d, *J* = 7.6 Hz, 1H), 6.17 (d, *J* = 7.6 Hz, 1H), 6.02 (d, *J* = 4.3 Hz, 1H), 4.45 (t, *J* = 5.2 Hz, 1H), 4.37-4.32 (m, 3H), 4.30 (dt, *J* = 5.2, 2.5 Hz, 1H).

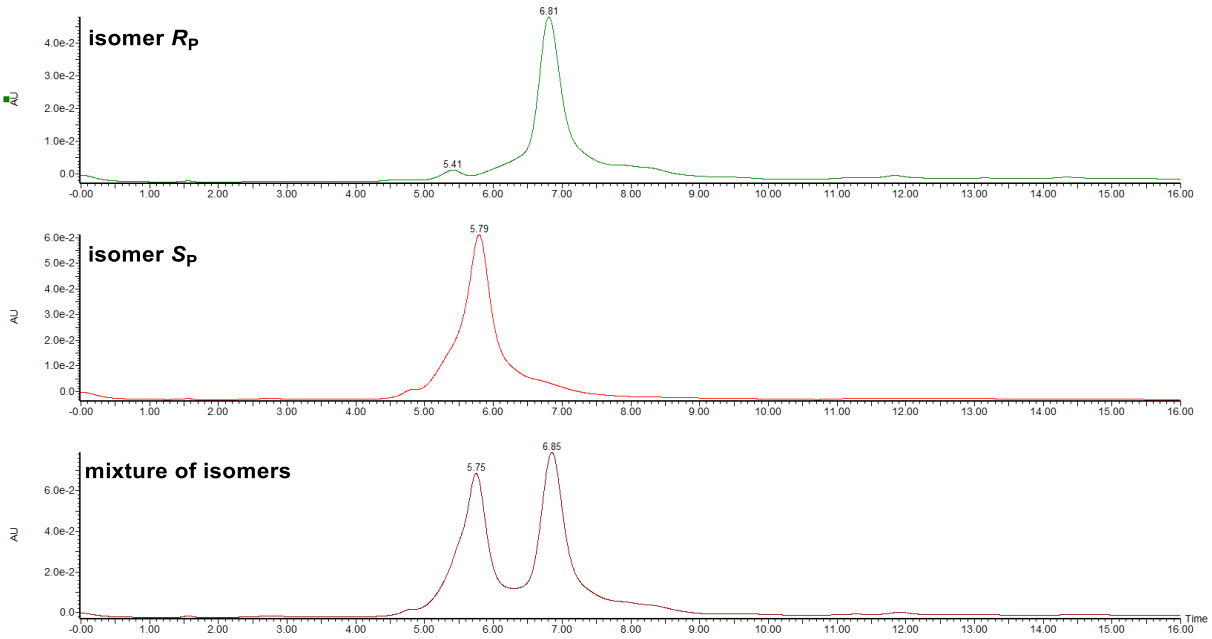
^{13}C NMR (150 MHz, D_2O) δ 166.2, 157.8, 141.9, 96.7, 89.1, 82.7 (d, *J* = 9.7 Hz), 74.3, 69.2, 64.4 (d, *J* = 5.9 Hz).

^{31}P NMR (162 MHz, D_2O) δ 43.2 (d, *J* = 27.1 Hz), -8.7 (d, *J* = 19.3 Hz), -23.5 (dd, *J* = 27.1, 19.3 Hz).

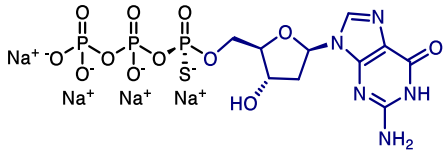
HRMS (ESI-TOF) m/z: calculated for $C_9H_{15}N_3O_{13}P_3S$ $[M-H]^-$: 497.9538, found: 497.9533.

Retention time: 5.79 min (*Method 2*)

LC trace for compound 28:



Compound (R_P)-29



5'-*O*-deoxyguanosine tetrasodium (*R*)-triphosphoro-α-thioate

Following the **General Procedure B** with slight modifications compound **(R_P)-29** was obtained from diphosphate precursor **9c** (169 mg, 0.2 mmol), (+)-Ψ* (112 mg, 0.26 mmol) and protected deoxyguanosine **S15** (148 mg, 0.4 mmol). Phosphate transfer step was performed using 8.0 equiv. of DBU. Deprotection was performed at 40 °C. After extraction, the aqueous phase was concentrated to ~3 mL, neutralized using 10% aq. AcOH and directly purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 40:60), followed by reverse-phase chromatography on C18-silica gel (1 M aq. TEAA/MeCN, from 100:0 to 95:5). Fractions containing product were pooled and lyophilized. Obtained solid was redissolved in minimal amount of water and the product was precipitated as sodium salt from 0.2 M $NaClO_4$ in acetone to afford 35 mg of the title compound after drying under high vacuum (**Yield = 29%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.16 (s, 1H), 6.32 (t, *J* = 6.8 Hz, 1H), 4.31-4.28 (m, 1H), 4.27-4.23 (m, 2H), 2.83 (dt, *J* = 13.7, 6.8 Hz, 1H), 2.51 (ddd, *J* = 13.7, 6.8, 3.4 Hz, 1H) (*One signal overlapping with D_2O*).

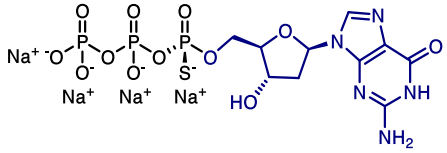
^{13}C NMR (150 MHz, D_2O) δ 159.0, 153.8, 151.4, 137.8, 116.1, 85.6 (d, *J* = 9.6 Hz), 83.6, 71.2, 65.7 (d, *J* = 6.1 Hz), 38.6.

^{31}P NMR (162 MHz, D_2O) δ 43.3 (d, *J* = 27.3 Hz), -5.2 (d, *J* = 19.2 Hz), -21.7 (dd, *J* = 27.3, 19.2 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_{10}H_{15}N_5O_{12}P_3S$ $[M-H]^-$: 521.9656, found: 521.9666.

Retention time: 9.78 min (*Method 2*)

Compound (S_P)-29



5'-*O*-deoxyguanosine tetrasodium (*S*)-triphosphoro-α-thioate

Following the **General Procedure B** with slight modifications compound **(S_P)-29** was obtained from diphosphate precursor **9c** (169 mg, 0.2 mmol), (–)-Ψ* (112 mg, 0.26 mmol) and protected deoxyguanosine **S15** (148 mg, 0.4 mmol). Phosphate transfer step was performed using 8.0 equiv. of DBU. Deprotection was performed at 40 °C. After extraction, the aqueous phase was concentrated to ~3 mL, neutralized using 10% aq. AcOH and directly purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 40:60), followed by reverse-phase chromatography on C18-silica gel (1 M aq. TEAA/MeCN, from 100:0 to 95:5). Fractions containing product were pooled and lyophilized. Obtained solid was redissolved in minimal amount of water and the product was precipitated as sodium salt from 0.2 M $NaClO_4$ in acetone to afford 39 mg of the title compound after drying under high vacuum (**Yield = 32%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.18 (s, 1H), 6.30 (t, *J* = 6.8 Hz, 1H), 4.83-4.79 (m, 1H), 4.31-4.21 (m, 3H), 2.81 (dt, *J* = 13.7, 6.8 Hz, 1H), 2.52 (ddd, *J* = 13.7, 6.8, 3.7 Hz, 1H).

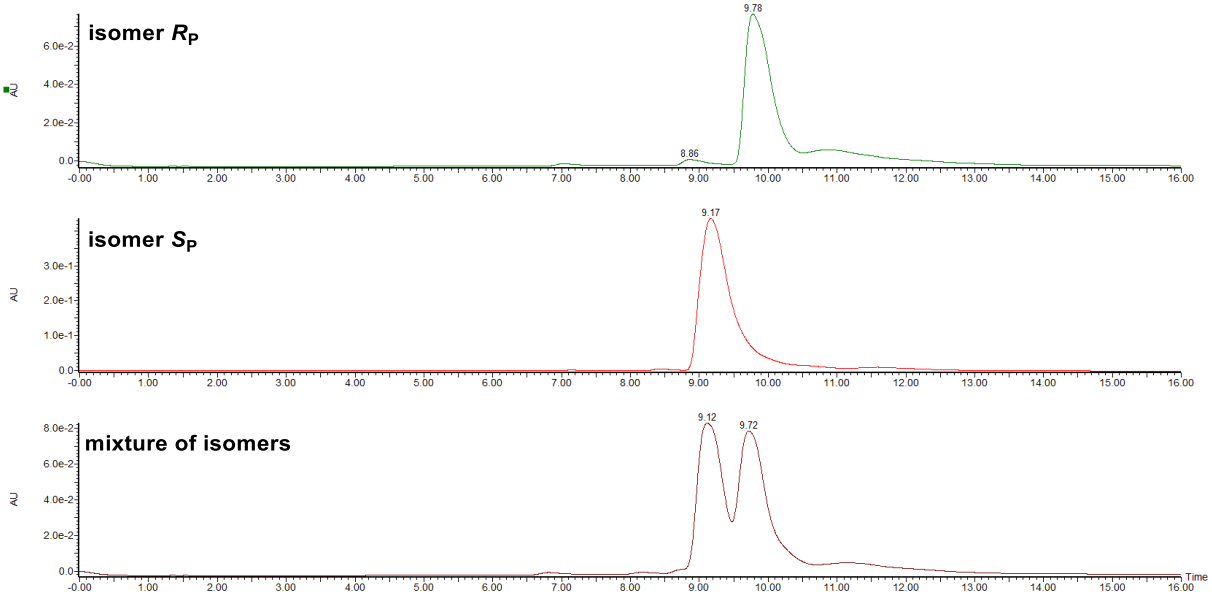
^{13}C NMR (150 MHz, D_2O) δ 159.1, 153.9, 151.3, 137.9, 116.2, 85.6 (d, *J* = 9.4 Hz), 83.6, 71.1, 65.6 (d, *J* = 6.3 Hz), 38.6.

^{31}P NMR (162 MHz, D_2O) δ 43.4 (d, *J* = 27.5 Hz), -5.8 (d, *J* = 20.1 Hz), -22.4 (dd, *J* = 27.5, 20.1 Hz).

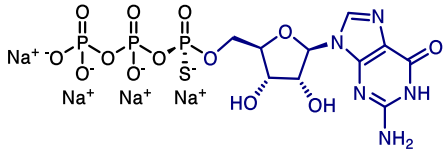
HRMS (ESI-TOF) m/z: calculated for $C_{10}H_{15}N_5O_{12}P_3S$ $[M-H]^-$: 521.9656, found: 521.9666.

Retention time: 9.17 min (*Method 2*)

LC trace for compound 29:



Compound (R_P)-30



5'-*O*-guanosine tetrasodium (*R*)-triphosphoro-α-thioate

Following the **General Procedure B** with slight modifications compound **(R_P)-30** was obtained from diphosphate precursor **9c** (169 mg, 0.2 mmol), (+)-Ψ* (112 mg, 0.26 mmol) and protected guanosine **S16** (196 mg, 0.4 mmol). Phosphate transfer step was performed using 8.0 equiv. of DBU. Deprotection was performed at 40 °C. After extraction, the aqueous phase was concentrated to ~3 mL, neutralized using 10% aq. AcOH and directly purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 40:60), followed by reverse-phase chromatography on C18-silica gel (1 M aq. TEAA/MeCN, from 100:0 to 95:5). Fractions containing product were pooled and lyophilized. Obtained solid was redissolved in minimal amount of water and the product was precipitated as sodium salt from 0.2 M $NaClO_4$ in acetone to afford 39 mg of the title compound after drying under high vacuum (**Yield = 31%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.20 (s, 1H), 5.93 (d, *J* = 5.9 Hz, 1H), 4.64-4.62 (m, 1H), 4.41-4.37 (m, 1H), 4.33 (ddd, *J* = 10.8, 7.9, 2.7 Hz, 1H), 4.27 (ddd, *J* = 10.8, 5.9, 3.0 Hz, 1H) (*One signal overlapping with D_2O*).

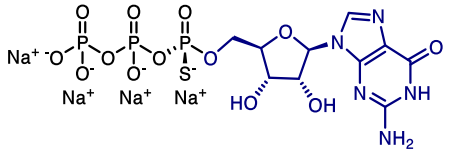
^{13}C NMR (150 MHz, D_2O) δ 159.1, 154.0, 151.7, 137.7, 116.1, 86.6, 83.7 (d, *J* = 9.3 Hz), 73.8, 70.3, 65.5 (d, *J* = 5.4 Hz).

^{31}P NMR (162 MHz, D_2O) δ 43.0 (d, *J* = 28.6 Hz), -5.8 (d, *J* = 19.9 Hz), -22.4 (dd, *J* = 28.6, 19.9 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_{10}H_{15}N_5O_{13}P_3S$ $[M-H]^-$: 537.9600, found: 537.9592.

Retention time: 8.92 min (*Method 2*)

Compound (S_P)-30



5'-*O*-guanosine tetrasodium (*S*)-triphosphoro-α-thioate

Following the **General Procedure B** with slight modifications compound **(S_P)-30** was obtained from diphosphate precursor **9c** (169 mg, 0.2 mmol), (–)-Ψ* (112 mg, 0.26 mmol) and protected guanosine **S16** (196 mg, 0.4 mmol). Phosphate transfer step was performed using 8.0 equiv. of DBU. Deprotection was performed at 40 °C. After extraction, the aqueous phase was concentrated to ~3 mL, neutralized using 10% aq. AcOH and directly purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 40:60), followed by reverse-phase chromatography on C18-silica gel (1 M aq. TEAA/MeCN, from 100:0 to 95:5). Fractions containing product were pooled and lyophilized. Obtained solid was redissolved in minimal amount of water and the product was precipitated as sodium salt from 0.2 M $NaClO_4$ in acetone to afford 41 mg of the title compound after drying under high vacuum (**Yield = 33%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.26 (s, 1H), 5.93 (d, *J* = 6.0 Hz, 1H), 4.82-4.79 (m, 1H), 4.64-4.61 (m, 1H), 4.41-4.37 (m, 1H), 4.34 (ddd, *J* = 10.8, 7.6, 3.0 Hz, 1H), 4.27 (dt, *J* = 10.8, 4.7 Hz, 1H).

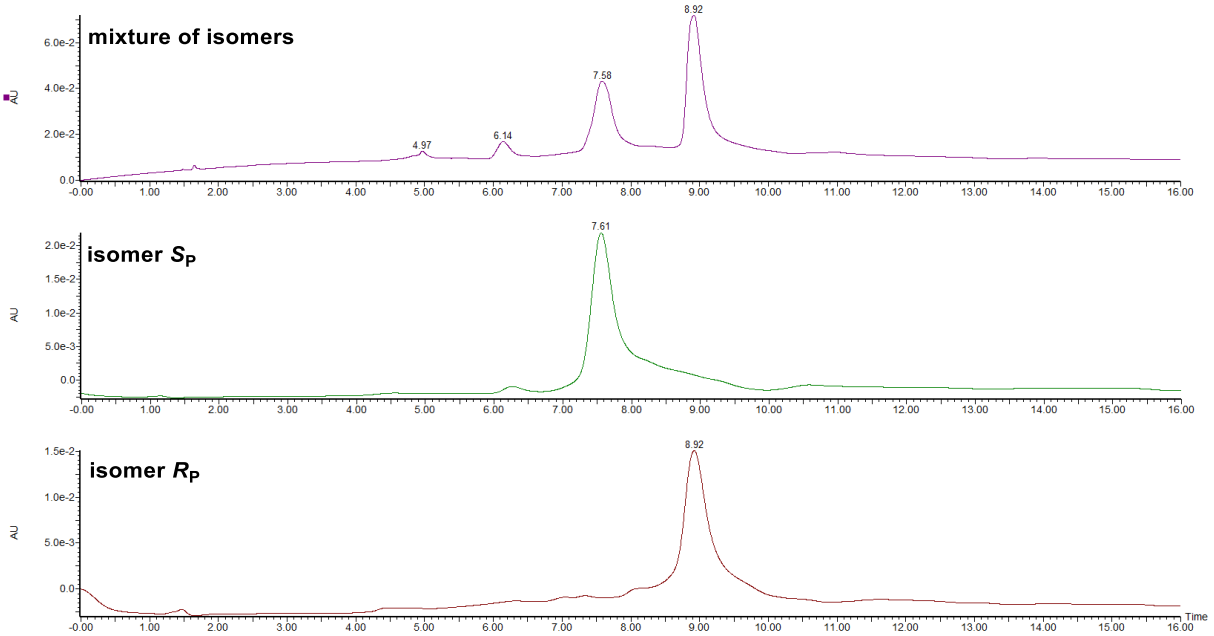
^{13}C NMR (150 MHz, D_2O) δ 159.1, 154.0, 151.7, 137.9, 116.2, 86.7, 83.8 (d, *J* = 9.3 Hz), 73.7, 70.4, 65.1 (d, *J* = 6.7 Hz).

^{31}P NMR (162 MHz, D_2O) δ 43.2 (d, *J* = 27.1 Hz), -5.8 (d, *J* = 20.0 Hz), -22.4 (dd, *J* = 27.1, 20.0 Hz).

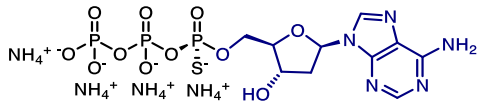
HRMS (ESI-TOF) m/z: calculated for $C_{10}H_{15}N_5O_{13}P_3S$ $[M-H]^-$: 537.9600, found: 537.9592.

Retention time: 7.61 min (*Method 2*)

LC trace for compound 30:



Compound (R_P)-31



5'-*O*-deoxyadenosine tetraammonium (*R*)-triphosphoro-α-thioate

Following the **General Procedure B** compound **(R_P)-31** was obtained from diphosphate precursor **9c** (169 mg, 0.2 mmol), (+)-Ψ* (112 mg, 0.26 mmol) and protected deoxyadenosine **S12** (225 mg, 0.4 mmol). Deprotection was performed at 40 °C. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 40:60) to afford 54 mg of the title compound after lyophilization (**Yield = 47%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.51 (s, 1H), 8.17 (s, 1H), 6.46 (t, *J* = 6.7 Hz, 1H), 4.34-4.30 (m, 1H), 4.27 (ddd, *J* = 10.9, 7.2, 3.5 Hz, 1H), 4.22 (ddd, *J* = 10.9, 6.5, 3.6 Hz, 1H), 2.82 (dt, *J* = 13.6, 6.7 Hz, 1H), 2.60 (ddd, *J* = 13.6, 6.7, 3.8 Hz, 1H) (*One signal overlapping with D_2O*).

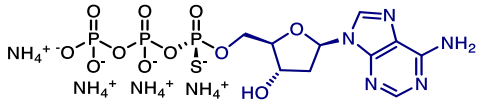
^{13}C NMR (150 MHz, D_2O) δ 155.3, 152.5, 148.5, 140.0, 118.4, 85.6 (d, *J* = 9.5 Hz), 83.6, 71.0, 65.6 (d, *J* = 6.2 Hz), 39.1.

^{31}P NMR (162 MHz, D_2O) δ 43.2 (d, *J* = 27.8 Hz), -8.5 (d, *J* = 20.2 Hz), -23.5 (dd, *J* = 27.8, 20.2 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_{10}H_{15}N_5O_{11}P_3S$ $[M-H]^-$: 505.9702, found: 505.9688.

Retention time: 6.63 min (*Method 1*)

Compound (S_P)-31



5'-*O*-deoxyadenosine tetraammonium (*S*)-triphosphoro-α-thioate

Following the **General Procedure B** compound **(S_P)-31** was obtained from diphosphate precursor **9c** (169 mg, 0.2 mmol), (–)-Ψ* (112 mg, 0.26 mmol) and protected deoxyadenosine **S12** (225 mg, 0.4 mmol). Deprotection was performed at 40 °C. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 40:60) to afford 59 mg of the title compound after lyophilization (**Yield = 51%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.50 (s, 1H), 8.11 (s, 1H), 6.42 (t, *J* = 6.7 Hz, 1H), 4.32-4.24 (m, 2H), 4.21-4.17 (m, 1H), 2.80 (dt, *J* = 13.6, 6.7 Hz, 1H), 2.59 (ddd, *J* = 13.6, 6.7, 3.8 Hz, 1H) (*One signal overlapping with D_2O*).

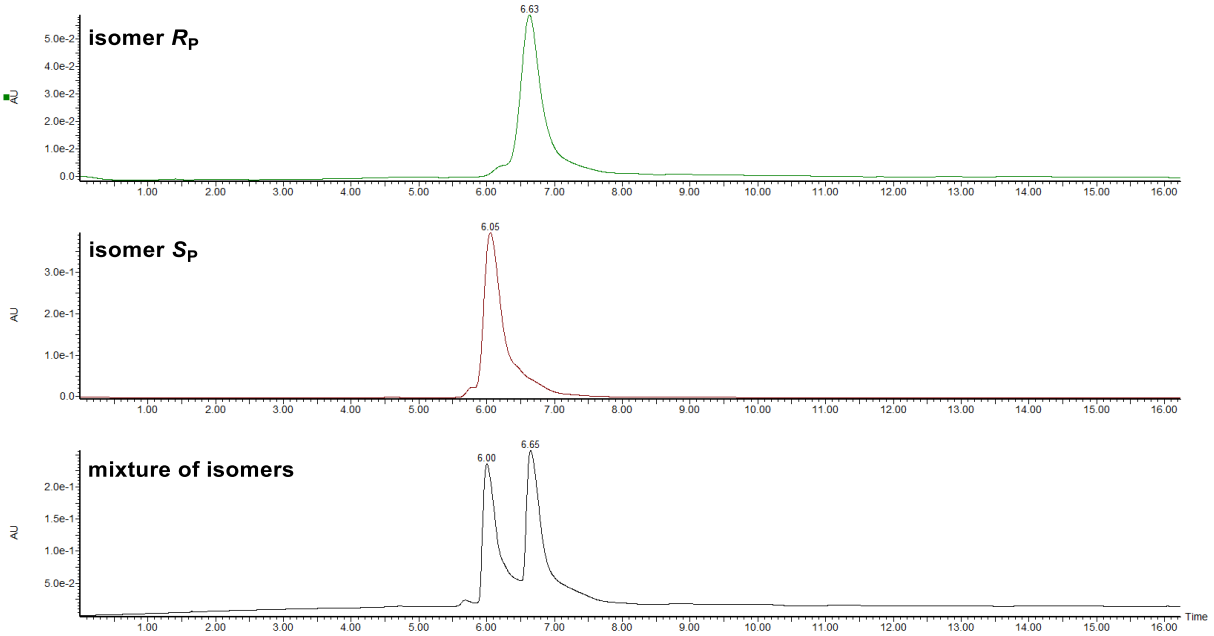
^{13}C NMR (150 MHz, D_2O) δ 155.2, 152.3, 148.4, 140.0, 118.3, 85.6 (d, *J* = 9.6 Hz), 83.6, 71.0, 65.5 (d, *J* = 6.4 Hz), 39.0.

^{31}P NMR (162 MHz, D_2O) δ 43.4 (d, *J* = 27.4 Hz), -7.4 (d, *J* = 20.7 Hz), -23.2 (dd, *J* = 27.4, 20.7 Hz).

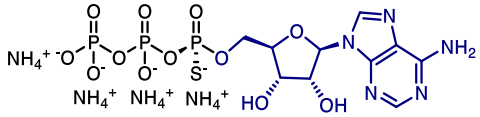
HRMS (ESI-TOF) m/z: calculated for $C_{10}H_{15}N_5O_{11}P_3S$ $[M-H]^-$: 505.9702, found: 505.9688.

Retention time: 6.05 min (*Method 1*)

LC trace for compound 31:



Compound (R_P)-32



5'-*O*-adenosine tetraammonium (*R*)-triphosphoro-*α*-thioate

Following the **General Procedure B** compound **(R_P)-32** was obtained from diphosphate precursor **9c** (169 mg, 0.2 mmol), (+)-Ψ* (112 mg, 0.26 mmol) and protected adenosine **S9** (273 mg, 0.4 mmol). Deprotection was performed at 40 °C. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 40:60) to afford 59 mg of the title compound after lyophilization (**Yield = 50%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.58 (s, 1H), 8.21 (s, 1H), 6.12 (d, *J* = 5.9 Hz, 1H), 4.62 (dd, *J* = 5.1, 3.6 Hz, 1H), 4.43-4.41 (m, 1H), 4.34 (ddd, *J* = 10.4, 7.5, 2.7 Hz, 1H), 4.28 (ddd, *J* = 11.9, 6.1, 2.7 Hz, 1H) (*One signal overlapping with D_2O*).

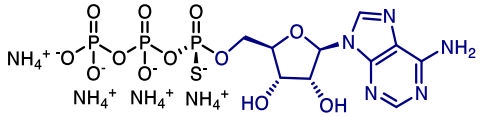
^{13}C NMR (150 MHz, D_2O) δ 155.5, 152.7, 149.0, 140.0, 118.5, 86.7, 83.9 (d, *J* = 9.7 Hz), 74.3, 70.4, 65.5 (d, *J* = 5.8 Hz).

^{31}P NMR (162 MHz, D_2O) δ 43.4 (d, *J* = 27.6 Hz), -10.9 (d, *J* = 19.6 Hz), -24.1 (dd, *J* = 27.6, 19.6 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_{10}H_{15}N_5O_{12}P_3S$ $[M-H]^-$: 521.9651, found: 521.9662.

Retention time: 5.85 min (*Method 1*)

Compound (S_P)-32



5'-*O*-adenosine tetraammonium (*S*)-triphosphoro-*α*-thioate

Following the **General Procedure B** compound **(S_P)-32** was obtained from diphosphate precursor **9c** (169 mg, 0.2 mmol), (–)-Ψ* (112 mg, 0.26 mmol) and protected adenosine **S9** (273 mg, 0.4 mmol). Deprotection was performed at 40 °C. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 40:60) to afford 61 mg of the title compound after lyophilization (**Yield = 52%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.63 (s, 1H), 8.18 (s, 1H), 6.12 (d, *J* = 5.7 Hz, 1H), 4.62-4.59 (m, 1H), 4.43-4.40 (m, 1H), 4.35 (ddd, *J* = 11.0, 7.7, 3.0 Hz, 1H), 4.28 (ddd, *J* = 11.7, 5.5, 3.0 Hz, 1H) (*One signal overlapping with D_2O*).

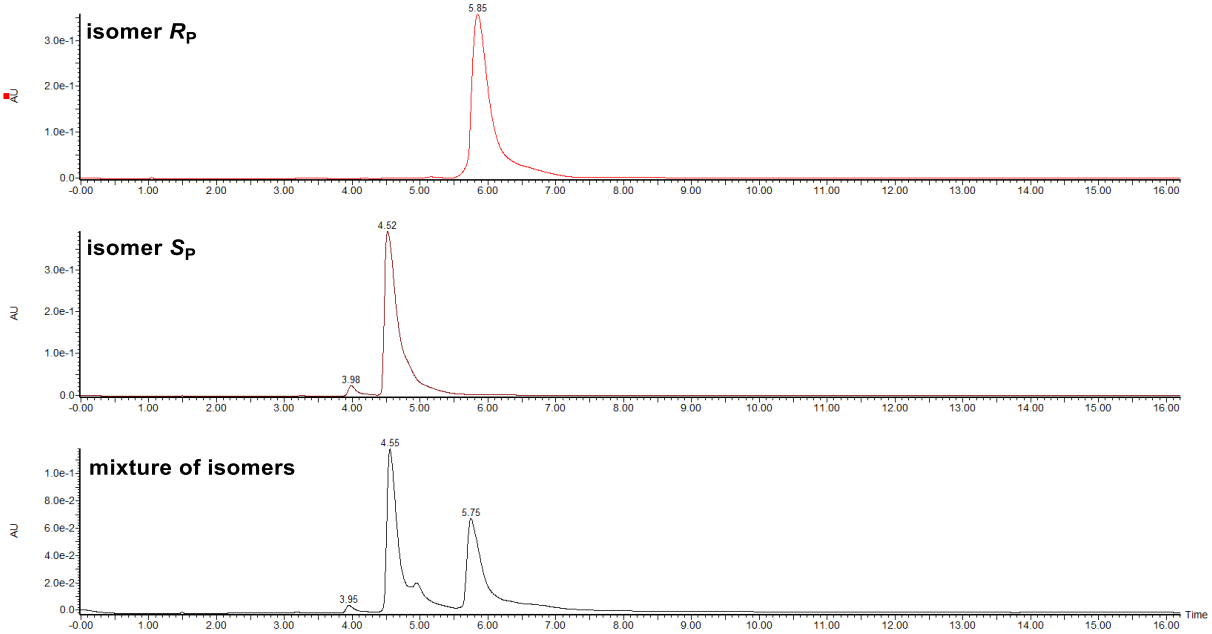
^{13}C NMR (150 MHz, D_2O) δ 155.4, 152.6, 148.9, 140.1, 118.4, 86.8, 83.8 (d, *J* = 9.5 Hz), 74.3, 70.4, 65.1 (d, *J* = 6.5 Hz).

^{31}P NMR (162 MHz, D_2O) δ 43.4 (d, *J* = 26.9 Hz), -8.5 (d, *J* = 20.1 Hz), -23.5 (dd, *J* = 26.9, 20.1 Hz).

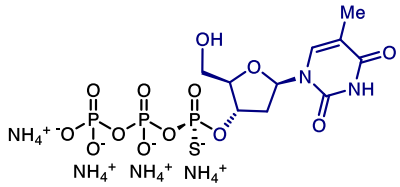
HRMS (ESI-TOF) m/z: calculated for $C_{10}H_{15}N_5O_{12}P_3S$ $[M-H]^-$: 521.9651, found: 521.9662.

Retention time: 4.52 min (*Method 1*)

LC trace for compound 32:



Compound (R_P)-33



3'-*O*-thymidine tetraammonium (*R*)-triphosphoro-α-thioate

Following the **General Procedure B** compound **(R_P)-33** was obtained from diphosphate precursor **9c** (169 mg, 0.2 mmol), (+)-Ψ* (112 mg, 0.26 mmol) and protected thymidine **S10** (138 mg, 0.4 mmol). Deprotection was performed at 40 °C. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 20:80) to afford 57 mg of the title compound after lyophilization (**Yield = 51%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 7.69 (s, 1H), 6.34 (t, *J* = 6.9 Hz, 1H), 5.09 (td, *J* = 6.9, 3.3 Hz, 1H), 4.31 (q, *J* = 3.7 Hz, 1H), 3.90-3.84 (m, 2H), 2.63 (ddd, *J* = 14.3, 6.9, 3.3 Hz, 1H), 2.48 (dt, *J* = 14.3, 6.9 Hz, 1H), 1.90 (s, 3H).

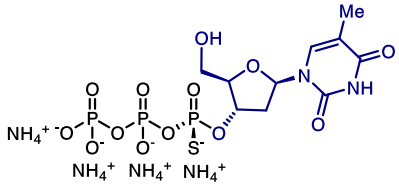
^{13}C NMR (150 MHz, D_2O) δ 166.5, 151.7, 137.6, 111.5, 85.5 (d, *J* = 5.8 Hz), 85.0, 75.6 (d, *J* = 6.0 Hz), 61.0, 37.5 (d, *J* = 3.9 Hz), 11.5.

^{31}P NMR (162 MHz, D_2O) δ 42.8 (d, *J* = 26.2 Hz), -10.0 (d, *J* = 19.3 Hz), -23.8 (dd, *J* = 26.2, 19.3 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_{10}H_{16}N_2O_{13}P_3S$ $[M-H]^-$: 496.9551, found: 496.9603.

Retention time: 8.45 min (*Method 3*)

Compound (S_P)-33



3'-*O*-thymidine tetraammonium (*S*)-triphosphoro-α-thioate

Following the **General Procedure B** compound **(S_P)-33** was obtained from diphosphate precursor **9c** (169 mg, 0.2 mmol), (–)-Ψ* (112 mg, 0.26 mmol) and protected thymidine **S10** (138 mg, 0.4 mmol). Deprotection was performed at 40 °C. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 20:80) to afford 60 mg of the title compound after lyophilization (**Yield = 53%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 7.69 (d, *J* = 1.1 Hz, 1H), 6.36 (dd, *J* = 7.1, 6.4 Hz, 1H), 5.10 (ddt, *J* = 10.0, 6.4, 3.1 Hz, 1H), 4.29 (q, *J* = 3.6 Hz, 1H), 3.91-3.85 (m, 2H), 2.66 (ddd, *J* = 14.4, 6.4, 3.1 Hz, 1H), 2.47 (dt, *J* = 14.4, 7.1 Hz, 1H), 1.90 (d, *J* = 1.1 Hz, 3H).

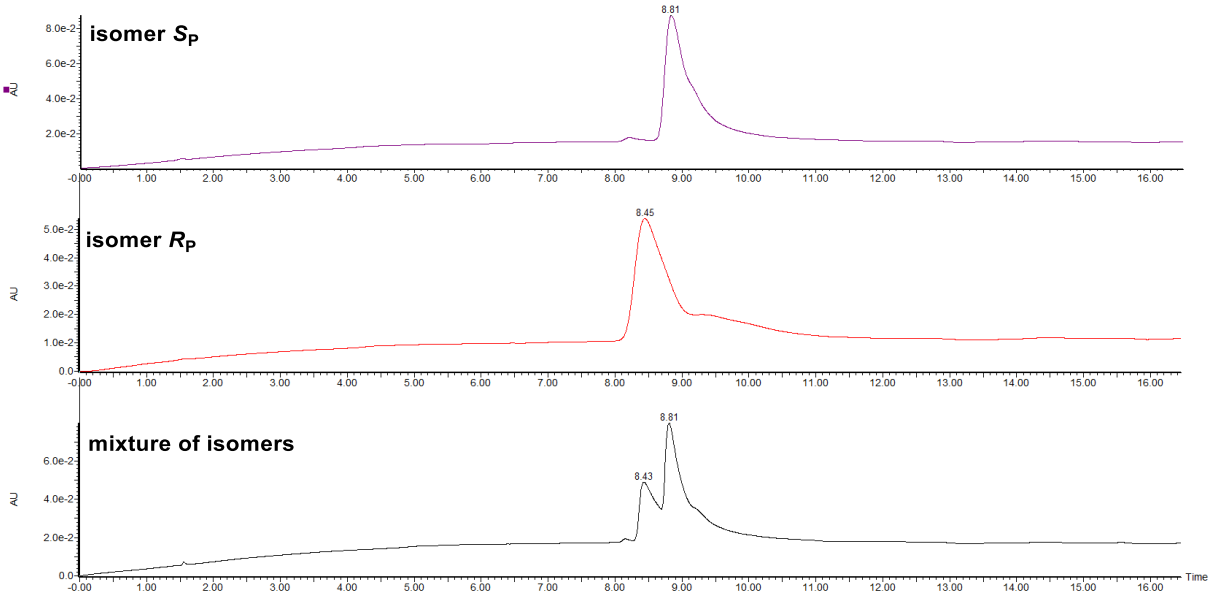
^{13}C NMR (150 MHz, D_2O) δ 166.5, 151.7, 137.7, 111.5, 85.7 (d, *J* = 6.3 Hz), 85.0, 75.7 (d, *J* = 6.0 Hz), 61.0, 37.4 (d, *J* = 3.7 Hz), 11.5.

^{31}P NMR (162 MHz, D_2O) δ 42.7 (d, *J* = 26.2 Hz), -7.8 (d, *J* = 20.1 Hz), -23.3 (dd, *J* = 26.2, 20.1 Hz).

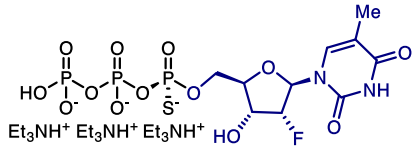
HRMS (ESI-TOF) m/z: calculated for $C_{10}H_{16}N_2O_{13}P_3S$ $[M-H]^-$: 496.9551, found: 496.9603.

Retention time: 8.81 min (*Method 3*)

LC trace for compound 33:



Compound (R_P)-34



5'-*O*-2'-fluorothymidine tris(triethylammonium) (*R*)-triphosphoro-α-thioate

Following modified **General Procedure B** compound **(R_P)-34** was obtained from diphosphate precursor **9c** (338 mg, 0.4 mmol), (+)-Ψ* (206 mg, 0.48 mmol) and protected 2'-fluorothymidine **S50** (94 mg, 0.2 mmol). Thiophosphate transfer reagent formation step was performed using 8.0 equiv. of DBU. Deprotection was performed at room temperature. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 25:75), followed by reverse-phase chromatography on C18-silica gel (1 M aq. TEAA/MeCN, from 100:0 to 90:10). Fractions containing product were pooled and lyophilized multiple times to afford 44 mg of the title compound (**Yield = 27%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 7.77 (s, 1H), 6.11 (d, *J* = 17.5 Hz, 1H), 5.25-5.14 (m, 1H), 4.61-4.54 (m, 1H), 4.48-4.43 (m, 1H), 4.35-4.29 (m, 2H), 3.20 (q, *J* = 7.3 Hz, 18H), 1.95 (s, 3H), 1.28 (t, *J* = 7.3 Hz, 27H).

^{13}C NMR (150 MHz, D_2O) δ 166.6, 151.4, 137.1, 111.6, 93.4 (d, *J* = 186.5 Hz), 87.6 (d, *J* = 34.7 Hz), 81.5 (dd, *J* = 9.6 Hz), 67.6 (d, *J* = 15.7 Hz), 64.1 (d, *J* = 5.8 Hz), 46.6, 11.7, 8.2.

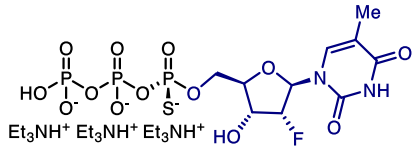
^{19}F NMR (376 MHz, D_2O) δ -206.7.

^{31}P NMR (162 MHz, D_2O) δ 42.8 (d, *J* = 28.9 Hz), -11.1 (d, *J* = 19.7 Hz), -24.3 (dd, *J* = 28.9, 19.7 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_{10}H_{15}FN_2O_{13}P_3S$ $[M-H]^-$: 514.9492, found: 514.9485.

Retention time: 7.02 min (*Method 1*)

Compound (S_P)-34



5'-*O*-2'-fluorothymidine tris(triethylammonium) (*S*)-triphosphoro-α-thioate

Following modified **General Procedure B** compound **(S_P)-34** was obtained from diphosphate precursor **9c** (338 mg, 0.4 mmol), (–)-Ψ* (206 mg, 0.48 mmol) and protected 2'-fluorothymidine **S50** (94 mg, 0.2 mmol). Thiophosphate transfer reagent formation step was performed using 8.0 equiv. of DBU. Deprotection was performed at room temperature. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 25:75), followed by reverse-phase chromatography on C18-silica gel (1 M aq. TEAA/MeCN, from 100:0 to 90:10). Fractions containing product were pooled and lyophilized multiple times to afford 52 mg of the title compound (**Yield = 32%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 7.75 (s, 1H), 6.11 (d, *J* = 17.8 Hz, 1H), 5.26-5.14 (m, 1H), 4.60-4.53 (m, 1H), 4.43-4.33 (m, 2H), 4.32-4.28 (m, 1H), 3.20 (q, *J* = 7.3 Hz, 18H), 1.97 (s, 3H), 1.28 (t, *J* = 7.3 Hz, 27H).

^{13}C NMR (150 MHz, D_2O) δ 166.6, 151.4, 137.2, 111.6, 93.3 (d, *J* = 186.5 Hz), 87.8 (d, *J* = 34.9 Hz), 81.4 (dd, *J* = 9.7 Hz), 67.8 (d, *J* = 15.7 Hz), 63.8 (d, *J* = 6.0 Hz), 46.6, 11.8, 8.2.

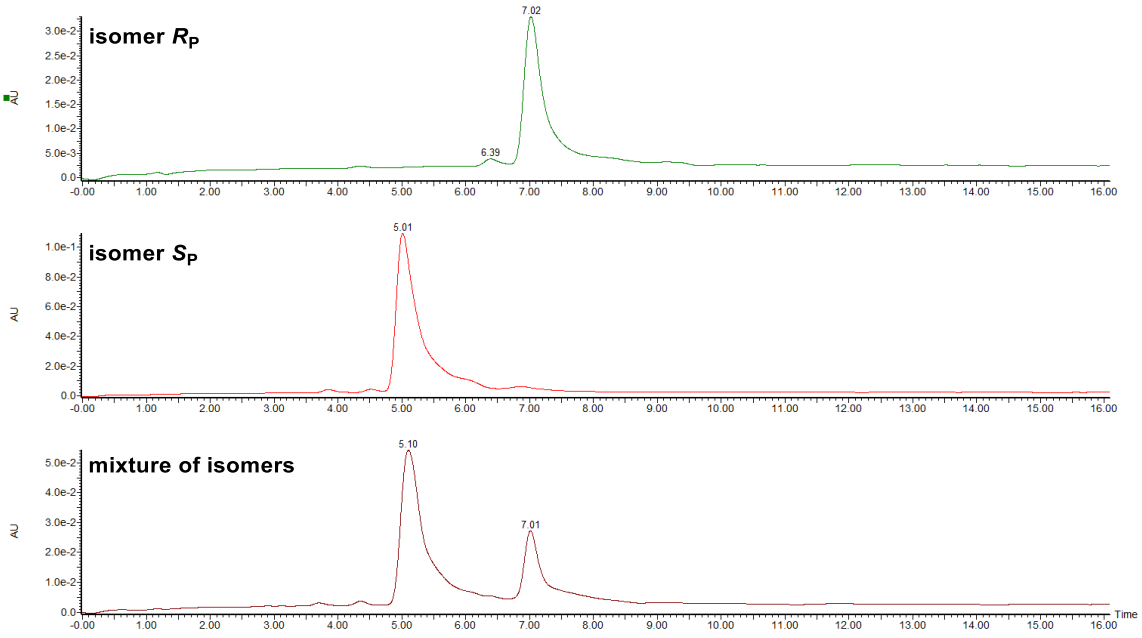
^{19}F NMR (376 MHz, D_2O) δ -206.3.

^{31}P NMR (162 MHz, D_2O) δ 43.5 (d, *J* = 27.0 Hz), -10.4 (d, *J* = 18.3 Hz), -23.0 (dd, *J* = 27.0, 18.3 Hz).

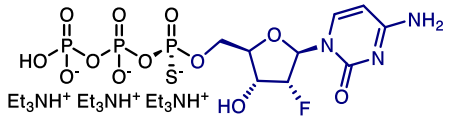
HRMS (ESI-TOF) m/z: calculated for $C_{10}H_{15}FN_2O_{13}P_3S$ $[M-H]^-$: 514.9492, found: 514.9485.

Retention time: 5.01 min (*Method 1*)

LC trace for compound 34:



Compound (R_P)-35



5'-*O*-2'-fluorocytidine tris(triethylammonium) (*R*)-triphosphoro-α-thioate

Following modified **General Procedure B** compound **(R_P)-35** was obtained from diphosphate precursor **9c** (338 mg, 0.4 mmol), (+)-Ψ* (206 mg, 0.48 mmol) and protected 2'-fluorocytidine **S38** (91 mg, 0.2 mmol). Thiophosphate transfer reagent formation step was performed using 8.0 equiv. of DBU. Deprotection was performed at room temperature. The crude product after work-up was neutralized using 10% aq. AcOH and purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 25:75), followed by reverse-phase chromatography on C18-silica gel (1 M aq. TEAA/MeCN, from 100:0 to 90:10). Fractions containing product were pooled and lyophilized multiple times to afford 53 mg of the title compound (**Yield = 33%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.13 (d, *J* = 7.7 Hz, 1H), 6.20 (d, *J* = 7.7 Hz, 1H), 6.09 (d, *J* = 16.7 Hz, 1H), 5.13 (dd, *J* = 52.2, 4.2 Hz, 1H), 4.55-4.46 (m, 2H), 4.35-4.30 (m, 2H), 3.20 (q, *J* = 7.3 Hz, 18H), 1.28 (t, *J* = 7.3 Hz, 27H).

^{13}C NMR (150 MHz, D_2O) δ 163.7, 153.8, 142.4, 95.9, 93.8 (d, *J* = 186.2 Hz), 88.3 (d, *J* = 34.2 Hz), 81.2 (d, *J* = 9.8 Hz), 67.2 (d, *J* = 15.9 Hz), 63.4 (d, *J* = 5.3 Hz), 46.6, 8.2.

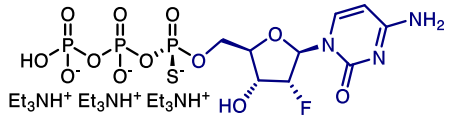
^{19}F NMR (376 MHz, D_2O) δ -206.0.

^{31}P NMR (162 MHz, D_2O) δ 43.2 (d, *J* = 28.0 Hz), -11.1 (d, *J* = 19.8 Hz), -24.3 (dd, *J* = 28.0, 19.8 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_9H_{14}FN_3O_{12}P_3S$ [M-H]$^-$: 499.9495, found: 499.9487.

Retention time: 8.94 min (*Method 2*)

Compound (S_P)-35



5'-*O*-2'-fluorocytidine tris(triethylammonium) (*S*)-triphosphoro-α-thioate

Following modified **General Procedure B** compound **(S_P)-35** was obtained from diphosphate precursor **9c** (338 mg, 0.4 mmol), (–)-Ψ* (206 mg, 0.48 mmol) and protected 2'-fluorocytidine **S38** (91 mg, 0.2 mmol). Thiophosphate transfer reagent formation step was performed using 8.0 equiv. of DBU. Deprotection was performed at room temperature. The crude product after work-up was neutralized using 10% aq. AcOH and purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 25:75), followed by reverse-phase chromatography on C18-silica gel (1 M aq. TEAA/MeCN, from 100:0 to 90:10). Fractions containing product were pooled and lyophilized multiple times to afford 63 mg of the title compound (**Yield = 39%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.10 (d, *J* = 7.6 Hz, 1H), 6.15 (d, *J* = 7.6 Hz, 1H), 6.09 (d, *J* = 17.1 Hz, 1H), 5.10 (dd, *J* = 52.2, 4.3 Hz, 1H), 4.51 (ddd, *J* = 23.4, 8.3, 4.3 Hz, 1H), 4.47-4.43 (m, 1H), 4.36-4.29 (m, 2H), 3.19 (q, *J* = 7.3 Hz, 18H), 1.27 (t, *J* = 7.3 Hz, 27H).

^{13}C NMR (150 MHz, D_2O) δ 165.7, 156.4, 141.9, 96.3, 93.9 (d, *J* = 185.9 Hz), 88.4 (d, *J* = 34.0 Hz), 81.0 (d, *J* = 9.9 Hz), 67.3 (d, *J* = 16.0 Hz), 63.3 (d, *J* = 5.9 Hz), 46.6, 8.2.

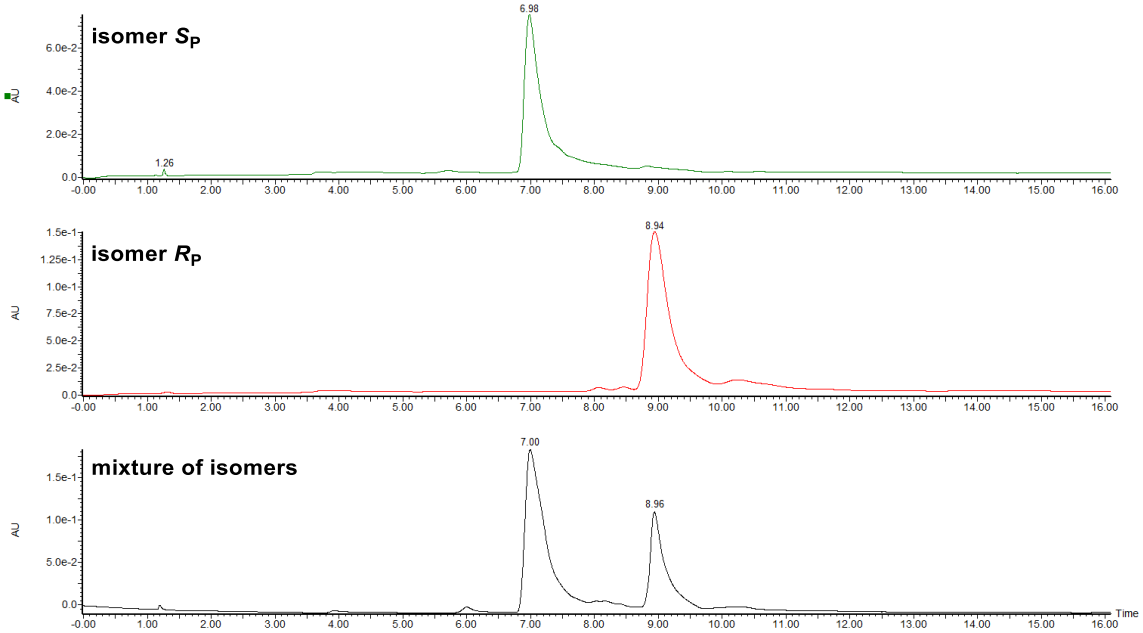
^{19}F NMR (376 MHz, D_2O) δ -205.6.

^{31}P NMR (162 MHz, D_2O) δ 43.3 (d, *J* = 27.3 Hz), -10.7 (d, *J* = 19.7 Hz), -24.1 (dd, *J* = 27.3, 19.7 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_9H_{14}FN_3O_{12}P_3S$ [M-H]$^-$: 499.9495, found: 499.9487.

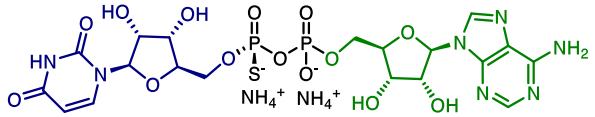
Retention time: 6.98 min (*Method 2*)

LC trace for compound 35:



8.3.3 Dinucleoside Di- and Trithiophosphates

Compound (R_P)-36



***P*^{1}-(5'-*O*-adenosine)-*P*^{2}-(5'-*O*-uridine) diammonium (*R*)-diphosphoro-2-thioate**

Following the **General Procedure C** compound **(R_P)-36** was obtained from protected adenosine monophosphate **S52** (152 mg, 0.2 mmol), (+)-Ψ* (128 mg, 0.3 mmol) and protected uridine **S8** (278 mg, 0.5 mmol). Deprotection was performed at 40 °C. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 20:80) to afford 77 mg of the title compound after lyophilization (**Yield = 55%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.45 (s, 1H), 8.19 (s, 1H), 7.79 (d, *J* = 8.1 Hz, 1H), 6.08 (d, *J* = 5.8 Hz, 1H), 5.84 (d, *J* = 4.8 Hz, 1H), 5.71 (d, *J* = 8.1 Hz, 1H), 4.79-4.76 (m, 1H), 4.54 (dd, *J* = 5.0, 3.7 Hz, 1H), 4.40-4.37 (m, 1H), 4.35 (ddd, *J* = 11.4, 4.6, 2.8 Hz, 1H), 4.31-4.18 (m, 6H).

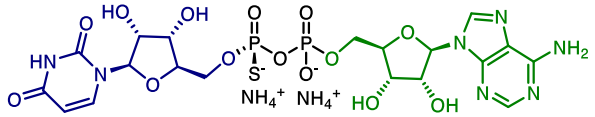
^{13}C NMR (150 MHz, D_2O) δ 165.7, 155.1, 152.4, 151.4, 148.9, 141.3, 139.9, 118.4, 102.2, 88.3, 86.9, 83.8 (d, *J* = 9.4 Hz), 83.0 (d, *J* = 9.7 Hz), 74.2, 73.9, 70.4, 69.7, 65.3 (d, *J* = 5.5 Hz), 64.8 (d, *J* = 6.5 Hz).

^{31}P NMR (162 MHz, D_2O) δ 43.5 (d, *J* = 27.7 Hz), -12.0 (d, *J* = 27.7 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_{19}H_{24}N_7O_{14}P_2S$ $[M-H]^-$: 668.0582, found: 668.0587.

Retention time: 7.82 min (*Method 1*)

Compound (S_P)-36



***P*^{1}-(5'-*O*-adenosine)-*P*^{2}-(5'-*O*-uridine) diammonium (*S*)-diphosphoro-2-thioate**

Following the **General Procedure C** compound **(S_P)-36** was obtained from protected adenosine monophosphate **S52** (152 mg, 0.2 mmol), (–)-Ψ* (128 mg, 0.3 mmol) and protected uridine **S8** (278 mg, 0.5 mmol). Deprotection was performed at 40 °C. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 20:80) to afford 90 mg of the title compound after lyophilization (**Yield = 64%, d.r. > 20:1**).

Preparative scale: Following the **General Procedure C** compound **(S_P)-36** was obtained from protected adenosine monophosphate **S52** (1.52 g, 2.0 mmol), (–)-Ψ* (1.28 g, 3.0 mmol) and protected uridine **S8** (2.78 g, 5.0 mmol). Deprotection was performed at 40 °C. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 20:80) to afford 1.01 g of the title compound after lyophilization (**Yield = 72%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.43 (s, 1H), 8.14 (s, 1H), 7.71 (d, *J* = 8.1 Hz, 1H), 6.06 (d, *J* = 5.9 Hz, 1H), 5.82 (d, *J* = 5.1 Hz, 1H), 5.67 (d, *J* = 8.1 Hz, 1H), 4.56 (dd, *J* = 5.0, 3.6 Hz, 1H), 4.39-4.37 (m, 1H), 4.31-4.28 (m, 1H), 4.28-4.20 (m, 6H) (*One signal overlapping with D_2O*).

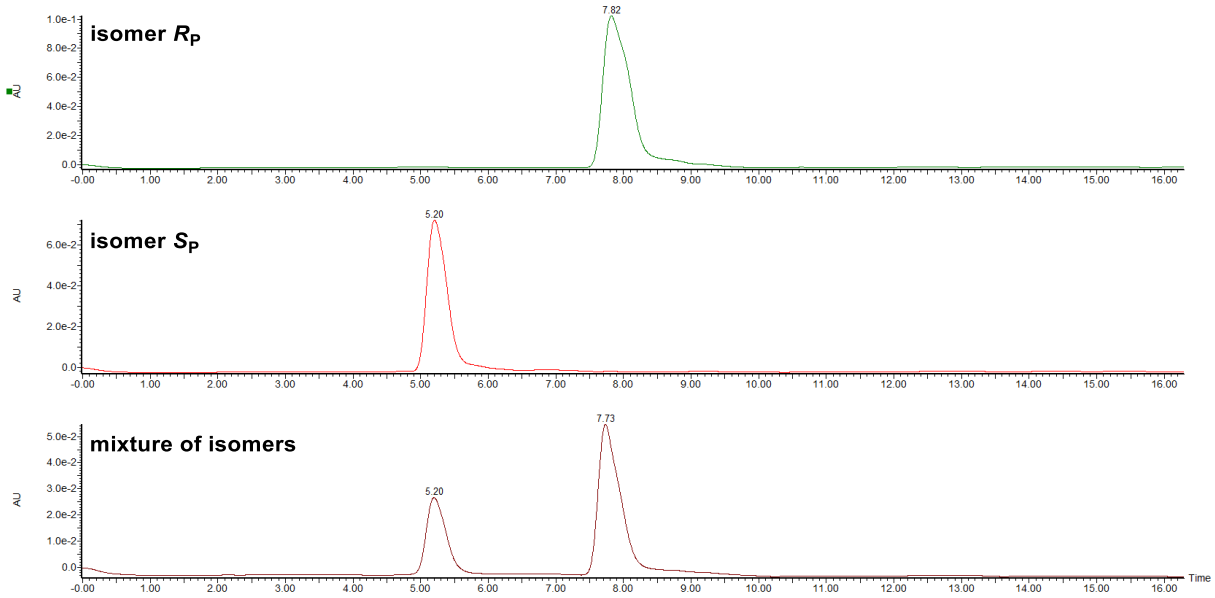
^{13}C NMR (150 MHz, D_2O) δ 165.6, 155.0, 152.3, 151.4, 148.9, 141.1, 139.8, 118.3, 102.2, 88.1, 86.7, 83.8 (d, *J* = 9.6 Hz), 83.0 (d, *J* = 10.1 Hz), 74.2, 74.0, 70.5, 69.7, 65.30 (d, *J* = 5.1 Hz), 64.26 (d, *J* = 5.4 Hz).

^{31}P NMR (162 MHz, D_2O) δ 43.2 (d, *J* = 25.8 Hz), -12.0 (d, *J* = 25.8 Hz).

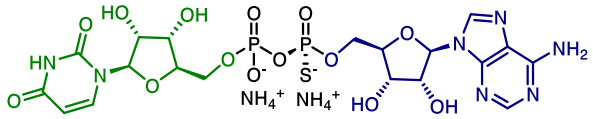
HRMS (ESI-TOF) m/z: calculated for $C_{19}H_{24}N_7O_{14}P_2S$ $[M-H]^-$: 668.0582, found: 668.0587.

Retention time: 5.20 min (*Method 1*)

LC trace for compound 36:



Compound (R_P)-37



***P^1*-(5'-*O*-adenosine)-*P^2*-(5'-*O*-uridine) diammonium (*R*)-diphosphoro-1-thioate**

Following the **General Procedure C** compound **(R_P)-37** was obtained from protected uridine monophosphate **S54** (127 mg, 0.2 mmol), (+)-Ψ* (128 mg, 0.3 mmol) and protected adenosine **S9** (342 mg, 0.5 mmol). Deprotection was performed at 40 °C. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 20:80) to afford 72 mg of the title compound after lyophilization (**Yield = 51%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.56 (s, 1H), 8.16 (s, 1H), 7.69 (d, *J* = 8.1 Hz, 1H), 6.08 (d, *J* = 5.8 Hz, 1H), 5.83 (d, *J* = 5.0 Hz, 1H), 5.70 (d, *J* = 8.1 Hz, 1H), 4.53 (dd, *J* = 5.0, 3.6 Hz, 1H), 4.42-4.39 (m, 1H), 4.33 (ddd, *J* =

11.6, 4.1, 2.4 Hz, 1H), 4.31-4.22 (m, 5H), 4.17 (ddd, *J* = 11.6, 5.3, 2.6 Hz, 1H) (*One signal overlapping with D_2O*).

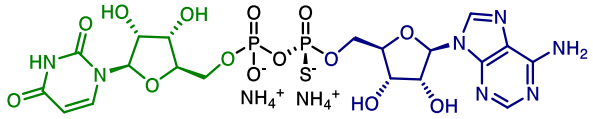
^{13}C NMR (150 MHz, D_2O) δ 165.6, 155.1, 152.4, 151.4, 148.9, 141.0, 140.0, 118.3, 102.3, 88.2, 87.0, 83.8 (d, *J* = 9.6 Hz), 83.1 (d, *J* = 9.4 Hz), 74.4, 74.0, 70.6, 69.6, 65.4 (d, *J* = 6.4 Hz), 65.0 (d, *J* = 5.2 Hz).

^{31}P NMR (162 MHz, D_2O) δ 43.5 (d, *J* = 27.3 Hz), -12.0 (d, *J* = 27.3 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_{19}H_{24}N_7O_{14}P_2S$ $[M-H]^-$: 668.0582, found: 668.0557.

Retention time: 7.11 min (*Method 1*)

Compound (S_P)-37



***P*^{1}-(5'-*O*-adenosine)-*P*^{2}-(5'-*O*-uridine) diammonium (*S*)-diphosphoro-1-thioate**

Following the **General Procedure C** compound **(S_P)-37** was obtained from protected uridine monophosphate **S54** (127 mg, 0.2 mmol), (–)-Ψ* (128 mg, 0.3 mmol) and protected adenosine **S9** (342 mg, 0.5 mmol). Deprotection was performed at 40 °C. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 20:80) to afford 79 mg of the title compound after lyophilization (**Yield = 56%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.51 (s, 1H), 8.16 (s, 1H), 7.66 (d, *J* = 8.1 Hz, 1H), 6.07 (d, *J* = 5.9 Hz, 1H), 5.82 (d, *J* = 5.1 Hz, 1H), 5.69 (d, *J* = 8.1 Hz, 1H), 4.79-4.76 (m, 1H), 4.54 (dd, *J* = 4.9, 3.3 Hz, 1H), 4.42-4.39 (m, 1H), 4.33-4.27 (m, 3H), 4.26-4.20 (m, 3H), 4.18-4.14 (m, 1H).

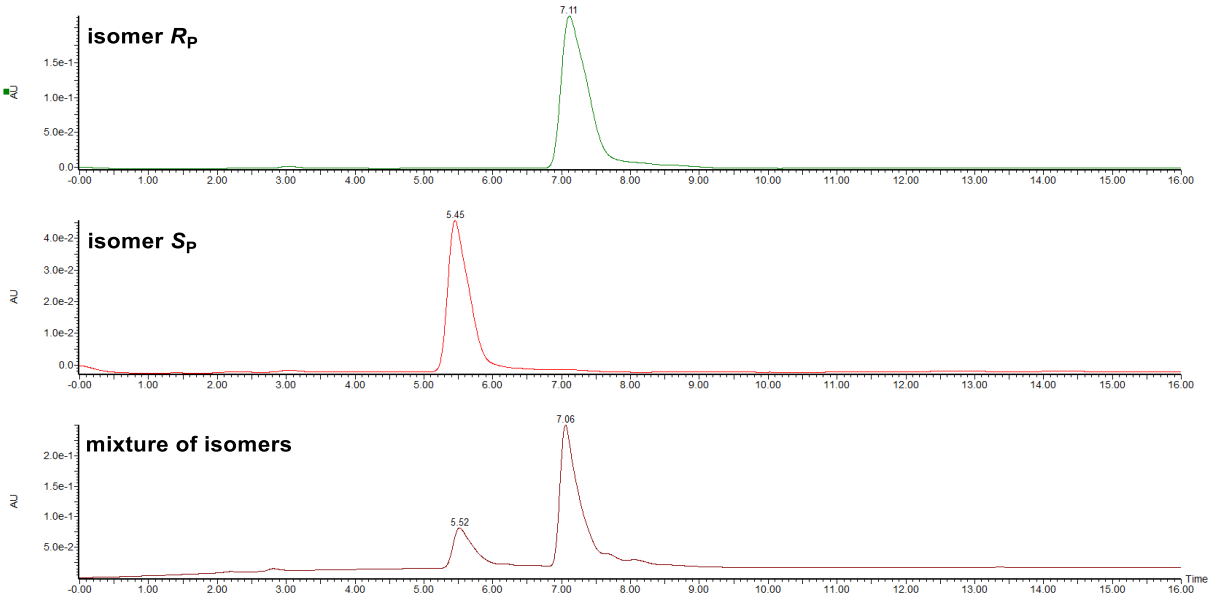
^{13}C NMR (150 MHz, D_2O) δ 165.6, 155.1, 152.4, 151.4, 149.0, 141.0, 139.9, 118.3, 102.3, 88.1, 86.7, 83.8 (d, *J* = 10.0 Hz), 83.1 (d, *J* = 9.6 Hz), 74.3, 73.9, 70.6, 69.6, 65.7 (d, *J* = 5.9 Hz), 64.9 (d, *J* = 5.2 Hz).

^{31}P NMR (162 MHz, D_2O) δ 43.1 (d, *J* = 26.1 Hz), -12.1 (d, *J* = 26.1 Hz).

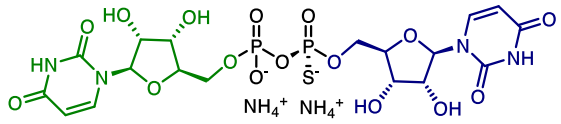
HRMS (ESI-TOF) m/z: calculated for $C_{19}H_{24}N_7O_{14}P_2S$ $[M-H]^-$: 668.0582, found: 668.0557.

Retention time: 5.45 min (*Method 1*)

LC trace for compound 37:



Compound (R_P)-38



***P*^{1},*P*^{2}-di-(5'-*O*-uridine) diammonium (*R*)-diphosphoro-1-thioate**

Following the **General Procedure C** compound **(R_P)-38** was obtained from protected uridine monophosphate **S54** (127 mg, 0.2 mmol), (+)-Ψ* (128 mg, 0.3 mmol) and protected uridine **S8** (278 mg, 0.5 mmol). Deprotection was performed at 40 °C. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 20:80) to afford 57 mg of the title compound after lyophilization (**Yield = 42%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.03 (d, *J* = 8.0 Hz, 1H), 7.96 (d, *J* = 8.2 Hz, 1H), 6.00-5.94 (m, 4H), 4.41-4.36 (m, 4H), 4.36-4.27 (m, 4H), 4.27-4.19 (m, 2H).

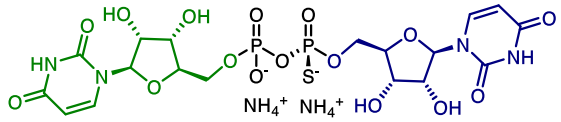
^{13}C NMR (150 MHz, D_2O) δ 165.65, 165.64, 151.27, 151.26, 141.4, 141.3, 102.24, 102.16, 88.0, 87.9, 82.8 (d, *J* = 9.4 Hz), 82.6 (d, *J* = 9.7 Hz), 73.38, 73.35, 69.31, 69.29, 64.54 (d, *J* = 5.9 Hz), 64.53 (d, *J* = 6.5 Hz).

^{31}P NMR (162 MHz, D_2O) δ 43.3 (d, *J* = 27.7 Hz), -12.1 (d, *J* = 27.7 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_{18}H_{23}N_4O_{16}P_2S$ $[M-H]^-$: 645.0310, found: 645.0323.

Retention time: 4.85 min (*Method 1*)

Compound (S_P)-38



***P*^{1},*P*^{2}-di-(5'-*O*-uridine) diammonium (*S*)-diphosphoro-1-thioate**

Following the **General Procedure C** compound **(S_P)-38** was obtained from protected uridine monophosphate **S54** (127 mg, 0.2 mmol), (–)-Ψ* (128 mg, 0.3 mmol) and protected uridine **S8** (278 mg, 0.5 mmol). Deprotection was performed at 40 °C. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 20:80) to afford 54 mg of the title compound after lyophilization (**Yield = 40%, d.r. > 20:1**).

Preparative scale: Following the **General Procedure C** compound **(S_P)-38** was obtained from protected uridine monophosphate **S54** (1.27 g, 2.0 mmol), (–)-Ψ* (1.28 g, 3.0 mmol) and protected uridine **S8** (2.78 g, 5.0 mmol). Deprotection was performed at 40 °C. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 20:80) to afford 0.87 g of the title compound after lyophilization (**Yield = 64%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.01 (dd, *J* = 8.1, 1.4 Hz, 1H), 7.94 (dd, *J* = 8.1, 1.4 Hz, 1H), 5.98-5.94 (m, 4H), 4.42-4.39 (m, 1H), 4.39-4.35 (m, 3H), 4.32-4.24 (m, 5H), 4.23-4.18 (m, 1H).

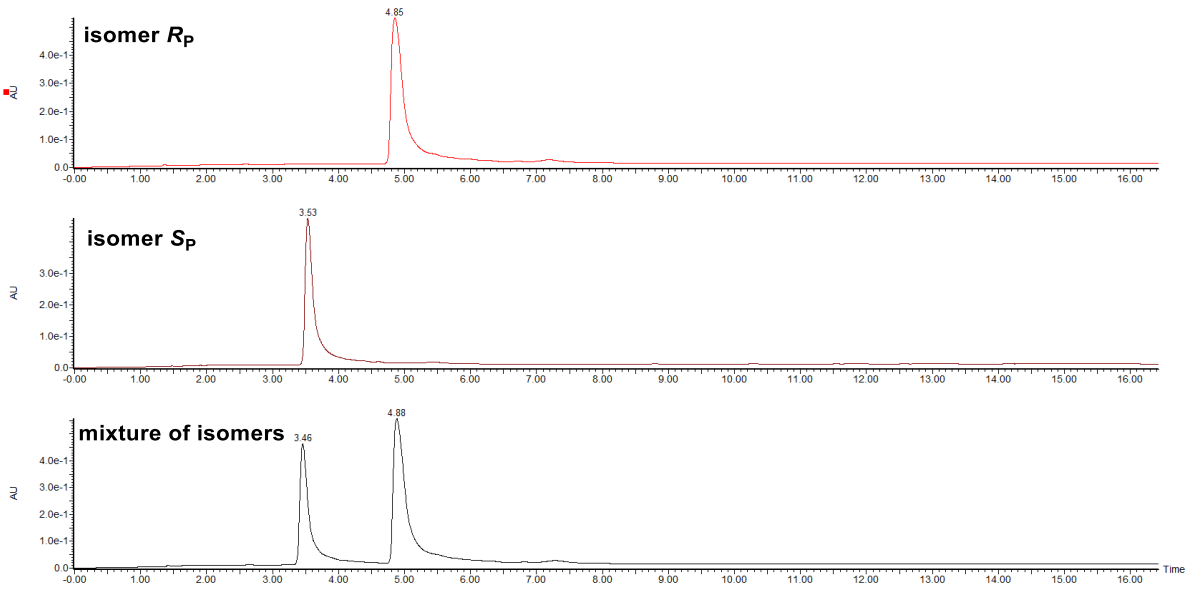
^{13}C NMR (150 MHz, D_2O) δ 165.63, 165.62, 151.2, 141.4, 141.3, 102.3, 102.2, 87.83, 87.77, 82.7 (d, *J* = 9.5 Hz), 82.6 (d, *J* = 10.0 Hz), 73.4, 73.3, 69.29, 69.28, 64.7 (d, *J* = 6.0 Hz), 64.5 (d, *J* = 5.6 Hz). (*One signal missing due to the overlap in the aromatic region*).

^{31}P NMR (162 MHz, D_2O) δ 43.1 (d, *J* = 26.3 Hz), -12.1 (d, *J* = 26.3 Hz).

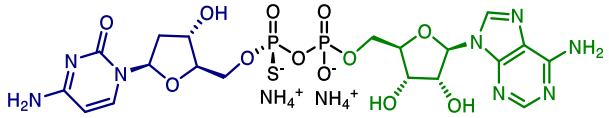
HRMS (ESI-TOF) m/z: calculated for $C_{18}H_{23}N_4O_{16}P_2S$ $[M-H]^-$: 645.0310, found: 645.0323.

Retention time: 3.53 min (*Method 1*)

LC trace for compound 38:



Compound (R_P)-39



***P*^{1}-(5'-*O*-adenosine)-*P*^{2}-(5'-*O*-deoxycytidine) diammonium (*R*)-diphosphoro-2-thioate**

Following the **General Procedure C** with slight modifications compound **(R_P)-39** was obtained from protected adenosine monophosphate **S52** (152 mg, 0.2 mmol), (+)-Ψ* (128 mg, 0.3 mmol) and protected deoxycytidine **S13** (217 mg, 0.5 mmol). Phosphate transfer step was performed using 5.0 equiv. of DBU. Deprotection was performed at 40 °C. The crude product after work-up was neutralized using 10% aq. AcOH and purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 20:80) to afford 93 mg of the title compound after lyophilization (**Yield = 68%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.46 (s, 1H), 8.21 (s, 1H), 7.87 (d, *J* = 7.7 Hz, 1H), 6.18 (t, *J* = 6.5 Hz, 1H), 6.10 (d, *J* = 5.7 Hz, 1H), 5.94 (d, *J* = 7.7 Hz, 1H), 4.55 (t, *J* = 4.5 Hz, 1H), 4.54-4.50 (m, 1H), 4.43-4.39 (m, 1H), 4.37-4.33 (m, 1H), 4.28-4.22 (m, 2H), 4.21-4.16 (m, 2H), 2.38 (dt, *J* = 13.6, 5.2 Hz, 1H), 2.21 (dt, *J* = 13.6, 6.5 Hz, 1H) (*One signal overlapping with D_2O*).

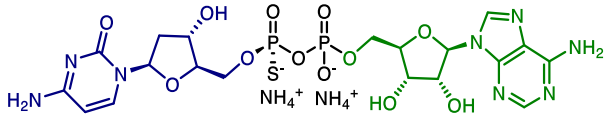
^{13}C NMR (150 MHz, D_2O) δ 162.9, 154.8, 153.5, 151.9, 148.9, 142.4, 140.1, 118.5, 95.7, 87.0, 86.1, 85.5 (d, *J* = 9.6 Hz), 83.8 (d, *J* = 9.4 Hz), 74.2, 70.6, 70.4, 65.4 (d, *J* = 5.6 Hz), 65.3 (d, *J* = 6.0 Hz), 39.5.

^{31}P NMR (162 MHz, D_2O) δ 43.5 (d, *J* = 27.4 Hz), -12.5 (d, *J* = 27.4 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_{19}H_{27}N_8O_{12}P_2S$ $[M+H]^+$: 653.0944, found: 653.0962.

Retention time: 6.38 min (*Method 1*)

Compound (S_P)-39



***P*^{1}-(5'-*O*-adenosine)-*P*^{2}-(5'-*O*-deoxycytidine) diammonium (*S*)-diphosphoro-2-thioate**

Following the **General Procedure C** with slight modifications compound **(S_P)-39** was obtained from protected adenosine monophosphate **S52** (152 mg, 0.2 mmol), (–)-Ψ* (128 mg, 0.3 mmol) and protected deoxycytidine **S13** (217 mg, 0.5 mmol). Phosphate transfer step was performed using 5.0 equiv. of DBU. Deprotection was performed at 40 °C. The crude product after work-up was neutralized using 10% aq. AcOH and purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3/water, from 0:100 to 20:80) to afford 85 mg of the title compound after lyophilization (**Yield = 62%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.46 (s, 1H), 8.19 (s, 1H), 7.83 (d, *J* = 7.7 Hz, 1H), 6.15 (t, *J* = 6.5 Hz, 1H), 6.08 (d, *J* = 5.8 Hz, 1H), 5.92 (d, *J* = 7.7 Hz, 1H), 4.59-4.55 (m, 1H), 4.54-4.50 (m, 1H), 4.41-4.38 (m, 1H), 4.28-4.23 (m, 2H), 4.23-4.17 (m, 3H), 2.35 (dt, *J* = 13.7, 4.8 Hz, 1H), 2.17 (dt, *J* = 13.7, 6.5 Hz, 1H) (*One signal overlapping with D_2O*).

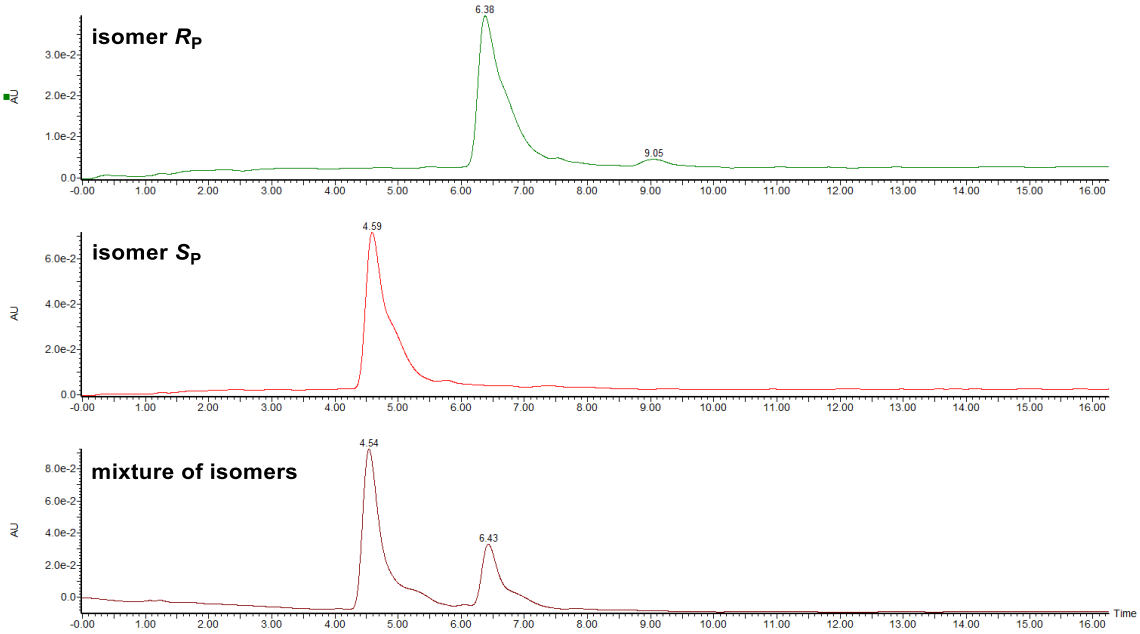
^{13}C NMR (150 MHz, D_2O) δ 162.3, 154.6, 152.8, 151.7, 148.9, 142.5, 140.1, 118.3, 95.6, 86.9, 86.0, 85.5 (d, *J* = 10.1 Hz), 83.9 (d, *J* = 9.5 Hz), 74.2, 70.7, 70.5, 65.6 (d, *J* = 5.8 Hz), 65.4 (d, *J* = 5.5 Hz), 39.6.

^{31}P NMR (162 MHz, D_2O) δ 43.2 (d, *J* = 25.6 Hz), -12.0 (d, *J* = 25.6 Hz).

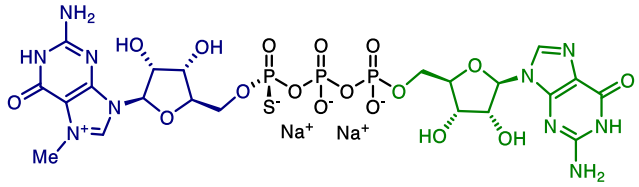
HRMS (ESI-TOF) m/z: calculated for $C_{19}H_{27}N_8O_{12}P_2S$ $[M+H]^+$: 653.0944, found: 653.0962.

Retention time: 4.59 min (*Method 1*)

LC trace for compound 39:



Compound (R_P)-40



***P*^{1}-(5'-*O*-7-methylguanosine)-*P*^{3}-(5'-*O*-guanosine) disodium (*R*)-triphosphoro-1-thioate**

A flame dried round bottom flask with a stir bar was charged with protected guanosine diphosphate **S60** (1.26 g, 1.0 mmol, 1.0 equiv.; 75% wt. purity) and the flask was capped with a septum. Anhydrous DMSO (10 mL) and DBU (0.90 mL, 6.0 mmol, 6.0 equiv.) were added and the mixture was stirred for 10 min. Subsequently, 3 Å molecular sieves (1.0 g) and (+)-Ψ* reagent (1.08 g, 2.5 mmol, 2.5 equiv.) were added and the reaction was stirred at room temperature for 1 h. After that time protected 7-methylguanosine **S56** (0.90 g, 2.0 mmol, 2.0 equiv.) was added, followed by another portion of DBU (1.05 mL, 7.0 mmol, 7.0 equiv.) and the mixture was stirred for another 5 h. Upon completion of the reaction, resulting mixture was filtered and the solid residue was washed with ~2 mL of DMSO. Filtrate was partitioned between six 50 mL centrifuge tubes containing a solution of 0.2 M $NaClO_4$ in acetone (40 mL) each. The resulting suspension was centrifuged at 1000 x *g* for 3 min. The supernatant was discarded, and the pellet was washed with acetone twice. The solid residues from each of the centrifuge tubes were redissolved in minimal amount of water,

combined and purified by ion exchange chromatography on DEAE Sephadex (gradient 1 M NH_4HCO_3/water, from 0:100 to 40:60). Fractions containing product were combined and the solvent was evaporated under reduced pressure (temp. ≤ 40 °C). The remaining solid was co-evaporated with water 3 times to remove residual buffer. The residue was redissolved in 40 mL 80% aq. AcOH and the resulting solution was stirred at 35 °C for 16 h. Subsequently, volatiles were removed under reduced pressure, the crude was redissolved in a mixture of MeOH/H_2O/Et_3N (20:5:1) (100 mL) and the solution was stirred at 30 ºC for 16 h. The reaction mixture was concentrated under reduced pressure (temp. ≤ 40 °C) ant the crude was purified by reverse-phase column chromatography on C-18 silica gel (gradient 1 M aq. TEAA/MeCN, from 100:0 to 95:5). Fractions containing product were combined and lyophilized 3 times to remove remaining buffer. Obtained solid was redissolved in minimal amount of water and the product was precipitated as sodium salt from 0.2 M $NaClO_4$ in acetone to afford 370 mg of the title compound after drying under high vacuum (**Yield = 43%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.00 (s, 1H), 5.86 (d, *J* = 2.2 Hz, 1H), 5.78 (d, *J* = 6.1 Hz, 1H), 4.60 (t, *J* = 5.6 Hz, 1H), 4.49-4.46 (m, 1H), 4.46-4.42 (m, 2H), 4.40-4.37 (m, 1H), 4.36-4.29 (m, 4H), 4.29-4.24 (m, 1H), 4.05 (s, 3H).

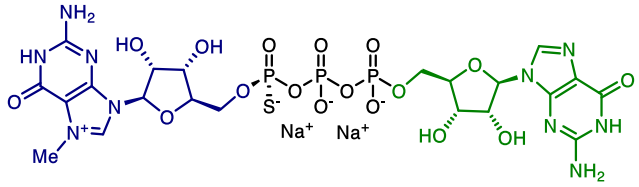
^{13}C NMR (150 MHz, D_2O) δ 162.8, 161.7, 158.8, 154.0, 151.3, 148.9. 136.9, 133.6 (br), 115.7, 108.8, 89.3, 86.4, 83.6 (d, *J* = 8.9 Hz), 82.9 (d, *J* = 9.7 Hz), 74.7, 74.1, 70.4, 68.5, 65.4 (d, *J* = 5.4 Hz), 63.9 (d, *J* = 6.5 Hz), 36.0.

^{31}P NMR (162 MHz, D_2O) δ 42.8 (d, *J* = 26.0 Hz), -11.6 (d, *J* = 20.1 Hz), -24.0 (dd, *J* = 26.0, 20.1 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_{21}H_{28}N_{10}O_{17}P_3S$ $[M-H]^-$: 817.0573, found: 817.0567.

Retention time: 9.63 min (*Method 2*)

Compound (S_P)-40



***P*^{1}-(5'-*O*-7-methylguanosine)-*P*^{3}-(5'-*O*-guanosine) disodium (*S*)-triphosphoro-1-thioate**

Compound **(S_P)-40** was obtained following analogous procedure as **(R_P)-40** using (–)-Ψ*. Purification by reverse-phase column chromatography on C-18 silica gel (gradient 1 M aq. TEAA/MeCN, from 100:0 to 95:5), followed by precipitation as a sodium salt afforded 353 mg of the title compound, after drying under high vacuum (**Yield = 41%, d.r. > 20:1**).

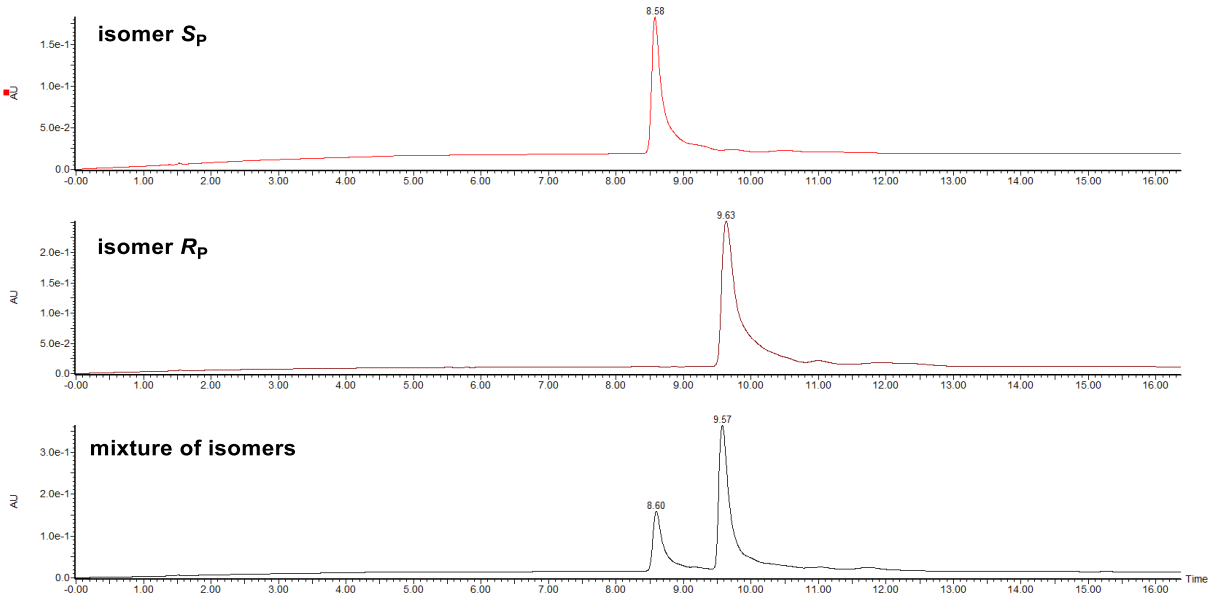
Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 8.01 (s, 1H), 5.88 (d, *J* = 3.0 Hz, 1H), 5.79 (d, *J* = 5.9 Hz, 1H), 4.66 (t, *J* = 5.5 Hz, 1H), 4.52-4.48 (m, 2H), 4.48-4.45 (m, 1H), 4.41-4.32 (m, 4H), 4.30-4.25 (m, 2H), 4.06 (s, 3H).

^{13}C NMR (150 MHz, D_2O) δ 162.8, 161.7, 158.8, 154.0, 151.3, 149.1. 137.2, 133.9 (br), 115.9, 108.9, 89.0, 86.6, 83.6 (d, *J* = 8.6 Hz), 83.3 (d, *J* = 9.7 Hz), 74.9, 73.8, 70.3, 68.9, 65.4 (d, *J* = 5.0 Hz), 64.4 (d, *J* = 5.7 Hz), 35.9.

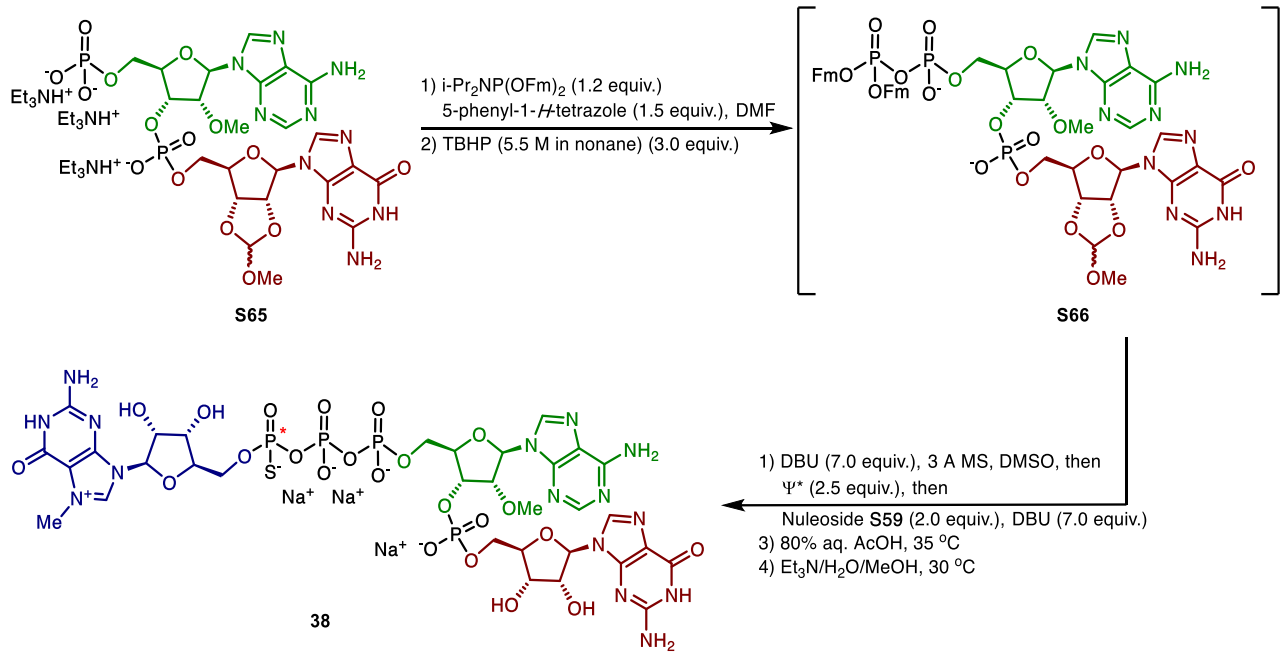
^{31}P NMR (162 MHz, D_2O) δ 43.3 (d, *J* = 25.5 Hz), -11.5 (d, *J* = 17.2 Hz), -24.1 (dd, *J* = 25.5, 27.2 Hz).
HRMS (ESI-TOF) m/z: calculated for $C_{21}H_{28}N_{10}O_{17}P_3S$ $[M-H]^-$: 817.0573, found: 817.0567.
Retention time: 8.58 min (*Method 2*)

LC trace for compound 40:



CleanCap® AG γ-thio-analogue (41)

*The calculated yields for CleanCap® AG γ-thio-analogues **41** include the yield of preparation of the diphosphate donor **S66***



Compound **S65** (1.56 g, 1.6 mmol, 1.0 equiv.) was dried by co-evaporation with anhydrous DMF and dissolved in anhydrous DMF (20 mL). i-$Pr_2NP(OFm)_2$ (1.27 g, 2.4 mmol, 1.5 equiv.) was added to the resulting solution,

followed by 5-phenyl-1-*H*-tetrazole (0.36 g, 2.4 mmol, 1.5 equiv.) and the reaction was stirred for 1 h at room temperature. Subsequently, *tert*-butyl hydroperoxide (5.5 M in nonane; 0.86 mL, 4.8 mmol, 3.0 equiv.) was added and the mixture was stirred for another 1 h. The reaction was quenched by the addition of Et_2O (250 mL) and the resulting suspension was sonicated for 10 min. Solvent was decanted and the remaining oily residue was redissolved in DCM (10 mL). Volatile components were evaporated under reduced pressure, and the residue was co-evaporated sequentially with MeCN and DCM to provide 2.72 g of crude compound **S66** (65% wt. purity as determined by quantitative ^{31}P NMR) (**NMR yield = 91%**).

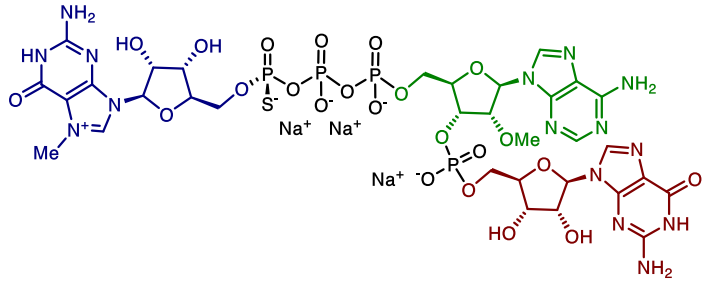
Compound **S66** was used in subsequent steps without any further purification.

(*Note*: *Due to low stability of the compound **S66** on silica we were not able to obtain analytically pure sample to gather analytical data. Compound **S66** was characterized only by crude ^{31}P NMR and HRMS*).

^{31}P NMR (162 MHz, DMSO-d_6) δ -1.9, -12.2 (d, *J* = 20.9 Hz), -12.6 (d, *J* = 20.9 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_{51}H_{50}N_{10}O_{18}P_3$ $[M-H]^-$: 1183.2523, found: 1183.2497.

Compound (R_P)-41



***P*^{1}-{5'-*O*-[2'-*O*-methyladenylyl-(3',5')-guanosine]}-*P*^{3}-(5'-*O*-7-methylguanosine) trisodium (*R*)-triphosphoro-3-thioate**

A flame dried round bottom flask with a stir bar was charged with crude protected guanosine diphosphate **S66** (1.82 g, 1.0 mmol, 1.0 equiv.; 65% wt. purity) (*obtained as described above*) and the flask was capped with a septum. Anhydrous DMSO (10 mL) and DBU (1.05 mL, 7.0 mmol, 7.0 equiv.) were added and the mixture was stirred for 10 min. Subsequently, 3 Å molecular sieves (1.0 g) and (+)-Ψ* reagent (1.08 g, 2.5 mmol, 2.5 equiv.) were added and the reaction was stirred at room temperature for 1 h. After that time protected 7-methylguanosine **S56** (0.90 g, 2.0 mmol, 2.0 equiv.) was added, followed by another portion of DBU (1.05 mL, 7.0 mmol, 7.0 equiv.) and the mixture was stirred for another 5 h. Upon completion of the reaction, the resulting mixture was filtered, and the solid residue was washed with ~2 mL of DMSO. Filtrate was partitioned between six 50 mL centrifuge tubes containing a solution of 0.2 M $NaClO_4$ in acetone (40 mL) each. The resulting suspension was centrifuged at 1000 x *g* for 3 min. Supernatant was discarded and the pellet was washed with acetone twice. The solid residues from each of the centrifuge tubes were redissolved in minimal amount of water, combined, and purified by ion exchange chromatography on DEAE Sephadex (gradient 1 M NH_4HCO_3/water, from 0:100 to 40:60). Fractions containing product were combined and the solvent was evaporated under reduced pressure (temp. ≤ 40 °C). The remaining solid was co-evaporated with water 3 times to remove residual buffer. The residue was redissolved in 80% aq. AcOH (40 mL), and the resulting solution was stirred at 35 °C for 16 h. Subsequently, the volatiles were removed under reduced pressure, the crude was redissolved in a mixture of MeOH/H_2O/Et_3N (20:5:1) (100 mL) and the solution was stirred at 30 °C for 16 h.

The reaction mixture was concentrated under reduced pressure (temp. ≤ 40 °C) ant the crude was purified by reverse-phase column chromatography on C-18 silica gel (gradient 1 M aq. TEAA/MeCN, from 100:0 to 95:5). Fractions containing product were combined and lyophilized 3 times to remove remaining buffer. Obtained solid was redissolved in minimal amount of water and the product was precipitated as sodium salt from 0.2 M $NaClO_4$ in acetone to afford 522 mg of the title compound after drying under high vacuum (**Yield = 38% from S65, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 9.04 (s, 1H), 8.36 (s, 1H), 8.06 (s, 1H), 7.95 (s, 1H), 6.00 (d, *J* = 5.5 Hz, 1H), 5.85-5.80 (m, 2H), 4.97-4.92 (m, 1H), 4.78-4.72 (m, 1H), 4.54-4.48 (m, 3H), 4.46-4.40 (m, 3H), 4.37-4.31 (m, 3H), 4.31-4.17 (m, 4H), 4.02 (s, 3H), 3.44 (s, 3H). (*Note: singlet at 9.04 ppm comes from an imidazolium cation hydrogen, which is partially exchangeable with deuterium in D_2O and may not appear on ^{1}H NMR spectrum or may appear with lower-than-expected intensity*).

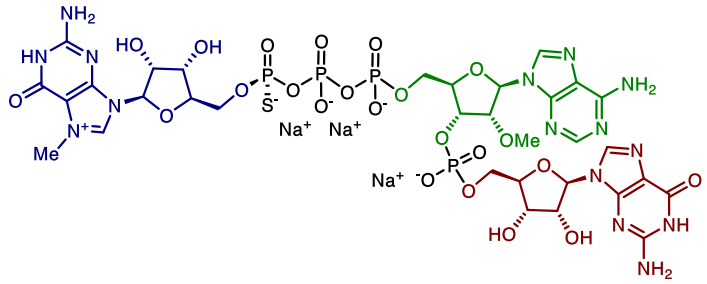
^{13}C NMR (150 MHz, D_2O) δ 158.0, 156.3, 155.9, 154.7, 153.1, 152.3, 150.9, 148.4, 148.0, 138.9, 137.0, 135.0 (br), 117.7, 115.6, 107.4, 89.1, 86.9, 84.4, 83.0 (d, *J* = 9.1 Hz), 82.42 (d, *J* = 9.5 Hz), 82.40 (d, *J* = 9.6 Hz), 81.5 (d, *J* = 4.2 Hz), 74.3, 72.9, 72.1 (d, *J* = 4.9 Hz), 69.8, 68.5, 64.60 (d, *J* = 4.6 Hz), 63.5 (d, *J* = 6.2 Hz), 57.5, 35.6.

^{31}P NMR (162 MHz, D_2O) δ 42.9 (d, *J* = 27.4 Hz), -1.0, -11.9 (d, *J* = 18.7 Hz), -24.2 (dd, *J* = 27.4, 18.7 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_{32}H_{42}N_{15}O_{23}P_4S$ $[M-H]^-$: 1160.1254, found: 1160.1228.

Retention time: 8.43 min (*Method 1*)

Compound (S_P)-41



***P*^{1}-{5'-*O*-[2'-*O*-methyladenylyl-(3',5')-guanosine]}-*P*^{3}-(5'-*O*-7-methylguanosine) trisodium (*S*)-triphosphoro-3-thioate**

Compound **(S_P)-41** was obtained following analogous procedure as **(R_P)-41** on 0.1 mmol scale and using (–)-Ψ*. Purification by reverse-phase column chromatography on C-18 silica gel (gradient 1 M aq. TEAA/MeCN, from 100:0 to 95:5), followed by precipitation as a sodium salt afforded 43 mg of the title compound, after drying under high vacuum (**Yield = 35% from S68, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 9.16 (s, 1H), 8.46 (s, 1H), 8.18 (s, 1H), 7.97 (s, 1H), 6.04 (d, *J* = 4.5 Hz, 1H), 5.88 (s, 1H), 5.81 (d, *J* = 4.5 Hz, 1H), 4.98-4.91 (m, 1H), 4.78-4.72 (m, 1H), 4.61-4.56 (m, 1H), 4.54-4.48 (m, 3H), 4.45-4.17 (m, 10H), 4.03 (s, 3H), 3.47 (s, 3H). (*Note: singlet at 9.16 ppm comes from an imidazolium*

cation hydrogen, which is partially exchangeable with deuterium in D_2O and may not appear on ^1H NMR spectrum or may appear with lower-than-expected intensity).

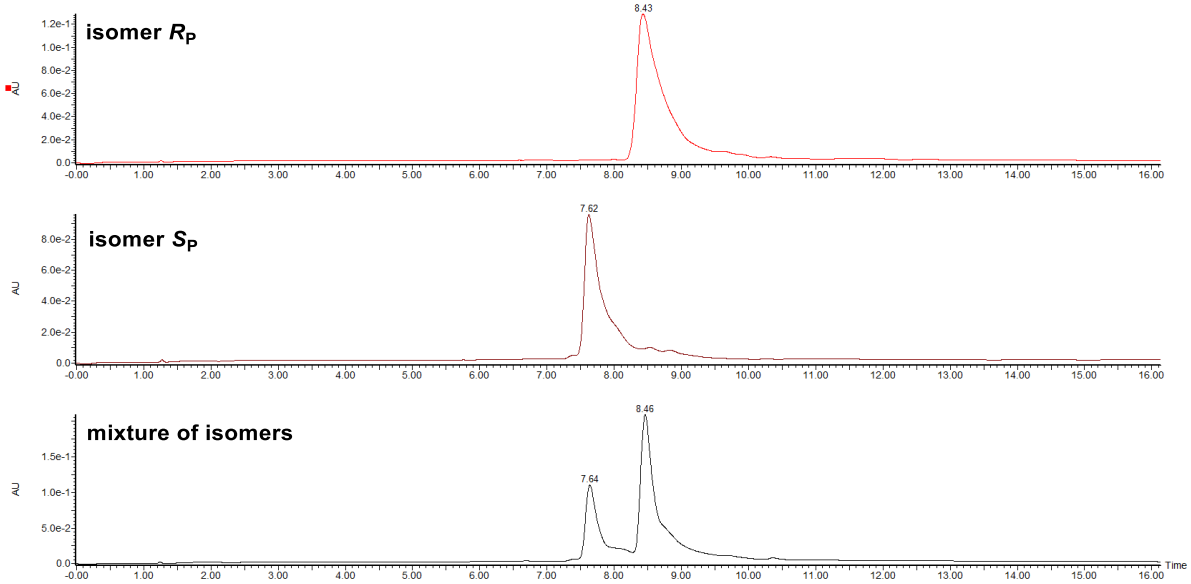
^{13}C NMR (150 MHz, D_2O) δ 157.7, 154.8, 153.8, 153.1, 152.3, 150.8, 149.0, 148.6, 147.6, 140.0, 137.0, 136.1, 117.7, 115.3, 107.2, 89.1, 87.0, 85.0, 83.4 (d, *J* = 9.3 Hz), 83.0 (d, *J* = 8.8 Hz), 82.3 (br), 81.6 (d, *J* = 3.5 Hz), 74.4, 73.0, 71.9 (d, *J* = 4.8 Hz), 69.7, 68.7, 64.6 (d, *J* = 4.9 Hz), 64.4, 63.8, 57.5, 35.6.

^{31}P NMR (162 MHz, D_2O) δ 43.2 (d, *J* = 26.6 Hz), -1.0, -11.8 (d, *J* = 18.8 Hz), -24.3 (dd, *J* = 26.6, 18.8 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_{32}H_{42}N_{15}O_{23}P_4S$ $[M-H]^-$: 1160.1254, found: 1160.1228.

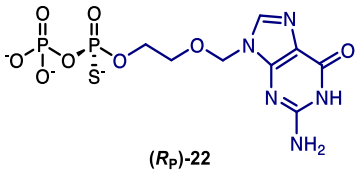
Retention time: 7.62 min (*Method 1*)

LC trace for compound 41:

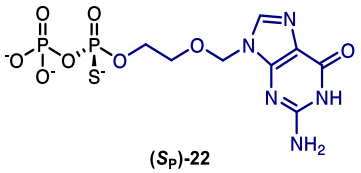


8.4 Confirmation of Enantiomeric Purity of Acyclovir α-Thiodiphosphate (22)

Enantiomers **(R_P)-22** and **(S_P)-22** were synthesized as described in section 8.3.1. Optical rotation values were measured in DMSO for triethylammonium salt of **22**.



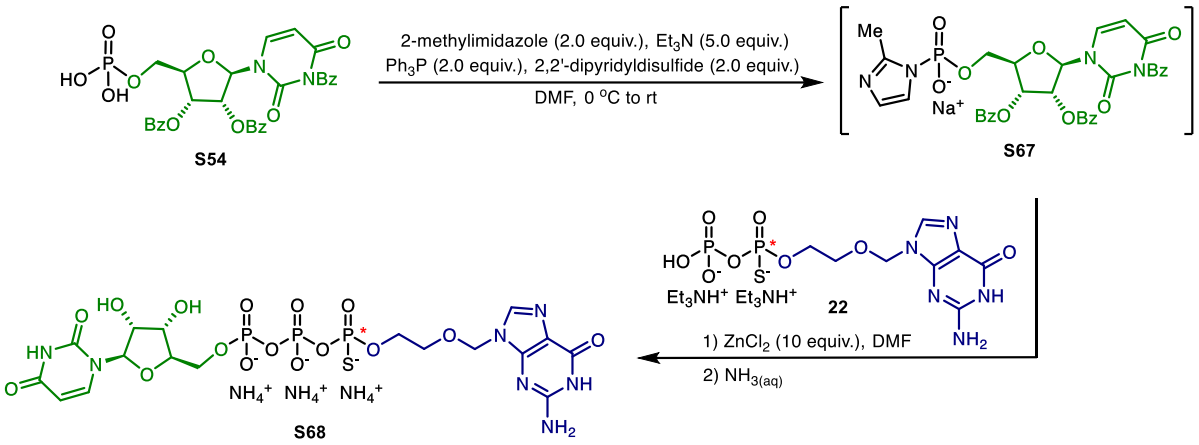
(R_P)-22
$[a]_D^{25}$ = +10.6



(S_P)-22
$[a]_D^{25}$ = -11.0

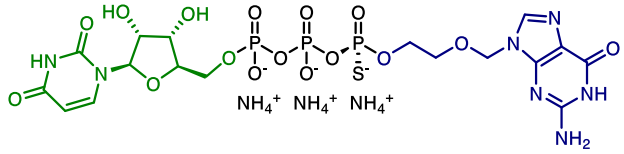
8.4.1 Derivatization Strategy

Despite multiple attempts we were unable to confirm enantiomeric purity of **22** by chiral HPLC (*see section 8.4.2 for details regarding examined LC method*). Therefore, compound **22** obtained using either (+)-Ψ* and (–)-Ψ* was derivatized using following procedure:



Protected uridine **S54** (0.64 g, 1.0 mmol, 1.0 equiv.) was dissolved in anhydrous DMF (5 mL) under argon atmosphere. The solution was cooled to 0 °C and anhydrous triethylamine (0.70 mL, 5.0 mmol, 5.0 equiv.) was added dropwise. After 5 min 2-methylimidazole (0.16 g, 2.0 mmol, 2.0 equiv.) and triphenylphosphine (0.52 g, 2.0 mmol, 2.0 equiv.) were added consecutively. The ice bath was removed, and the reaction was stirred for 10 min. Subsequently, 2,2'-dipyridyldisulfide (0.44 g, 2.0 mmol, 2.0 equiv.) was added and the reaction was stirred at room temperature for 3 h. After that time, the reaction was quenched by addition of anhydrous Et_2O (300 mL). The resulting heterogeneous mixture was centrifuged at 1000 x *g* for 3 min, the supernatant was discarded, and the solid residue was redissolved in minimal amount of anhydrous DMF. The solution was added to premade 0.2 M $NaClO_4$ solution in Et_2O/EtOAc/Et_3N (5:10:1; 40 mL). The resulting suspension was centrifuged at 1000 x *g* for 3 min, the supernatant was discarded, and the pellet was washed twice with Et_2O/EtOAc (1:2). The resulting solid was dried under high vacuum to provide 0.50 g of a crude compound **S67**, which was used in the next step without any further purification.

Compound (R_P)-S68



***P*^{1}-(5'-*O*-uridine)-*P*^{3}-(5'-*O*-acycloguanosine) triammonium (*R*)-triphosphoro-3-thioate**

Compound **(R_P)-22** (ammonium form) obtained as described in section 8.3.1 was transformed into bis(triethylammonium) salt by loading on a short C18 silica gel column and eluting with 1M triethylammonium acetate. The residual buffer was removed by multiple lyophilization.

Bis(triethylammonium) salt of compound **(R_P)-22** (60 mg, 0.1 mmol, 1.0 equiv.) was dried by co-evaporation with anhydrous DMF at 55 °C. Subsequently, crude compound **S67** (108 mg, 0.15 mmol, 1.5 equiv.) was added, followed by premade solution of 0.2 M $ZnCl_2$ in anhydrous DMF (5.0 mL) and the reaction was stirred under argon atmosphere for 2 days. After that time, the resulting solution was added dropwise to Et_2O (100 mL). The resulting suspension was centrifuged at 1000 x *g* for 3 min, the supernatant was discarded, and the oily residue was treated with deionized water to initiate precipitation. The suspension was centrifuged at 1000 x *g* for 3 min, the supernatant was discarded, and the pellet was redissolved in DMSO (5.0 mL). Concentrated aq. NH_3 (20 mL) was added, and the mixture was stirred overnight at room temperature. Subsequently, the reaction mixture was concentrated to ~ 5 mL under reduced pressure and added dropwise to a solution of 0.2 M $NaClO_4$ in acetone (40 mL). The resulting suspension was centrifuged at 1000 x *g* for 3 min. The supernatant was discarded, and the pellet was washed with acetone twice. The crude product was purified by ion exchange chromatography on DEAE Sephadex (gradient 1 M NH_4HCO_3/water, from 0:100 to 40:60). Fractions containing product were combined and the solvent was evaporated under reduced pressure (temp. ≤ 40 °C). The solid residue was dissolved in minimal amount of water and lyophilized to provide 26 mg of the title compound (**Yield = 34%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 7.93 (s, 1H), 7.85 (d, *J* = 8.1 Hz, 1H), 5.90 (d, *J* = 5.2 Hz, 1H), 5.88 (d, *J* = 8.1 Hz, 1H), 5.51 (s, 2H), 4.38-4.34 (m, 1H), 4.32 (t, *J* = 5.2 Hz, 1H), 4.27-4.19 (m, 3H), 4.19-4.12 (m, 2H), 3.80 (t, *J* = 4.5 Hz, 2H).

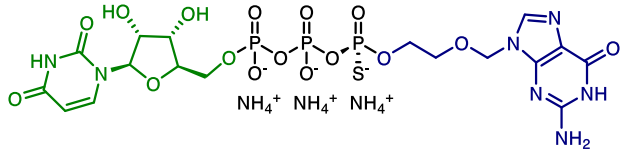
^{13}C NMR (150 MHz, D_2O) δ 166.2, 159.0, 154.1, 151.8, 151.6, 141.4, 139.9, 116.0, 102.5, 88.3, 83.2 (d, *J* = 9.2 Hz), 73.8, 72.7, 69.6, 68.2 (d, *J* = 8.8 Hz), 65.3 (d, *J* = 6.1 Hz), 65.0 (d, *J* = 5.5 Hz).

^{31}P NMR (162 MHz, D_2O) δ 43.3 (d, *J* = 26.1 Hz), -11.6 (d, *J* = 19.2 Hz), -24.1 (dd, *J* = 26.1, 19.2 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_{17}H_{25}N_7O_{16}P_3S$ $[M+H]^+$: 708.0291, found: 708.0300.

Retention time: 8.78 min (*Method 2*)

Compound (S_P)-S68



***P*^{1}-(5'-*O*-uridine)-*P*^{3}-(5'-*O*-acycloguanosine) triammonium (*S*)-triphosphoro-3-thioate**

Compound **(S_P)-S68** was obtained following analogous procedure as **(R_P)-S68** on 0.1 mmol scale using **(S_P)-22** (obtained as described in section 8.3.1). Purification by DEAE Sephadex (gradient 1 M NH_4HCO_3/water, from 0:100 to 40:60), afforded 23 mg of the title compound, after lyophilization (**Yield = 30%, d.r. > 20:1**).

Physical state: white solid

^{1}H NMR (600 MHz, D_2O) δ 7.94 (s, 1H), 7.84 (d, *J* = 6.9 Hz, 1H), 5.92 - 5.86 (m, 2H), 5.52 (s, 2H), 4.38-4.30 (m, 2H), 4.28-4.19 (m, 3H), 4.19-4.12 (m, 2H), 3.81 (t, *J* = 4.5 Hz, 2H).

^{13}C NMR (150 MHz, D_2O) δ 166.2, 159.0, 154.1, 151.8, 151.6, 141.3, 139.9, 116.0, 102.5, 88.3, 83.1 (d, *J* = 9.0 Hz), 73.7, 72.6, 69.6, 68.1 (d, *J* = 8.8 Hz), 65.4 (d, *J* = 5.8 Hz), 65.0 (d, *J* = 4.9 Hz).

^{31}P NMR (162 MHz, D_2O) δ 43.6 (d, *J* = 26.0 Hz), -11.6 (d, *J* = 18.6 Hz), -24.1 (dd, *J* = 26.0, 18.6 Hz).

HRMS (ESI-TOF) m/z: calculated for $C_{17}H_{25}N_7O_{16}P_3S$ $[M+H]^+$: 708.0291, found: 708.0300.

Retention time: 8.78 min (*Method 2*)

Diastereomeric purities of **(R_P)-S68** and **(S_P)-S68** were confirmed by comparison of their ^{31}P NMR spectra (Fig. S15).

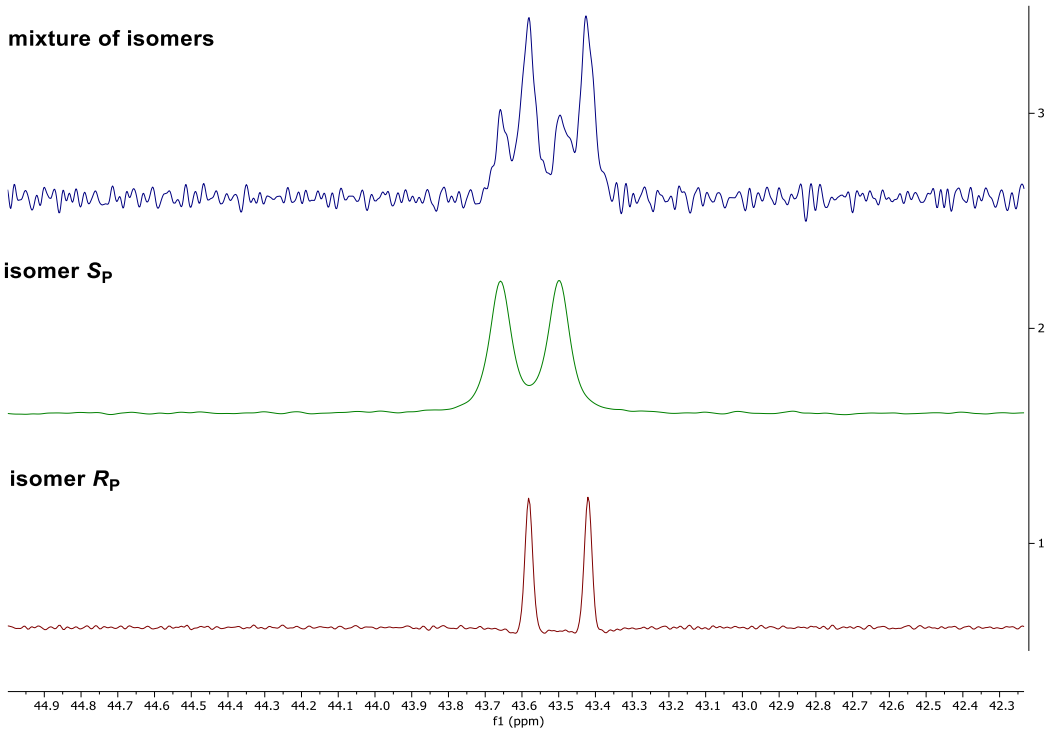


Figure S15. Comparison of ^{31}P NMR for the obtained epimers of **S68**.

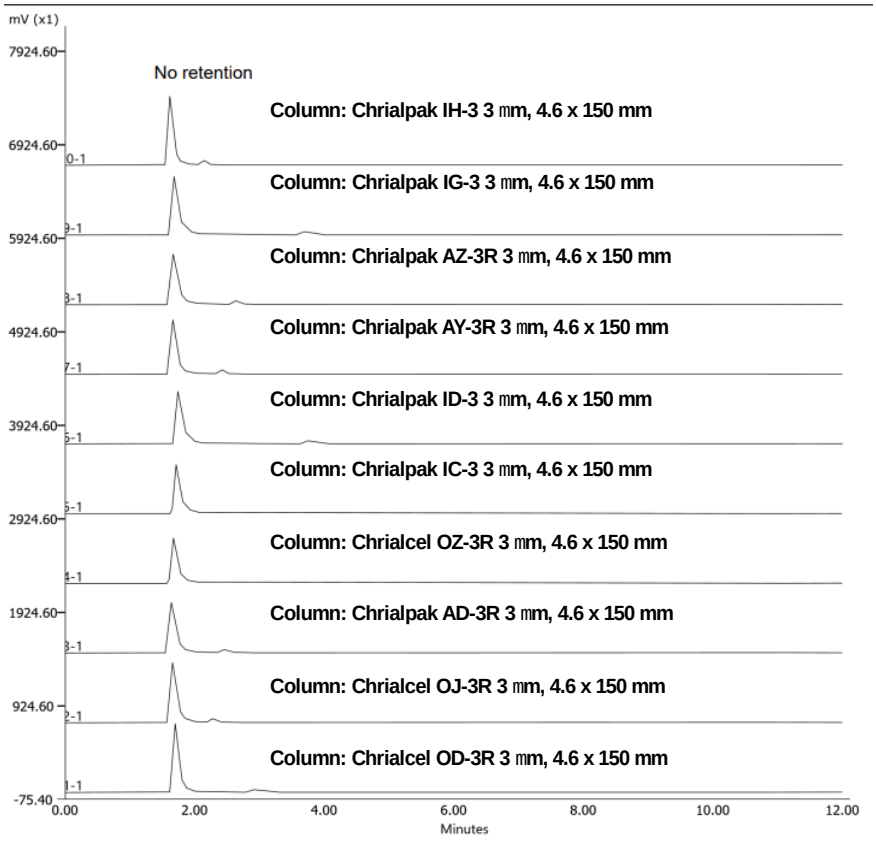
8.4.2 Chiral LC Methods Screening

Polysaccharide-based chiral screening in RP mode (acidic)

Solvent A: 0.05% TFA in MeCN/H_2O (5:95)
Solvent B: 0.05% TFA in MeCN/H_2O (95:5)
Flow rate: 1.2 mL/min
Temperature: 30 °C

Table S7. HPLC method gradient.

time (min)	Solvent A (%)	Solvent B (%)
0	100	0
6	100	0
10	0	100
12	0	100

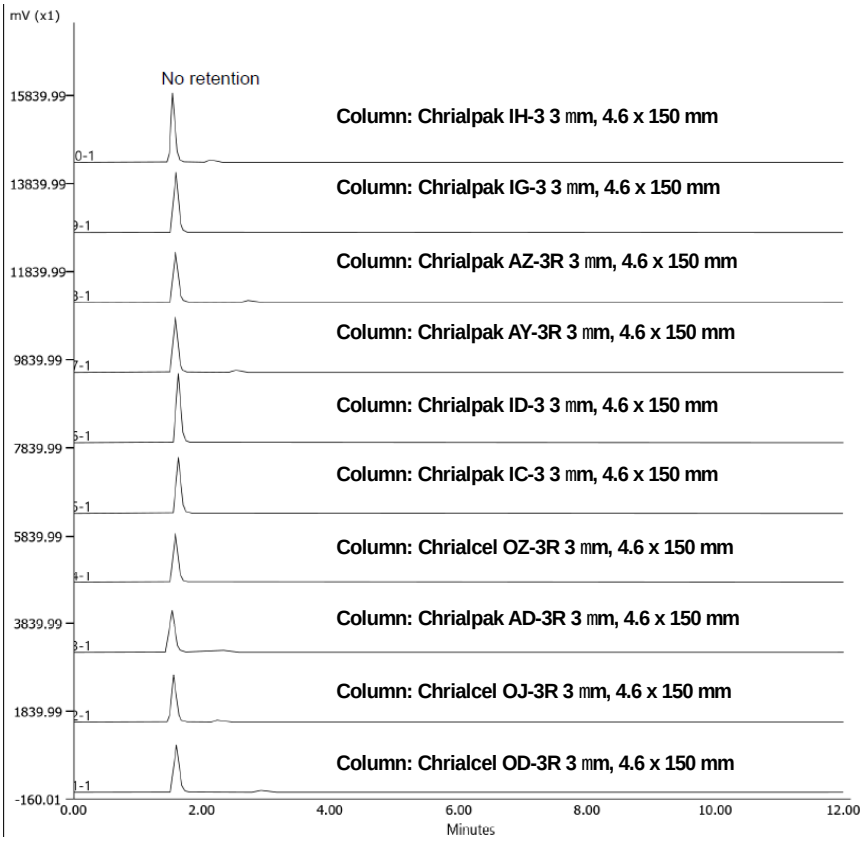


Polysaccharide-based chiral screening in RP mode (neutral)

Solvent A: 0.01 M ammonium acetate in MeCN/H_2O (5:95)
Solvent B: 0.01 M ammonium acetate in MeCN/H_2O (95:5)
Flow rate: 1.2 mL/min
Temperature: 30 °C

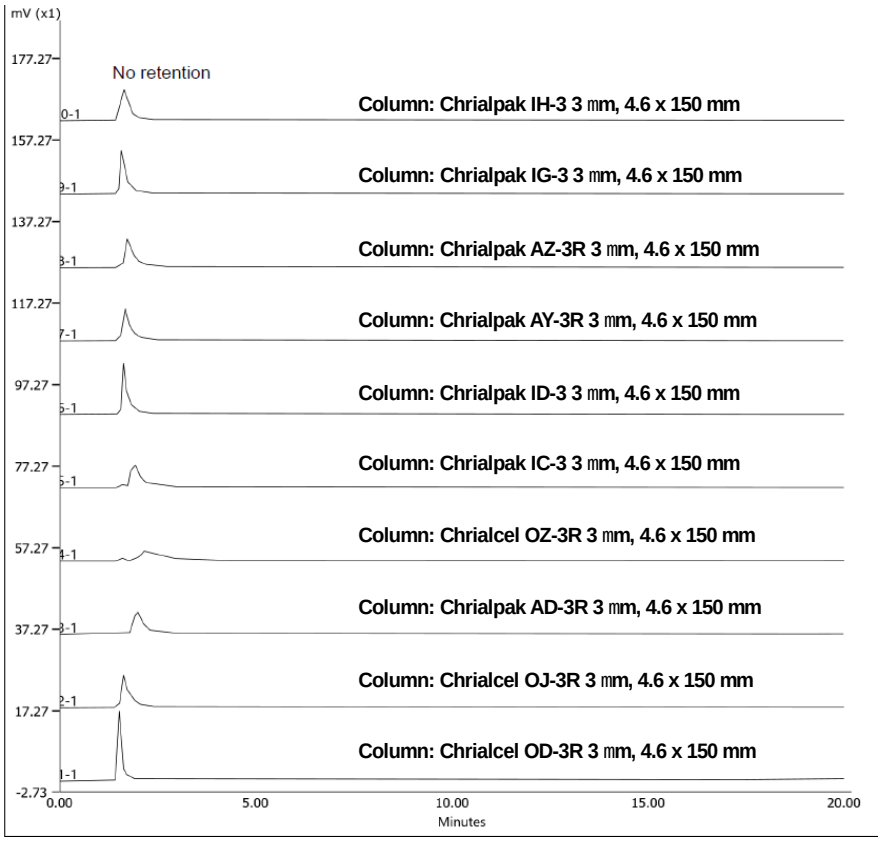
Table S8. HPLC method gradient.

time (min)	Solvent A (%)	Solvent B (%)
0	100	0
6	100	0
10	0	100
12	0	100



Polysaccharide-based chiral screening in HILIC mode (neutral)

Solvent A: 0.01 M ammonium acetate in MeCN/H_2O (5:95)
Solvent B: 0.01 M ammonium acetate in MeCN/H_2O (95:5)
Flow rate: 1.2 mL/min
Temperature: 30 ºC
Method: isocratic A:B (95:5); time = 20 min

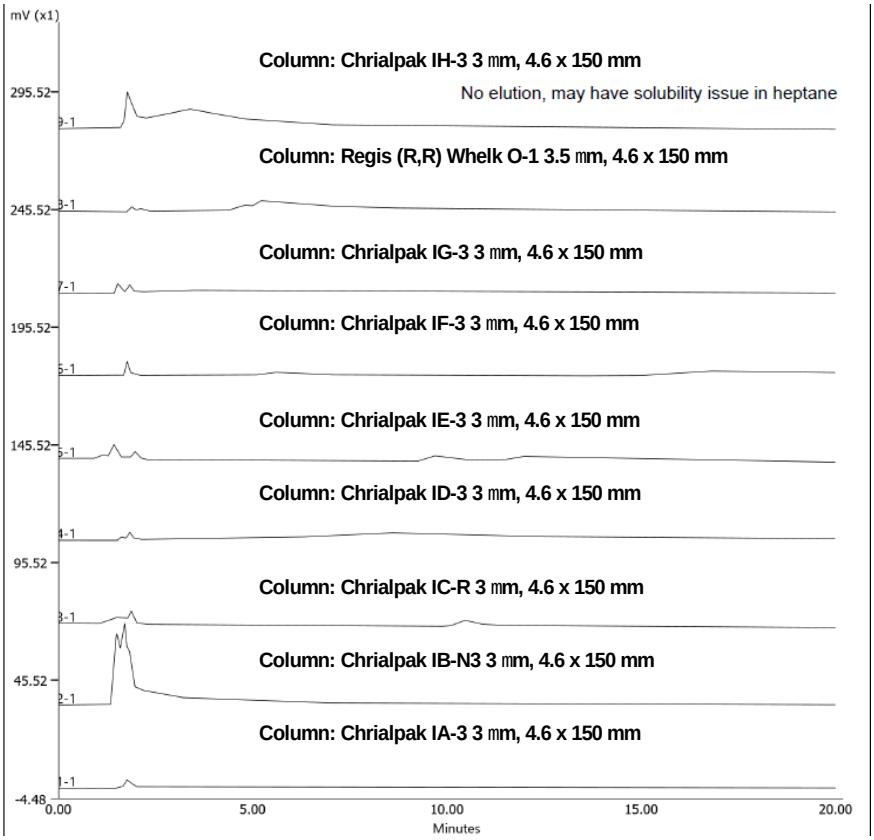


Polysaccharide-based chiral screening in NP mode

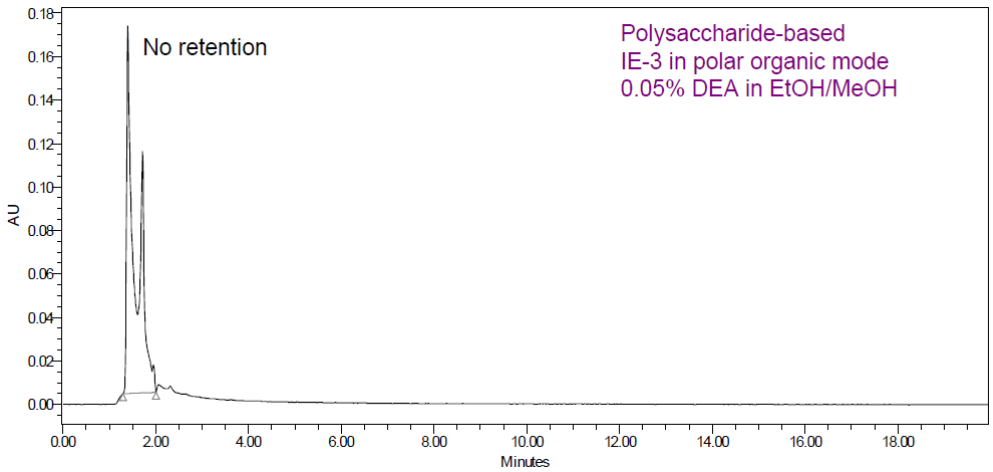
Solvent A: 0.05% diethanolamine in heptane
Solvent B: 0.05% diethanolamine in MeOH/EtOH (1:1)
Flow rate: 1.2 mL/min
Temperature: 30 ºC

Table S9. HPLC method gradient.

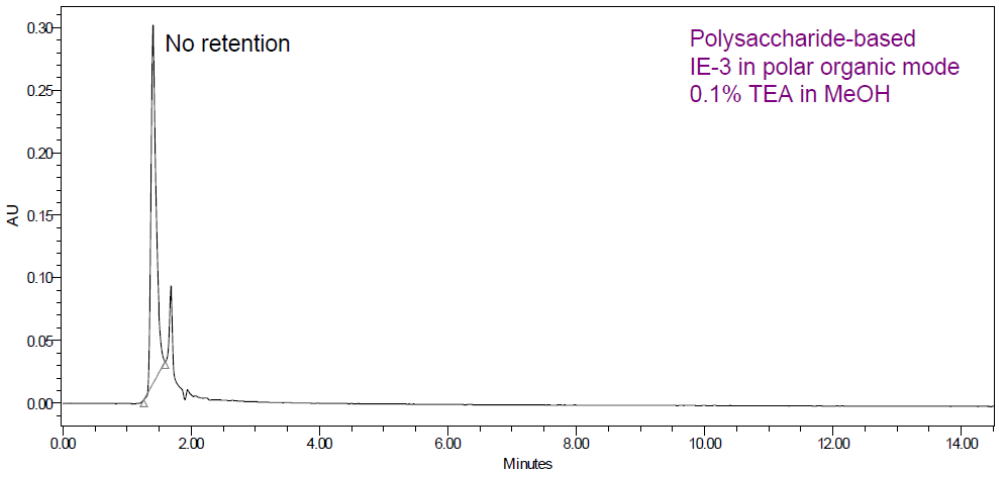
time (min)	Solvent A (%)	Solvent B (%)
0	60	40
15	40	60
20	40	60



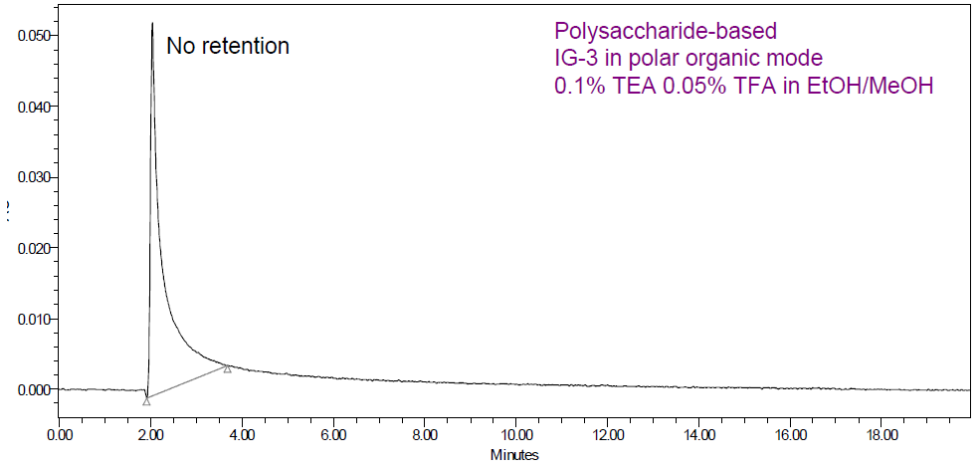
Solvent A: 0.05% diethanolamine in heptane
Solvent B: 0.05% diethanolamine in MeOH/EtOH (1:1)
Flow rate: 1.0 mL/min
Temperature: 40 ºC
Column: Chiralpak IE-3, 3 µm, 4.6 x 150 mm
Method: isocratic 100% solvent B, time = 20 min



Solvent A: 0.1% triethylamine in MeOH
Flow rate: 1.0 mL/min
Temperature: 40 °C
Column: Chiralpak IE-3, 3 µm, 4.6 x 150 mm
Method: isocratic 100% solvent A, time = 15 min

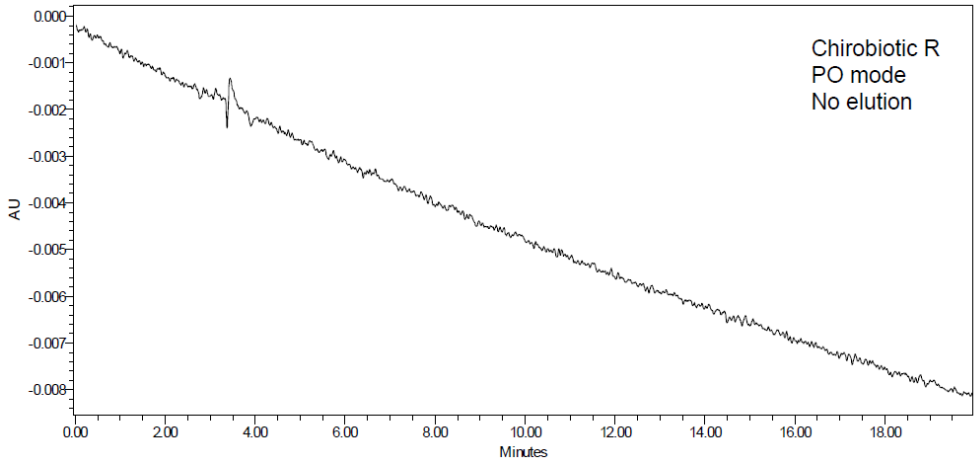


Solvent A: 0.1% triethylamine, 0.05% TFA in MeOH/EtOH (1:1)
Flow rate: 1.0 mL/min
Temperature: 25 °C
Column: Chiralpak IG-3, 3 µm, 4.6 x 150 mm
Method: isocratic 100% solvent A, time = 20 min



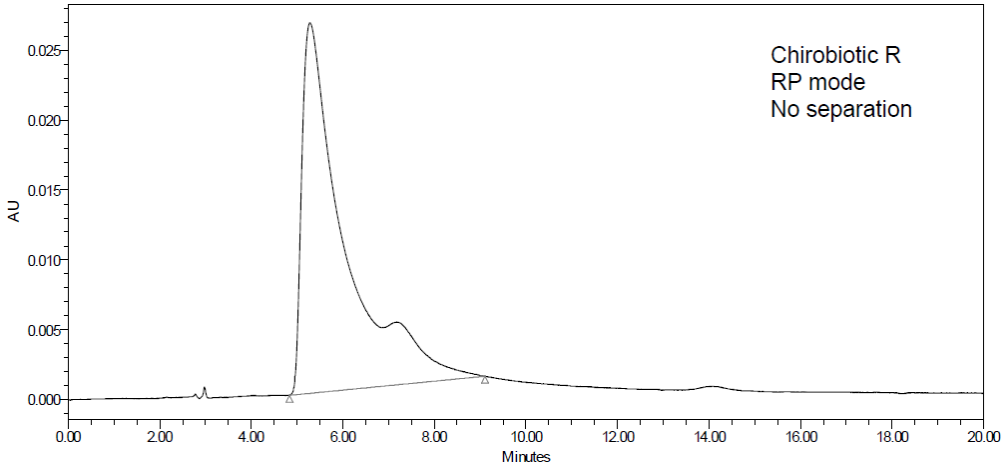
Chrobiotic chiral screening in polar organic mode

Solvent A: 0.1% triethylamine, 0.05% TFA in MeOH
Flow rate: 1.0 mL/min
Temperature: 25 ºC
Column: Chirobiotic R, 5 µm, 4.6 x 250 mm
Method: isocratic 100% solvent A, time = 20 min

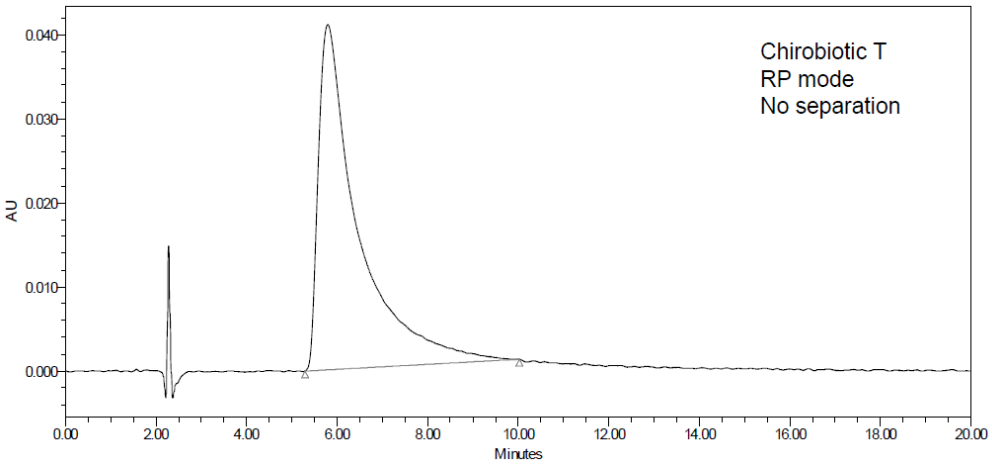


Chrobiotic chiral screening in RP mode

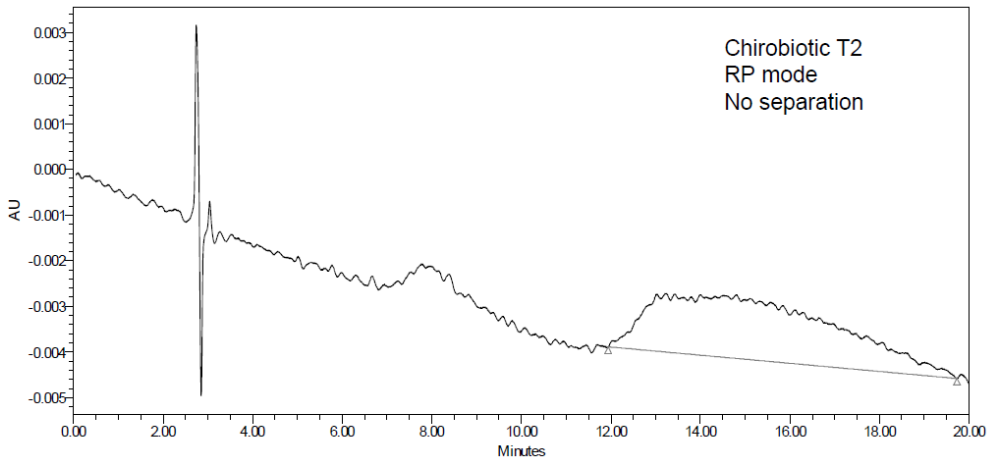
Solvent A: H_2O
Solvent B: 0.02 M ammonium acetate in H_2O/MeOH (20:80)
Flow rate: 1.0 mL/min
Temperature: 25 ºC
Column: Chirobiotic R, 5 µm, 4.6 x 250 mm
Method: isocratic solvent A:B (1:1), time = 20 min



Solvent A: H_2O
Solvent B: 0.02 M ammonium acetate in H_2O/MeOH (20:80)
Flow rate: 0.8 mL/min
Temperature: 25 ºC
Column: Chirobiotic T, 5 µm, 4.6 x 150 mm
Method: isocratic solvent A:B (1:9), time = 20 min

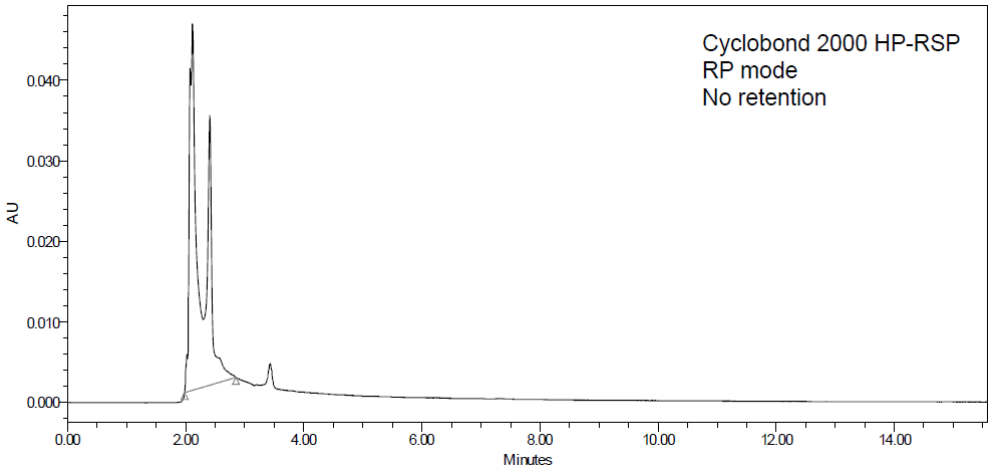


Solvent A: H_2O
Solvent B: 0.02 M ammonium acetate in H_2O/MeOH (20:80)
Flow rate: 1.0 mL/min
Temperature: 25 ºC
Column: Chirobiotic T2, 5 µm, 4.6 x 250 mm
Method: isocratic solvent A:B (4:1), time = 20 min

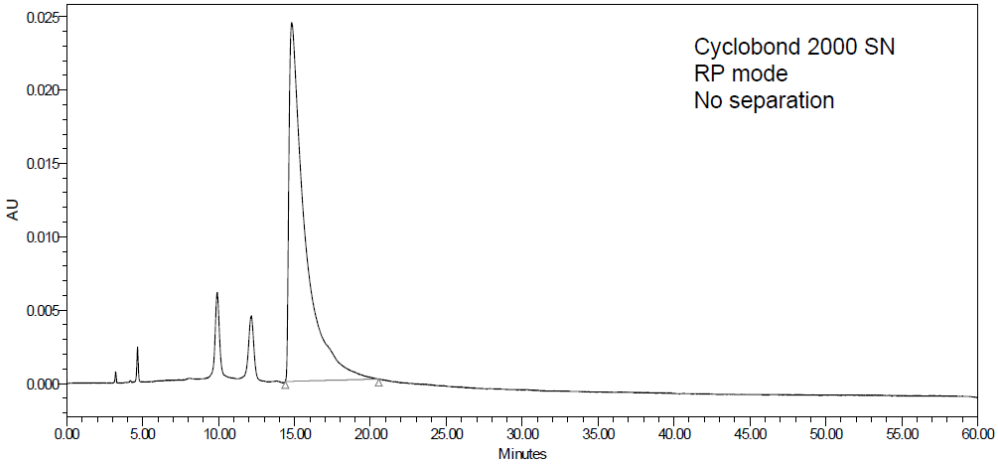


Cyclobond chiral screening in RP mode

Solvent A: H_2O
Solvent B: 0.02 M ammonium acetate in H_2O/MeOH (20:80)
Flow rate: 1.0 mL/min
Temperature: 25 ºC
Column: Astec Cyclobond 2000 HP-RSP, 5 µm, 4.6 x 250 mm
Method: isocratic solvent A:B (1:1), time = 15 min

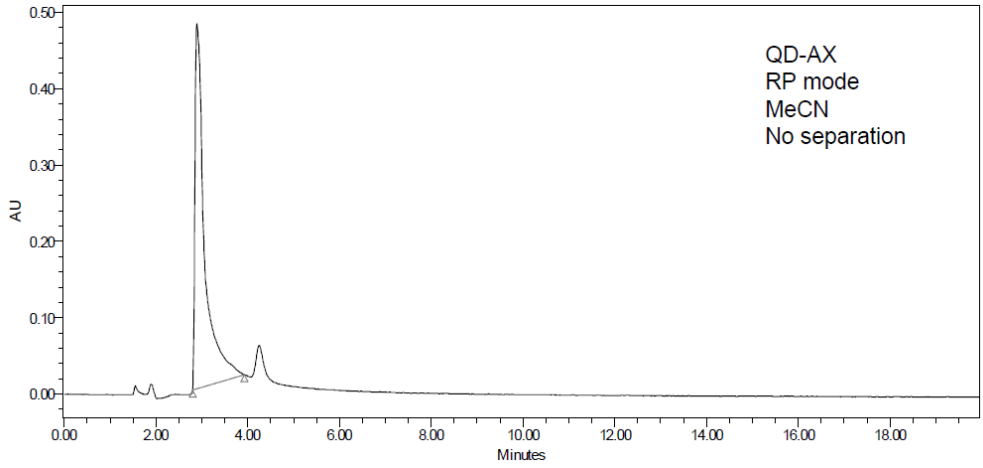


Solvent A: H_2O
Solvent B: 0.02 M ammonium acetate in H_2O/MeOH (20:80)
Flow rate: 1.0 mL/min
Temperature: 25 ºC
Column: Astec Cyclobond 2000 SN, 5 µm, 4.6 x 250 mm
Method: isocratic solvent A:B (1:3), time = 60 min

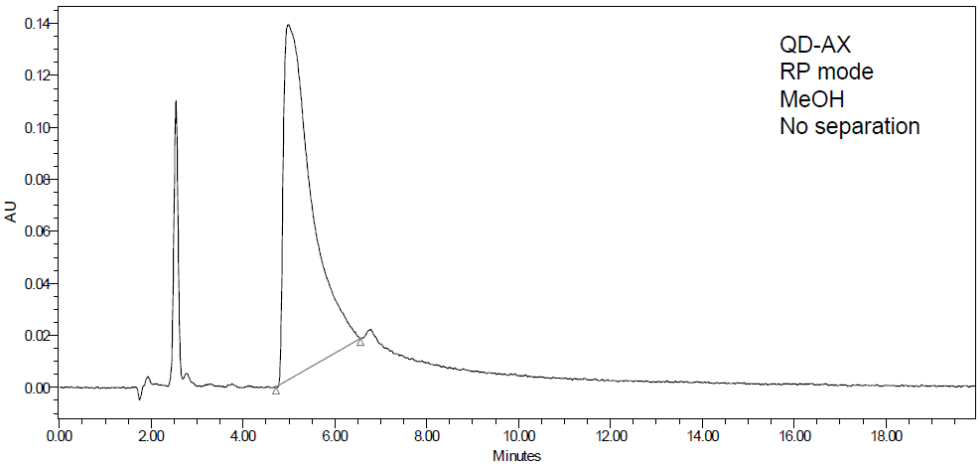


Chiralpak chiral screening in RP mode

Solvent A: 0.02 M ammonium acetate in H_2O
Solvent B: H_2O/MeCN (10:90)
Flow rate: 1.0 mL/min
Temperature: 25 ºC
Column: Chiralpak QD-AX, 5 µm, 4.6 x 250 mm
Method: isocratic A:B (1:9), time = 20 min



Solvent A: 0.02 M ammonium acetate in H_2O
Solvent B: MeOH
Flow rate: 1.0 mL/min
Temperature: 25 ºC
Column: Chiralpak QD-AX, 5 µm, 4.6 x 250 mm
Method: isocratic A:B (1:4), time = 20 min



9. Biophysical Studies of AG CleanCap® γ-thio Analogues

Susceptibility of AG CleanCap® γ-thio analogues to hydrolysis by hDcpS enzyme

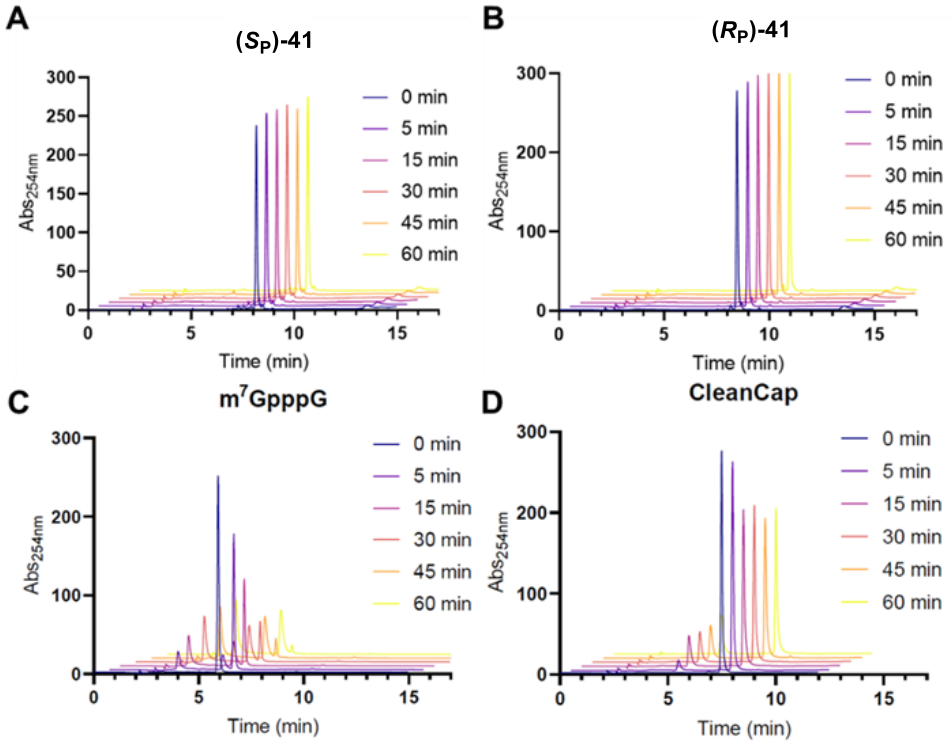


Figure S16. Representative HPLC profiles from hDcpS-susceptibility assay. Both S_P (**A**) and R_P (**B**) isomers of the of **41** remained intact for the duration of the experiment. Reference compounds m^7GpppG (**C**) and m^7GpppA$_m$pG (**D**) were cleaved between γ- and β- phosphates to yield 7-methylguanosine 5'-monophosphate (m^7GMP) and the corresponding downstream nucleotide (GDP or 5'-ppA$_m$pG, respectively).

Capping efficiency studies and susceptibility of AG CleanCap® γ-thio capped RNAs to decapping by DCP2 enzyme

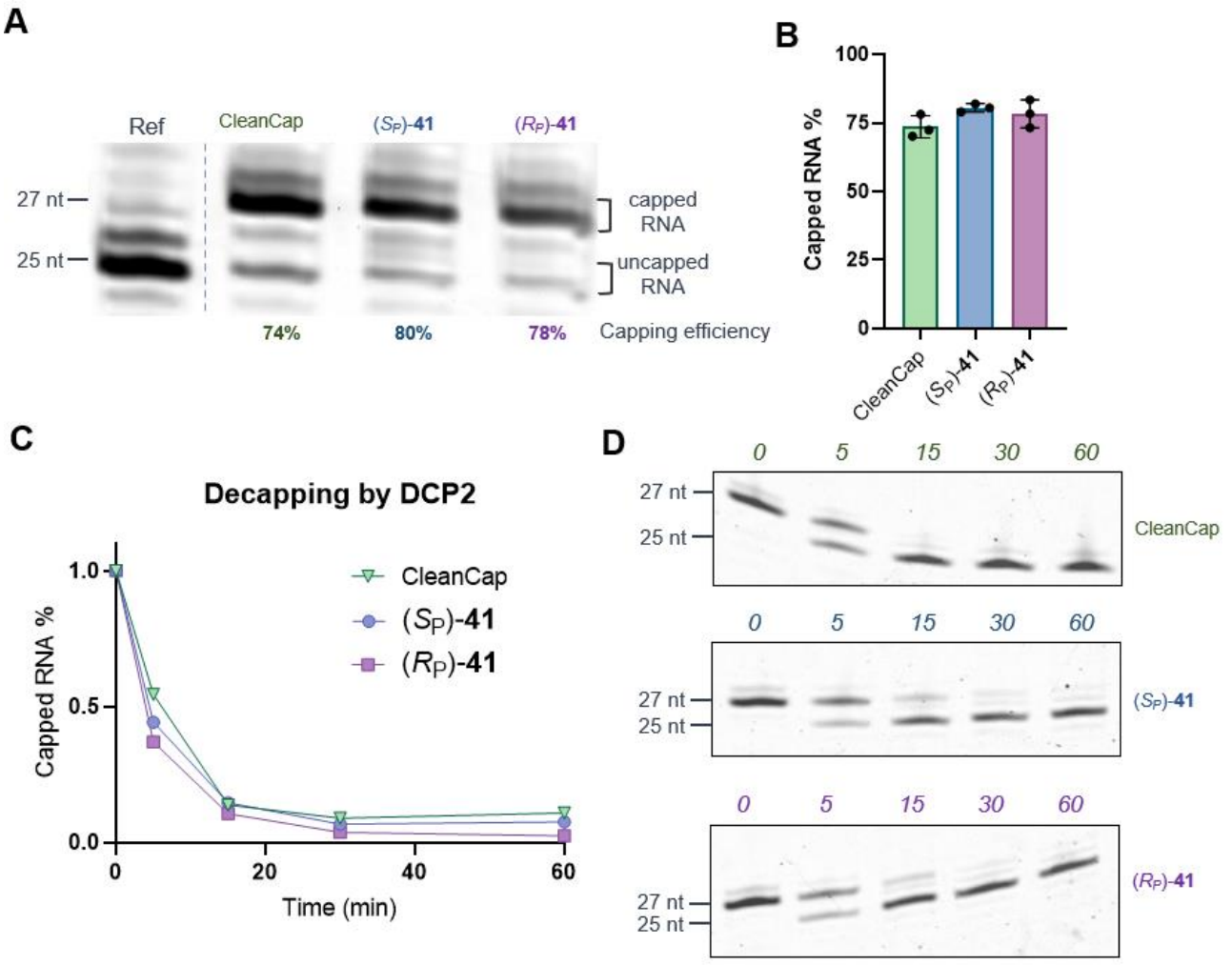


Figure S17. Incorporation into mRNA (capping) efficiency of (S_P)- and (R_P)-**41** and susceptibility of resulting short capped RNAs to DCP2 pyrophosphatase. (**A**) Representative polyacrylamide gel analysis of RNAs obtained by transcription in vitro by T7 polymerase in the presence of investigated cap analogs, where bands corresponding to capped and uncapped RNAs are separated due to difference in size (for unedited image see Fig. S18). (**B**) Comparison of capping efficiency values reveals that CleanCap reagent and (S_P)- and (R_P)-**41** are equally well incorporated into RNA by T7. Bars represent capping percentages (mean values ±S.D. from three independent transcriptions and six densitometric measurements of RNA bands). (**C**) PAGE analysis of differently capped RNA samples at various incubation times with DCP2 enzyme (mean values ±S.D. from duplicate experiment). (**D**) The decapping progress is based on densitometric measurements of bands corresponding to capped RNAs (upper band) and decapped RNAs (bottom band) at different time points (representative data) (for unedited image see Fig. S19).

Fluorescence quenching titration (FQT) binding assay

Murine eukaryotic translation initiation factor 4E (meIF4E, residues 28–217) was incubated in HEPES buffer (50 mM HEPES/KOH buffer pH 7.20 containing 100 mM KCl, 0.5 mM EDTA and 1 mM DTT) for approximately 20 min at 20 °C before each titration experiment. meIF4E solution (1400 µl, 0.1 µM) was titrated with 1 µl aliquots of given ligand in a 1-minute cycles (30 seconds for adding the ligand and equilibration, followed by 30 seconds of fluorescence data collection). Ligand solutions were prepared in the

same buffer as used for meIF4E incubation (1, 2, 5, 10, 20, 50, 100, 200, 500 μM and 1, 2 mM concentrations). meIF4E fluorescence was measured at 337 nm (10 nm bandwidth) upon excitation at 280 nm (5 nm bandwidth). Gentle stirring was applied throughout the whole experiment. Fluorescence values were corrected for inner filter effect and sample dilution. The equilibrium association constants (KAS) were determined by fitting a theoretical fluorescence quenching model to the experimental data points, as described previously,[22] using Origin 2022b software. KAS values were calculated as weighted averages based on three independent experiments. Weights were taken from corresponding standard deviations squared. KD values were calculated as KAS-1.

Hydrolysis of cap analogs by human DcpS

Each compound was incubated at 25 μM in 50 mM Tris/HCl pH 7.6 buffer containing 200 mM KCl and 5 mM $MgCl_2$ with 10 nM of human hDcpS at 37°C for 1 h and shaking at 75 x *g*. For the purpose of HPLC analysis aliquots were taken from the reaction mixture at different time points (5, 15, 30 45 and 60 min) and enzyme was deactivated by 2x dilution and heating at 98 °C for 5 min. Samples were analyzed by RP-HPLC on a Supelcosil LC-18-T column (4.6 × 250 mm, 5 μm, flow rate 1.3 mL/min), using a linear gradient of MeOH in phosphate buffer (0.1 M KH_2PO_4 / K_2HPO_4 pH 6.0). Absorption was monitored at 254 nm. Experiments were performed in duplicate.

In vitro transcription of 35 nt RNA

Capped RNAs were generated on an annealed DNA template containing T7 G φ 6.5 promoter sequence (CAGTAATACGACTCACTATA) followed by a 35-nt-long sequence (GGGGAAGCGGGCATGCGGCCAGCCATAGCCGATCA). In vitro transcription reaction (40 μl) was incubated for 2.5 h at 37 °C and contained RNA polymerase buffer (40 mMTris HCl pH 7.9, 20 mM $MgCl_2$, 1 mM DTT, 2 mM spermidine), 1 U/μl RiboLock RNase inhibitor (ThermoFisher Scientific), 5 mM of ATP/UTP/CTP, 4 mM of GTP, 8 mM of cap analogue, 40 ng/μl od DNA template, 2 U/100 μl inorganic pyrophosphatase (Sigma-Aldrich) and 0.125 mg/ml of T7 polymerase. After 2.5h incubation, 1 U/μl of DNase was added to the reaction to remove DNA template and the reaction as further incubated for 30 minutes at 37 °C. RNAs were first purified on Monarch RNA Cleanup columns (New England BioLabs) and later on HPLC using OligoClarity 3μm Phenomenex column at 55 °C at 1 ml/min flow on Agilent Technologies Series 1200 apparatus. Linear gradient of 10% to 70% in 30 minutes of 200 mM TEAA pH 7.0/ACN 1/1v in 100 mM TEAA pH 7.0 buffer was used. Both capped and uncapped fractions were collected together for further capping efficiency studies. Collected RNAs were freeze dried twice. Transcripts were analyzed on 15% polyacrylamide/7 M urea gels/1x TBE and stained with SYBR Gold. Sample concentrations were determined spectrophotometrically. Three independent transcriptions were performed.

DNAzyme treatment and capping efficiency determination

35 nt RNAs after HPLC purification and freeze drying were subjected to DNazyme treatment in order to remove heterogeneous 3' ends. RNA (1 µM) was incubated in 50 mM $MgCl_2$ and 50 mMTris-HCl pH 8.0 buffer with 1 µM concentration of DNAzyme 10-23 (TGATCGGCTAGGCTAGCTACAACGAGGCT-GGCCGC), [23,24] for 2 minutes at 95 °C followed by 1h incubation at 37 °C. Reaction was stopped by adding EDTA (0.2 M) 12.5 µl/100 µl reaction. RNAs were again purified on Monarch RNA Cleanup columns and analyzed on 15% PAA/7 M urea gels in TBE buffer. Capping efficiency values were calculated based on densitometric measurements of bands corresponding to capped and uncapped RNAs as described before[25]. Mean capping efficiency values were determined based on analysis of samples from three independent *in vitro* transcriptions. Remaining RNA samples after DNAzyme treatment were purified on HPLC as described above using slower elution gradient (10% to 30% in 30 minutes of 200 mM TEAA pH 7.0/ACN 1/1v in 100 mM TEAA pH 7.0) to separate capped RNA from the uncapped fraction. ImageJ software was used to process gel images and calculate band intensity.

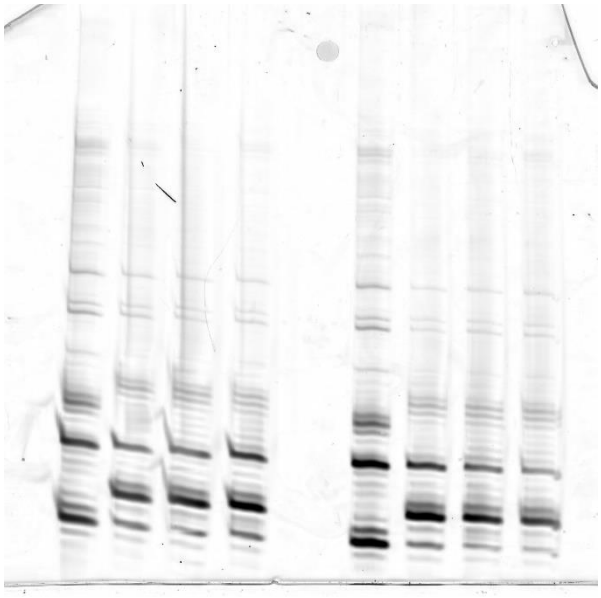


Figure S18. Uncropped image of polyacrylamide gel analysis of RNAs from Fig. S17a.

Decapping by DCP2 enzyme

Purified 25 nt IVT RNAs obtained after DNAzyme treatment were subjected to decapping by DPC2 enzyme. Prior to the experiment, enzyme was diluted in 1 ml of buffer (0.5M Tris HCl pH 8.0/0.5M NH_4Cl/0.1% Igepal). To the reaction mixture (29 µl) containing buffer (final concentration in 30 µl of reaction: 0.5M Tris HCl pH 8.0/0.5M NH_4Cl/0.1% Igepal, 2 mM $MnCl_2$, 5 mM $MgCl_2$, 1 mM DTT) and RNA substrate (25 ng) 1 µl of DCP2 solution was added (final concentration of enzyme in the reaction was 5 nM). Decapping reaction progress was monitored at different time points (0, 5, 15, 30 and 60 minutes). Reaction was stopped by diluting 5 µl of the reaction mixture in 5 µL of 2x Loading Dye and freezing in liquid N_2. Samples were then stored at -18 °C and brought to room temperature before PAGE analysis. Decapping progress for each RNA sample was

calculated based on two independent enzymatic experiments. ImageJ software was used to process gel images and calculate band intensity.

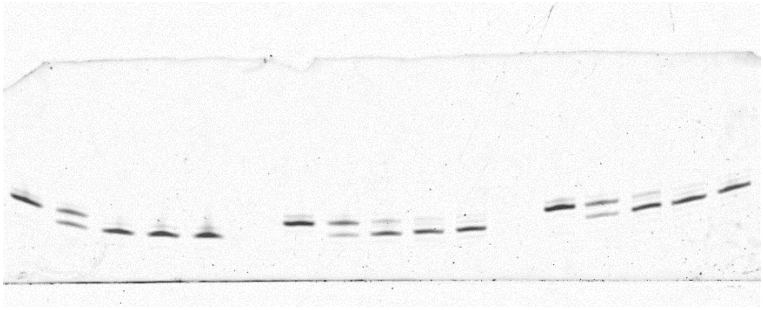


Figure S19. Uncropped image of polyacrylamide gel analysis of RNAs from Fig. S17d.

In vitro transcription of Gaussia Luciferase mRNAs

Differently capped Gaussia Luciferase mRNAs were generated on template of pJET_T7_Gluc_128A plasmid digested with restriction enzyme AarI (ThermoFisher Scientific). In vitro transcription reaction (20 µl) contained buffer (40 mM Tris-HCl pH 7.9, 20 mM $MgCl_2$, 1 mM DTT, and 2 mM spermidine) with 0.125 mg/ml T7 RNA polymerase, 1 U/µl RiboLock RNase Inhibitor, 4 mM ATP/UTP/CTP, 2 mM GTP, 4 mM of cap analog, 0.002 U/µl inorganic pyrophosphatase and 40 ng/µl of the DNA template. After 2.5 h of incubation at 37 °C, 1 U/µl of DNase was added to the reaction to remove DNA template and the reaction as further incubated for 30 minutes at 37 °C. As described for 35 nt RNAs, mRNAs were purified both on Monarch Cleanup columns and HPLC [RNASep™ Prep – RNA Purification Column (ADS Biotec) at 55 °C, 20 to 25% linear gradient of elution buffer over 20 minutes at 0.9 min/ml flow]. mRNA was later precipitated from eluted fractions using 1.2 volumes of isopropanol with 0.3 M NaOAc (pH 5.2) and 1 µl/ml of glycogen, followed by a washing step with 80% ethanol. mRNA pellets were dried under reduced pressure and dissolved in RNase-free water. Quality of the transcript was evaluated on native 1% 1× Tris-Borate-EDTA (TBE) agarose gel. Concentration of the final sample was determined spectrophotometrically.

Translation efficiency studies in Rabbit Reticulocyte Lysate system

Translation efficiency experiments were carried out in RRL system (Promega). 4 µl of RRL was diluted with 5 µl of buffer (40 µM amino acid mixture Δcysteine, 40 µM amino acid mixture Δmethionine, 2 mM $MgCl_2$, 380 mM potassium acetate). Lysate mixture was incubated in V-bottomed 96-well plate for 1 h at 30 °C with gentle stirring (50 x *g*), 9 µl of mixture per well. After incubation 1 µl of mRNA dilution (0.25, 0.125, 0.0625 or 0.0312 ng/µl) was added to each well. Reaction was once again incubated for 1 h at 30 °C and quenched by putting the plate in a -80 °C freezer. Before measurements, the plates were brought to room temperature. To detect luminescence, 50 µl of 10 ng/ml of h-coelenterazine (NanoLight) in PBS buffer was added to 10 µl of thawed translation mixture. Luminescence was measured using a Synergy H1 (BioTek) microplate reader. Translation efficiency experiments were performed in triplicates for each differently capped mRNA. Each mRNA concentration point was also measured in triplicate.

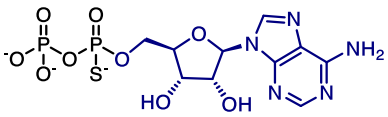
10. Inhibition of ecto-NTPDases by Nucleotide Thioisosteres

10.1. Results of inhibition assays at NTPDase-1, -2, -3 and -8

NTPDases catalyze the hydrolysis of nucleoside tri- and diphosphates yielding nucleoside monophosphates. The adenosine derivatives **20**, **32** and **S69**[26] were tested at a concentration of 20 µM for inhibition of recombinant human NTDPase1 (CD39), NTPDase-2, NTPDase-3 and NTPDase-8 using the malachite green assay.

Malachite green assay

The enzyme activity assay was performed as previously described with a few modifications[27]. The reaction buffer contained 10 mM HEPES (pH = 7.4), 2 mM $CaCl_2$ and 1 mM $MgCl_2$ in a final volume of 50 µL in transparent 96-well half area plates. Human recombinant COS-7 cell membrane preparations expressing CD39, NTPDase-2, NTPDase-3, or NTPDase-8, respectively, were preincubated with the test compound at 37°C upon gentle shaking for 5 min[28,29]. The amount of enzyme preparation was adjusted to ensure 10 – 20 % of substrate conversion. The reaction was initiated by the addition of 50 µM ATP for CD39 (K_m (CD39) = 17 µM), and 100 µM of ATP for NTPDases-2, -3 and -8 [K_m (NTPDase-2) = 70 µM; K_m (NTPDase-3) = 75 µM; and K_m (NTPDase-8) = 81 µM][30,31]. After 15 min of incubation at 37 °C with gentle shaking, the reaction was stopped by adding the detection reagents (20 µL of 0.6 mM malachite green solution, and 30 µL of 20 mM ammonium molybdate solution, in 1.5 M sulfuric acid). The released (inorganic) phosphate was quantified after 20 min of gentle shaking at 25°C by measuring the absorption of the malachite green-phosphomolybdate complex at 600 nm using a BMG PheraStar FS plate reader (BMG Labtech GmbH, Ortenberg, Germany). The corrected absorption was calculated by subtracting the absorption of the negative control samples, which had been incubated with denatured enzyme (90°C, 10 min).



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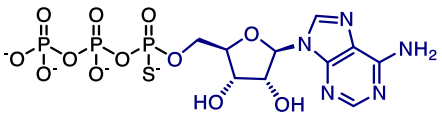

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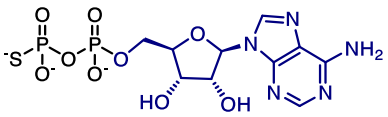


S69

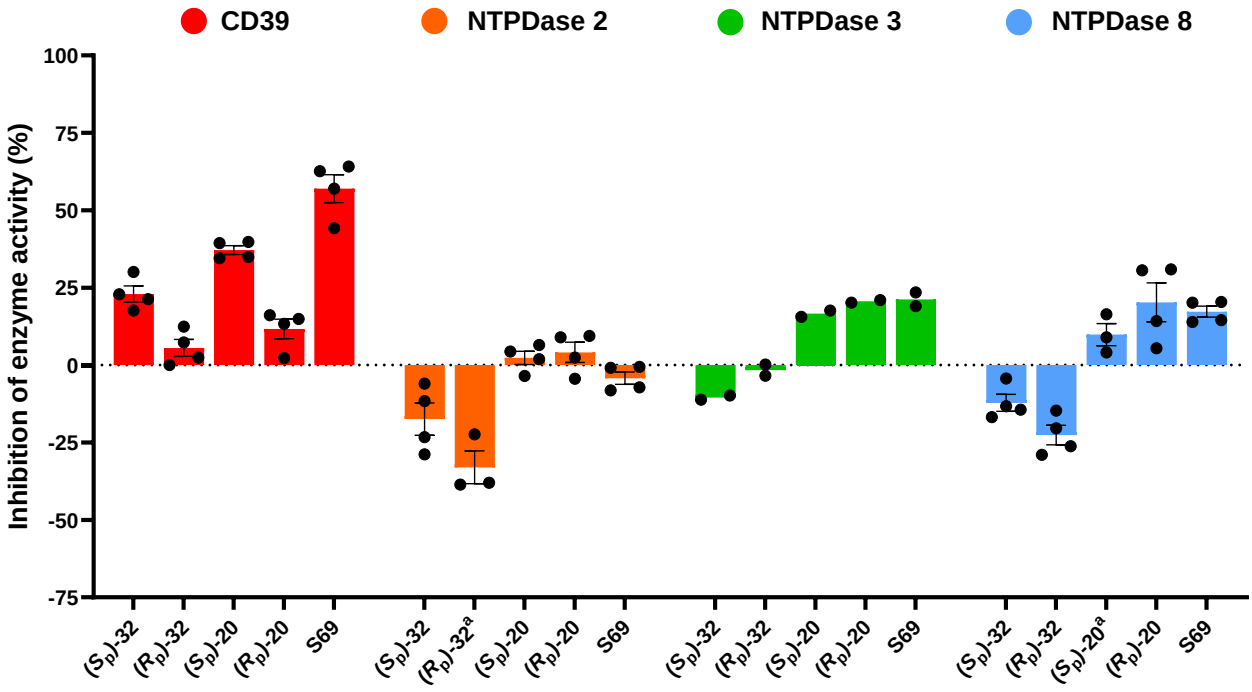


Figure S20. Comparison of the inhibition effect of ecto-NTPDases by ADP and ATP thioisosteres (20 µM) with the malachite green assay. The substrate concentration was 50 µM for CD39 (NTPDase 1) and 100 µM for NTPDases-2, -3 and -8. The results are presented as mean value ± SEM from four independent experiments for CD39 (NTPDase 1), NTPDase 3 and NTPDase 8 (n = 4). For NTPDase 2 the results are presented as mean value from two independent experiments (n = 2). Black dots represent results of each independent experiment. [a]The results for these entries are presented as mean value ± SEM from three independent experiments (n = 3).

The inhibition effect of enzyme activity varies significantly between R_P and S_P isomers of the studied compounds (i.e. ***(R_P)-32*** *enhances the enzymatic activity of NTDPase 2 much stronger than isomer* ***(S_P)-32****). Thus, it is evident that stereochemical configuration at α-thiophosphate moiety has significantly affects biological properties of nucleotide thioisosteres.*

The most potent inhibitor β-thioADP (**S69**) was further investigated to determine a concentration-response curve.

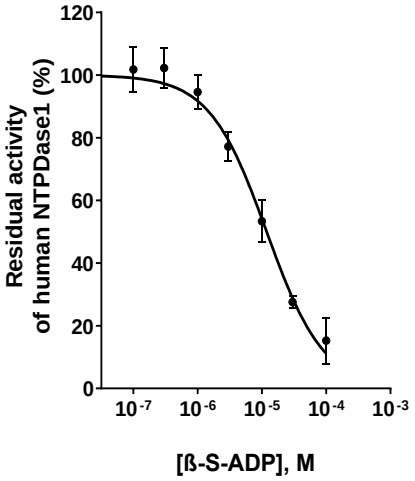


Figure S21. Concentration-dependent inhibition of CD39 by compound **S69**. An IC_{50} value of 11.9 µM and a K_i value of 3.02 µM was determined as mean value ± S.E.M from three independent experiments (n = 3).

S69 shows potency in the same range as other reported CD39 inhibitors, e.g. the standard inhibitor ARL67156[32].

10.2. Inhibition potency at ecto-5'-nucleotidase (CD73)

CD73 catalyzes the hydrolysis of nucleoside monophosphates yielding the nucleosides. AMP is the main substrate, yielding immunosuppressive adenosine. CD73 is a proposed target for the immunotherapy of cancer[33]. Preliminary screening was performed in a radioligand binding assay using the competitive inhibitor radioligand [^{3}H]PSB-17230 and membrane preparations of human breast cancer cells (MDA-MB-231) which express a high level of CD73.

CD73 radioligand binding assay

Membrane preparations of MDA-MB-231 cells, a human triple-negative breast cancer cell line with high CD73 expression, were obtained as previously described[34]. Competition binding assays were performed in 25 mM Tris and 25 mM NaH_2PO_4 buffer (pH 7.4). The final assay volume of 400 µl contained 4 µl of test compound, 100 µl of [^{3}H]PSB-17230 in buffer (1 nM final concentration), and 100 µl of cell membrane preparation (5 µg of protein). Non-specific binding was determined in the presence of the unlabeled CD73 inhibitor PSB-12379 (2 µM)[35]. The incubation was started by the addition of membrane preparation and was performed at 25 °C for 100 min. It was terminated by rapid vacuum filtration through GF/B glass-fiber filters using a Brandel 48-well harvester (Brandel, Gaithersburg, MD, USA). The filters were rinsed three times with approximately 3 mL each of ice-cold 25 mM Tris buffer (pH 7.4) to separate bound from free radioligand. The filters were punched out and transferred to scintillation vials. Luma Safe scintillation cocktail (2.5 mL) was added, and after 9 h of incubation, the samples were counted for 1 min each using a liquid scintillation counter (efficiency: 53%). K_i values were calculated using equations for one-site competition as implemented in GraphPad Prism 8.0.1.

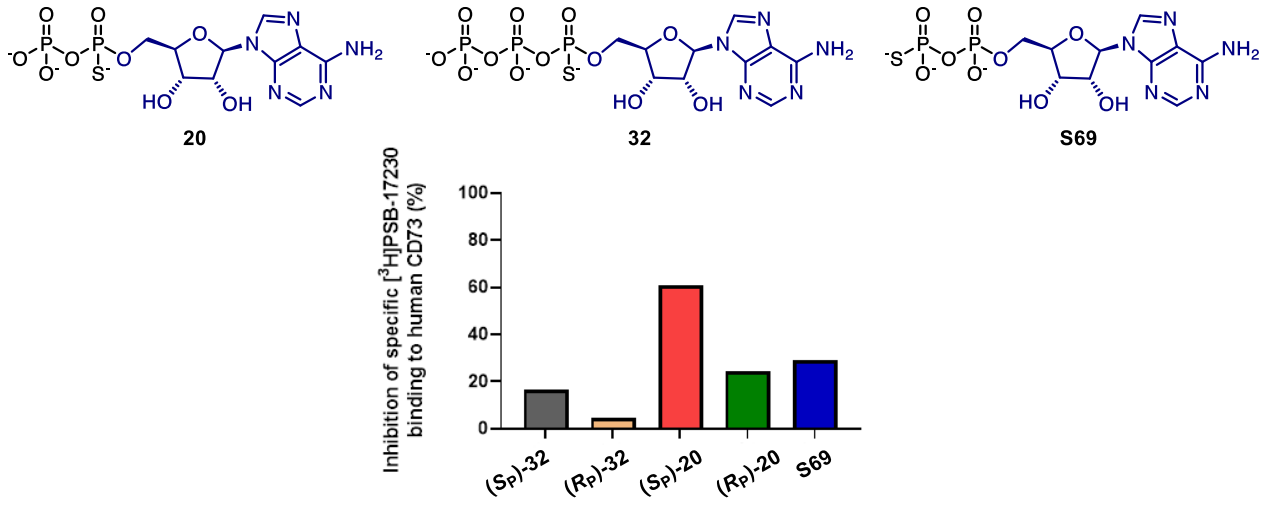


Figure S22. Preliminary screening: Effect of adenine thionucleotides (20 µM) on specific radioligand binding of [^{3}H]PSB-17230 to human CD73 expressed in triple-negative breast cancer cells (MDA-MB-231).

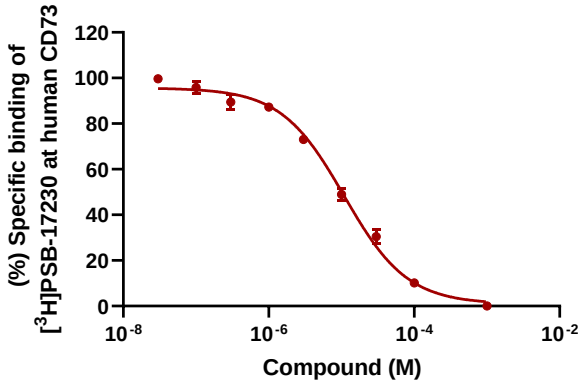


Figure S23. Concentration-dependent inhibition of radioligand binding to CD73 expressed in triple-negative breast cancer cells (MDA-MB-231) by compound **(*S*P)-20**. A K_i value of 0.891 ± 0.034 µM was determined as mean value ± S.E.M form three independent experiments (n = 3).

At a high concentration, only **(*S*P)-20** showed significant inhibition of radioligand binding to CD73. This indicates a higher affinity of the thio-ADP analogs as compared with the thio-ATP analogs, and that CD73 binding is stereoselective. From subsequent concentration-inhibition curves, a K_i value of 0.891 ± 0.034 µM was calculated (see Fig. S23). Thus, the α-(*S*P)-thio-ADP modification may be beneficial for high CD73 affinity.

10.3. Inhibition of nucleotide pyrophosphatase/phosphodiesterase 1 (NPP1)

NPP1 has a wide range of nucleotide substrates. One of its main substrates is ATP which is hydrolyzed to AMP. Other substrates are also hydrolyzed the respective nucleoside 5'-monophosphates[36]. Previous attempts to investigate nucleotidic scaffolds as competitive NPP1 inhibitors have shown promising results, with some of the compounds displaying high potency and selectivity[37-39].

In this study, testing was performed on nucleotide tioisosteres **12**, **20**, **24**, **32**, and **S69** using two different assay setups employing the artificial substrate *p*-nitrophenyl-5'-thymidine-monophosphate (*p*-Nph-5'-TMP) or the natural substrate ATP. The reaction buffer had the following composition: HEPES (10 mM, pH = 7.4), $CaCl_2$ (0.5 mM), and $ZnCl_2$ (0.01 mM).

p-Nph-5'-TMP is a standard substrate suitable for colorimetric determination of NPP activity. The produced *p*-nitrophenolate has an intense yellow color which shows maximum absorption at 405 nm[40]. Initial screening was performed at a compound concentration of 20 µM with 300 µM substrate and 0.9 µg NPP1/well. After 60 min of incubation at 37 °C, 10 µL of 0.1 M NaOH was added to terminate the reaction. Absorbance was measured on Mithras LB 940 continuous plate reader using MikroWin software for data acquisition.

Capillary electrophoresis (CE) assays were performed to measure ATP hydrolysis as described[41]. A polyacrylamide coated capillary [30 cm (20 cm effective length) × 50 µm (id), × 360 µm (od) from Chromatographie Service GmbH (Langerwehe, Germany)] was used. Soluble NPP1 (0.5 µg) was incubated with 100 µM ATP for 30 min at 37 °C either with or without inhibitor, followed by boiling the samples at

95 °C for 10 min. After cooling the plate briefly on ice, samples were electrokinetically injected into the HPLC column for 30 s with a voltage of –6 kV. Finally, analytes were separated by applying a separation voltage of –15 kV, and the nucleotides were detected with a UV detector at an absorbance of 254 nm. IC_{50} values were obtained by determining concentration-dependent inhibition at different concentrations (100 – 0.001 μM) versus both the substrates.

Results of NPP1 inhibition

Uridine derivatives **(*S*P)-12** and **(*S*P)-24** showed promising inhibition of NPP1 with similar IC_{50} values in both assay systems, versus both the substrates. The adenine derivatives **20**, **32** and **S69** were somewhat less potent in inhibiting the hydrolysis of *p*-NPh-5'-TMP. The most potent thioisostere **(*S*P)-24** (K_i value 0.226 μM vs. the artificial substrate, 0.109 μM vs. ATP as a substrate) was evaluated in more detail proving that it acted as a competitive NPP1 inhibitor versus both substrates.

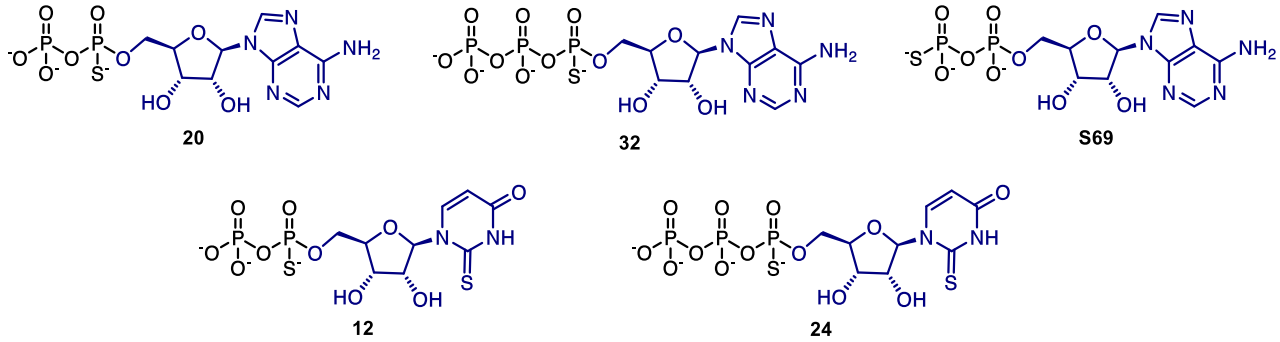


Table S10. Inhibition of nucleotide thioisosteres towards NPP1 versus *p*-NPh-5'-TMP and ATP.

Compound	*vs. p*-NPh-5'-TMP (300 μM)[a]			*vs.* ATP (100 μM)[b]	
	Inhibition at 20 μM (%)	IC_{50} ± SEM (μM)	K_i ± SEM (μM)	IC_{50} ± SEM (μM)	K_i ± SEM (μM)
(*R*P)-20	66 ± 1	8.13 ± 0.85	3.45 ± 0.36	N.D.	N.D.
(*S*P)-20	5 ± 1	> 100	-	N.D.	N.D.
(*R*P)-32	60 ± 2	12.7 ± 1.9	5.40 ± 0.80	N.D.	N.D.
(*S*P)-32	52 ± 4	19.8 ± 2.5	8.42 ± 1.06	N.D.	N.D.
S69	55 ± 1	18.5 ± 4.2	7.87 ± 1.87	N.D.	N.D.
(*R*P)-12	32 ± 2	57.4 ± 2.5	24.4 ± 1.1	N.D.	N.D.
(*S*P)-12	76 ± 1	3.88 ± 0.50	1.65 ± 0.21	4.02 ± 0.48	0.304 ± 0.036
(*R*P)-24	45 ± 3	21.1 ± 4.2	9.82 ± 0.63	N.D.	N.D.
(*S*P)-24	89 ± 2	0.532 ± 0.214	0.226 ± 0.091	1.44 ± 0.038	0.109 ± 0.002

[a]*p*-NPh-5'-TMP (K_m = 222 ± 44 μM; ref. 41). [b]ATP (Km = 8.17 ± 1.45 μM; ref. 41). N.D. - not determined

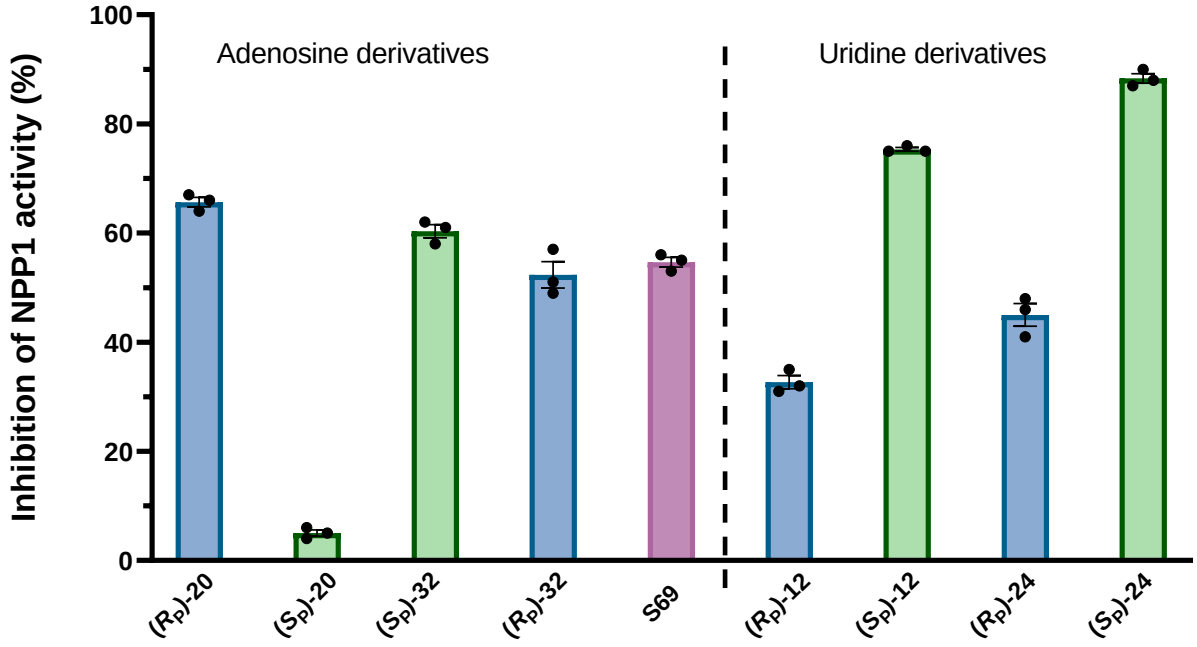


Figure S24. Comparison of the inhibition effect on NPP1 by nucleotide thioisosteres (20 μM) versus *p*-NPh-5'-TMP (300 μM). The results are presented as mean value ± SEM from three independent experiments (n = 3). Black dots represent results of each independent experiment.

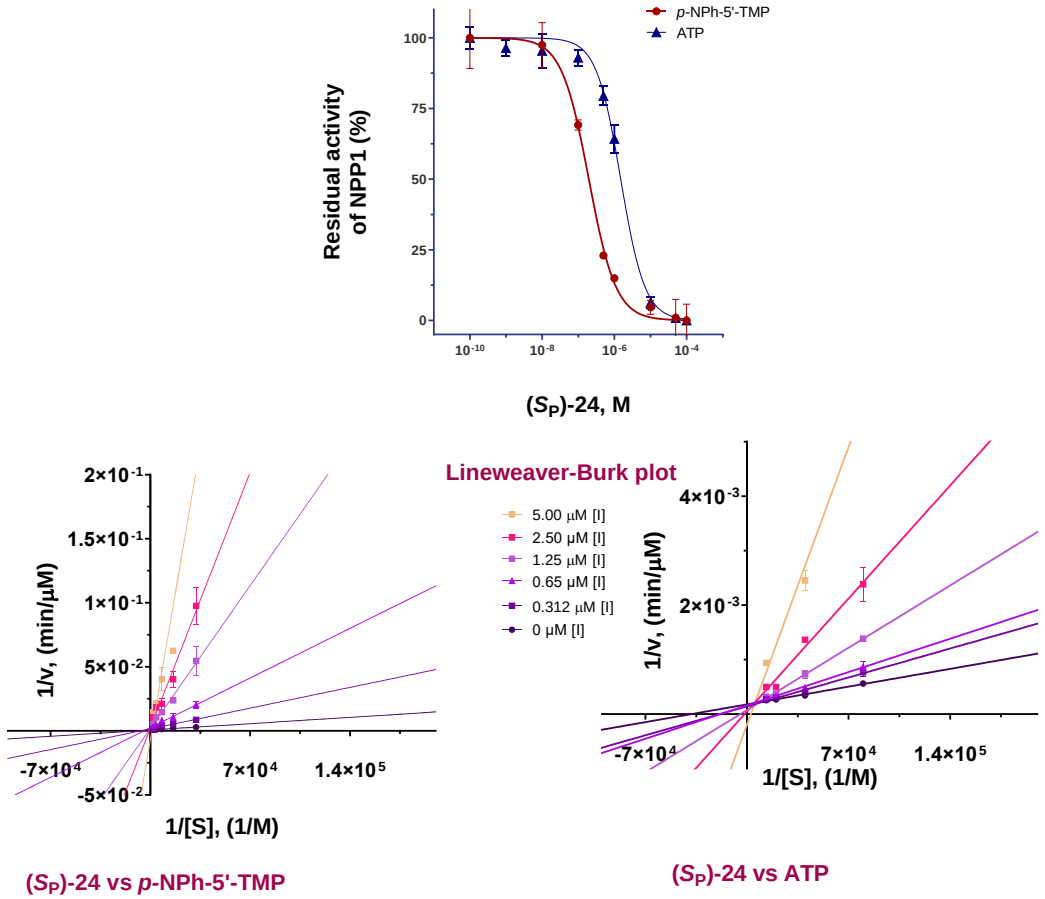


Figure S25. Inhibition profile of **(S$_P$)-24** versus NPP1. **Top**. Concentration-dependent inhibition of NPP1 by **(S$_P$)-24** vs. *p*-NPh-5'-TMP and ATP as substrates. **Bottom**. Determination of mechanism of inhibition versus both substrates, *p*-NPh-5'-TMP and ATP. Three independent assays were performed in duplicates. The data is represented as mean values ± SEM.

11. Stability of Nucleotide Thioisosteres towards Enzymatic Hydrolysis

Nucleotide thioisosteres **12**, **20**, **24**, **32**, and **S69** were investigated for stability versus nucleotide pyrophosphatase/phosphodiesterase-1 (NPP1, CD203a), tissue non-specific alkaline phosphatase (TNAP) and nucleoside triphosphate diphosphohydrolase-1 (CD39). The samples were analyzed using a Sciex P/ACE MDQ capillary electrophoresis system.

Quantification by capillary electrophoresis with UV detection (CE-UV)

Table S11. Assay operation of capillary electrophoresis with UV detection

Component	**Volume**	**End Concentration**
Test compound/substrate, 100 µM in water	30 µl	20 µM
Enzyme (NPP-1/TNAP/CD39)	30 µl/2 µl	4.48 µg NPP1 0.15 µg TNAP 1.30 µg CD39
Reaction buffer (instead of the substrate)	90 µl	-
Total	150 µl	
Incubate the mixture for **30/45/60 min (CD39/NPP1/TNAP)** at **37°C** and **125 x *g***.		
Terminate the enzyme reaction by heating at **90°C** and **125 x *g*** for **10 min**.		
Measure the mixture by using CE (capillary electrophoresis).		

Instrument	Capillary electrophoresis with DAD-detection system, Sciex P/ACE™ MDQ
Software	32 Karat software
Capillary	FS-polyacrylamide coated (ID 50 µm; AD 360 µm), 30 cm (20 cm eff. length)
Buffer	50 mM Phosphate, pH 6.5
Rinse	40 psi, 1 min with water and 40 psi, 1 min with 50 mM phosphate buffer pH 6.5
Inject	-6 kV, **30 sec**
Separation	0.17 ramp, reverse polarity, -15 kV, 5 min
Detection	254 nm

The capillary electrophoresis-based method was previously optimized for analyzing the enzymatic hydrolysis of nucleotides. The nucleotides were separated and quantified by UV detection at 254 nm.

Different reaction buffers were utilized for different enzymes: 10 mM HEPES (pH 7.4), 0.5 mM CaCl2, 0.01 mM ZnCl2 for NPP1 (in a final volume of 150 µL); 10 mM HEPES (pH 7.4), 0.5 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.01 mM $ZnCl_2$ for TNAP (in a final volume of 150 µL); and 10 mM HEPES (pH 7.4), 2 mM CaCl2, 1 mM MgCl2 for CD39 (in a final volume of 150 µL). For the testing, recombinant NPP1 expressed in insect cells (4.48 µg), recombinant human alkaline phosphatase (0.15 µg) or recombinant CD39 expressed in COS-7 cells (1.30 µg) were mixed with 20 µM of either the test compounds or the natural substrates (ATP, ADP, UTP, and UDP) and incubated with gentle shaking at 37°C for 30 min (CD39), 45 min (NPP1) or 60 min (TNAP). The enzyme reaction was terminated by heating the assay plate at 90°C for 10 min with gentle shaking. After cooling on ice, the samples were analyzed using a P/ACE MDQ capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA).

11.1. Stability toward enzymatic hydrolysis by NPP1

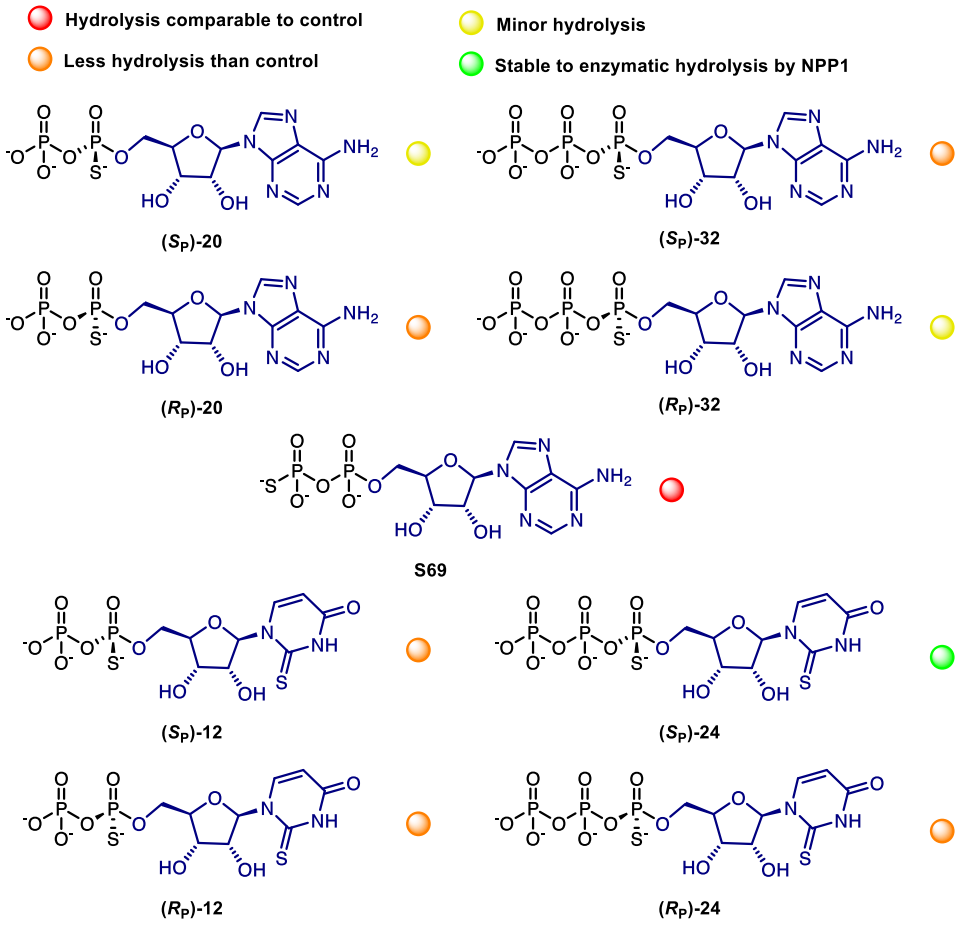


Figure S26. The stability of evaluated nucleotide thioisosteres towards enzymatic hydrolysis by NPP1 compared to their natural nucleotides

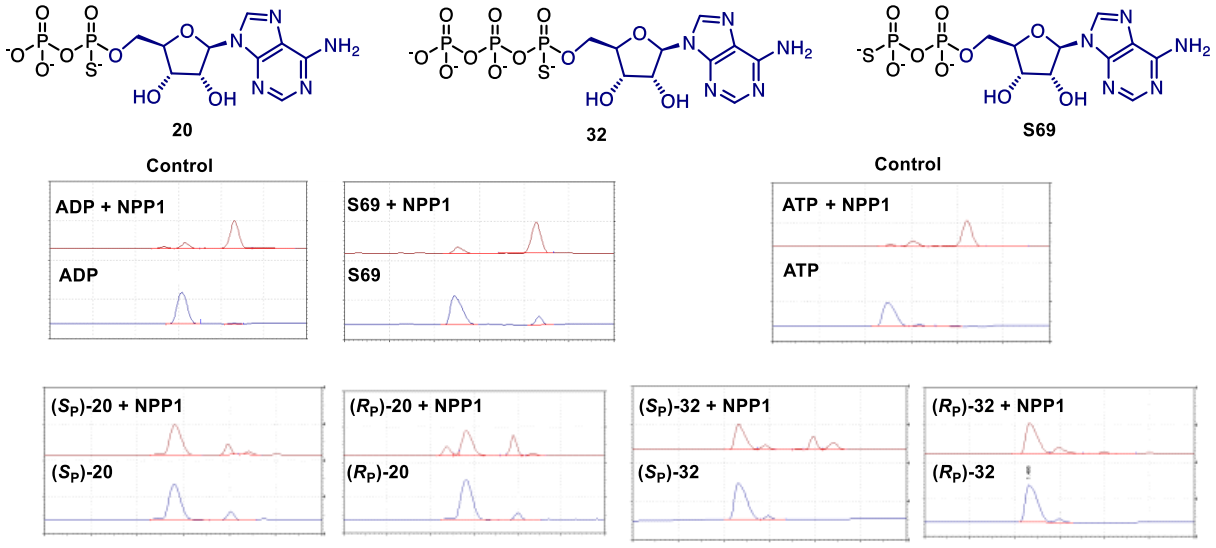


Figure S27. Electropherogram showing hydrolysis of thio-ATP and -ADP analogs by NPP1. 20 µM of either compound or substrate (ATP and ADP) were incubated without (blue) or with NPP1 (red) for 45 min. Two independent experiments were performed each in duplicate.

Even at high concentration of NPP1, only partial hydrolysis of the α-thio-ADP and ATP analogs was observed. Nucleotide tioisosteres **(*S*P)-20** and **(*R*P)-32** were particularly stable, and notably more resistant to hydrolysis than their diastereoisomers. Thus, these α-thioisosteres of ATP and ADP could act as inhibitors of NPP1.

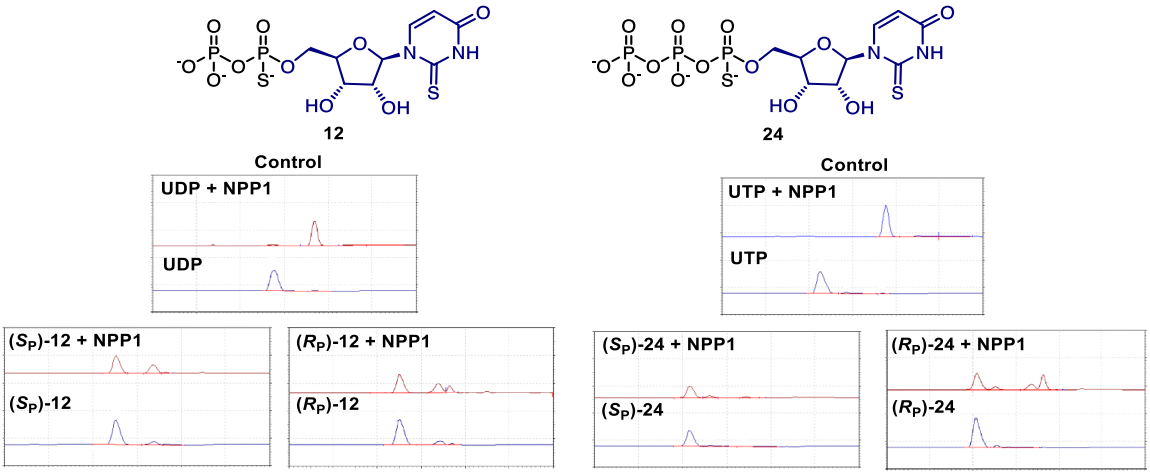


Figure S28. Electropherogram showing hydrolysis of 2-thioUDP and 2-thioUTP analogs by NPP1. 20 µM of either compound or substrate (UTP and UDP) were incubated without (blue) or with NPP1 (red) for 45 min. Two independent experiments were performed each in duplicate.

The investigated 2-thiouridine nucleotide thioisosteres were significantly more stable than the natural substrates UTP and UDP. The UTP analog **(*S*P)-24** showed almost no degradation under the applied conditions.

11.2. Stability toward enzymatic hydrolysis by TNAP

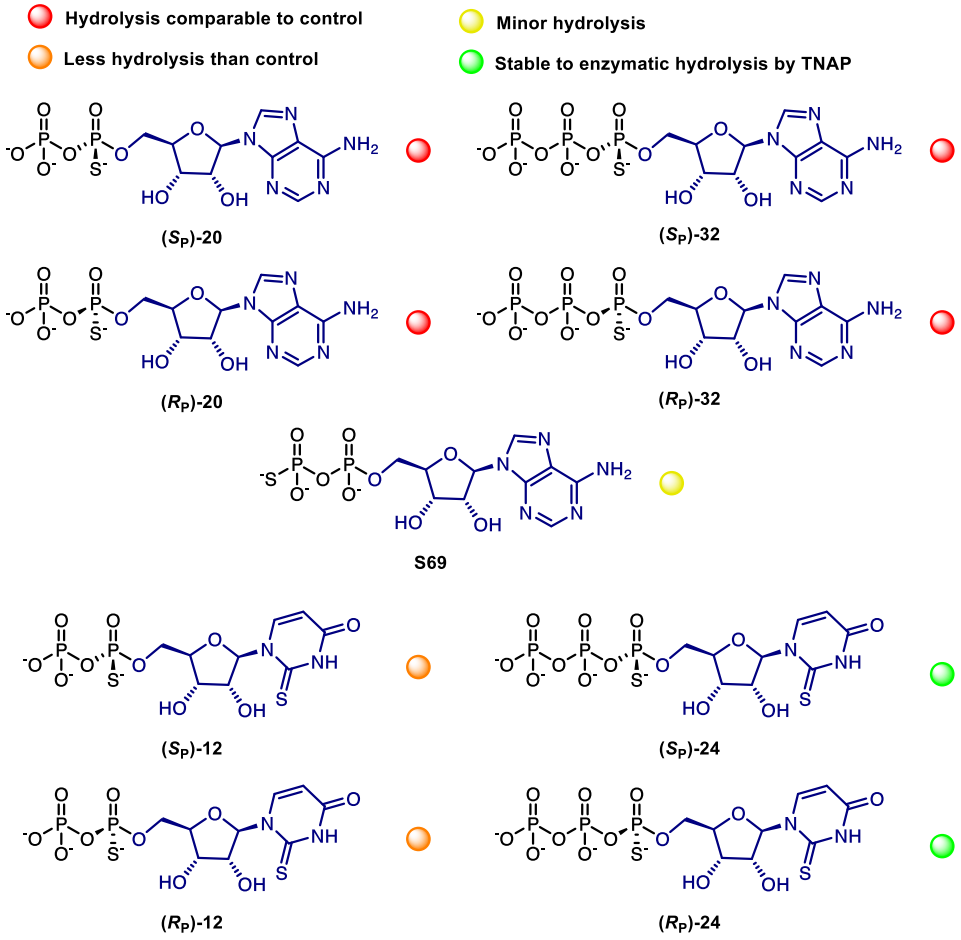


Figure S29. The stability of evaluated nucleotide thioisosteres towards enzymatic hydrolysis by TNAP compared to their natural nucleotides

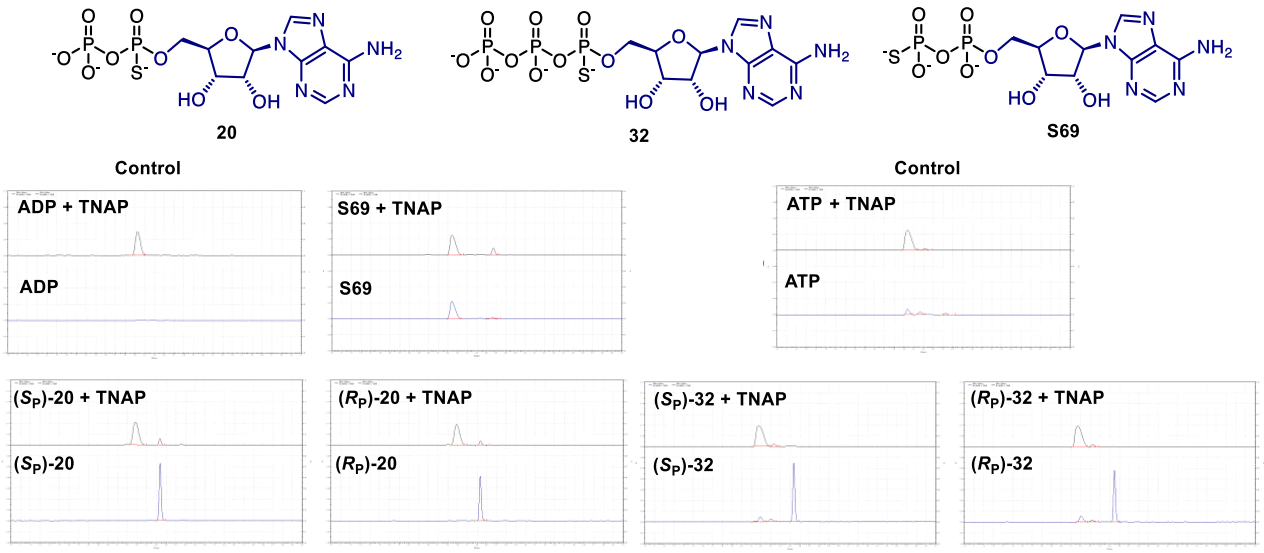


Figure S30. Electropherogram showing hydrolysis of thio-ATP and -ADP analogs by TNAP. 20 µM of either compound or substrate (ATP and ADP) were incubated without (blue) or with TNAP (black) for 60 min. Two independent experiments were performed each in duplicate.

α-Thioisosteres of ATP and ADP were hydrolyzed by TNAP yielding the corresponding thio-AMP derivatives which accumulated (ca. 60-70% formation of thio-AMP after 1 h of incubation under the applied conditions). It appeared that they could not be further hydrolyzed due to the presence of the thiophosphate group. In contrast, ATP and ADP, tested as controls, disappeared almost completely presumably being dephosphorylated to adenosine.

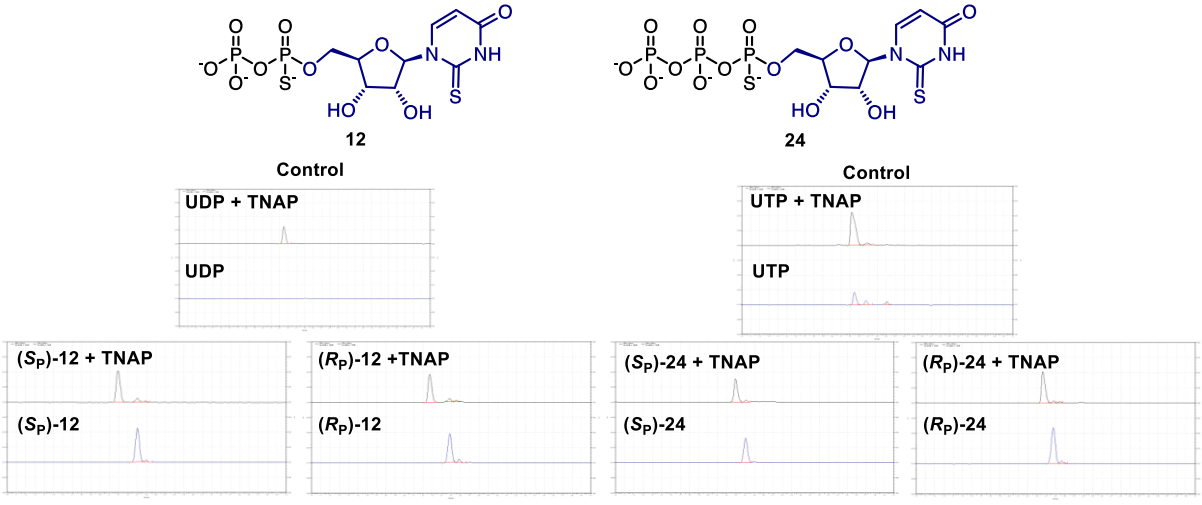


Figure S31. Electropherogram showing hydrolysis of 2-thioUDP and 2-thioUTP analogs by TNAP. 20 µM of either compound or substrate (UTP and UDP) were incubated without (blue) or with TNAP (black) for 60 min. Two independent experiments were performed each in duplicate.

Similar to adenosine derivatives, uridine thioisosteres were hydrolyzed only to the corresponding thioUMP analogues.

11.3. Stability toward enzymatic hydrolysis by CD39

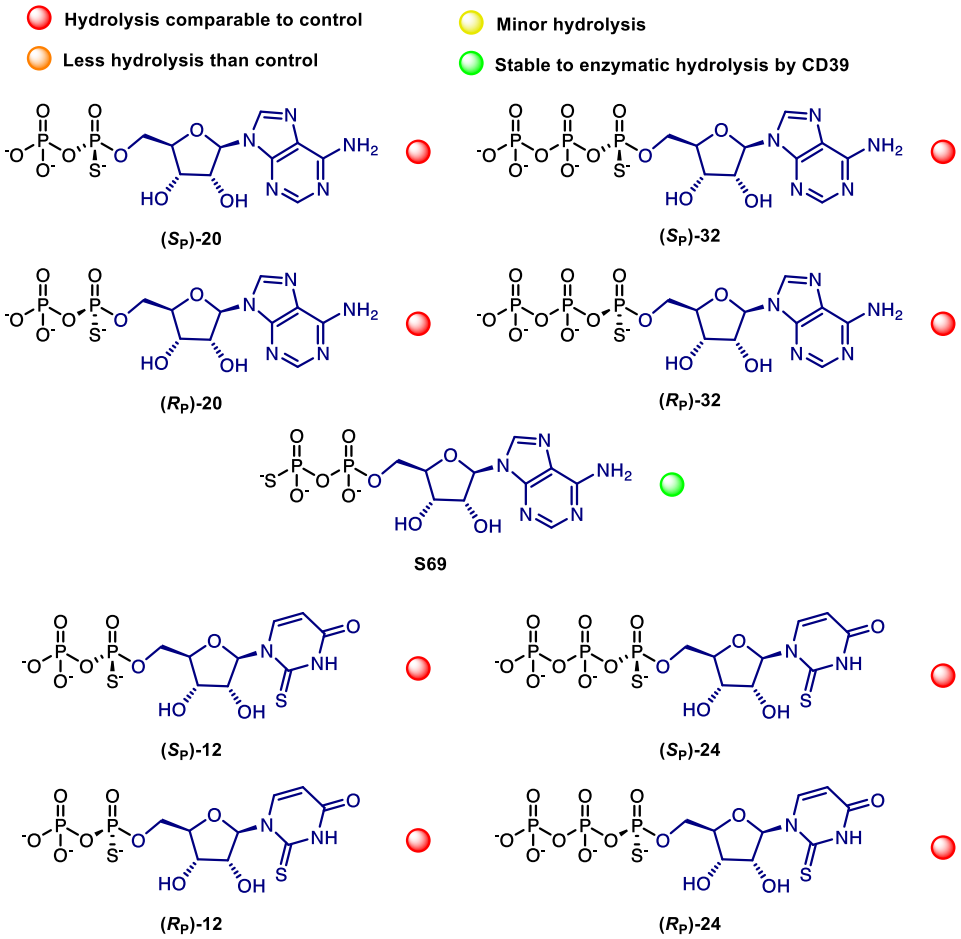


Figure S32. The stability of evaluated nucleotide thioisosteres towards enzymatic hydrolysis by CD39 compared to their natural nucleotides

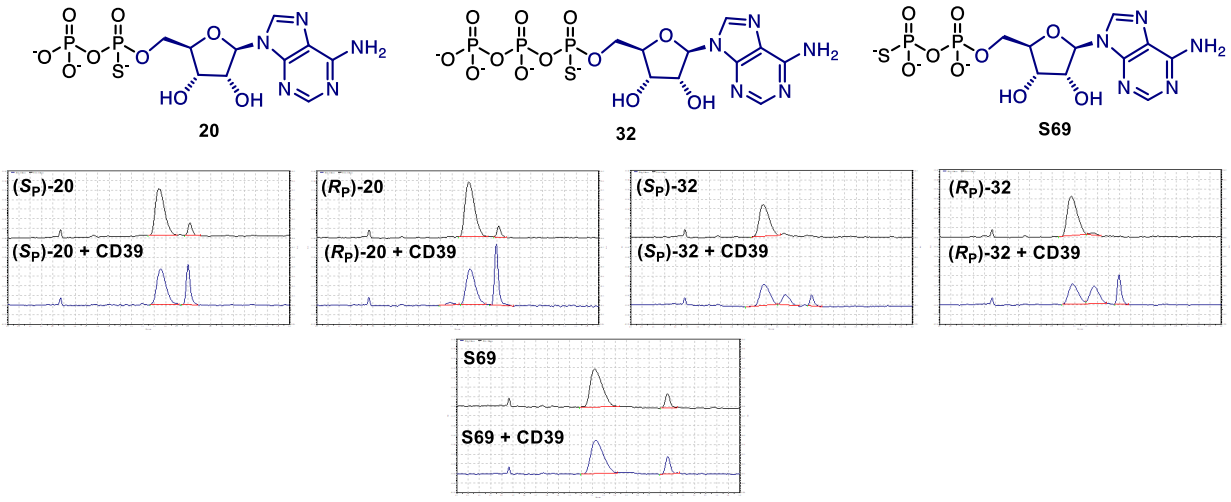


Figure S33. Electropherogram showing hydrolysis of thio-ATP and -ADP analogs by CD39. 20 µM of either compound or substrate (ATP and ADP) were incubated without (black) or with CD39 (blue) for 30 min. Two independent experiments were performed each in duplicate.

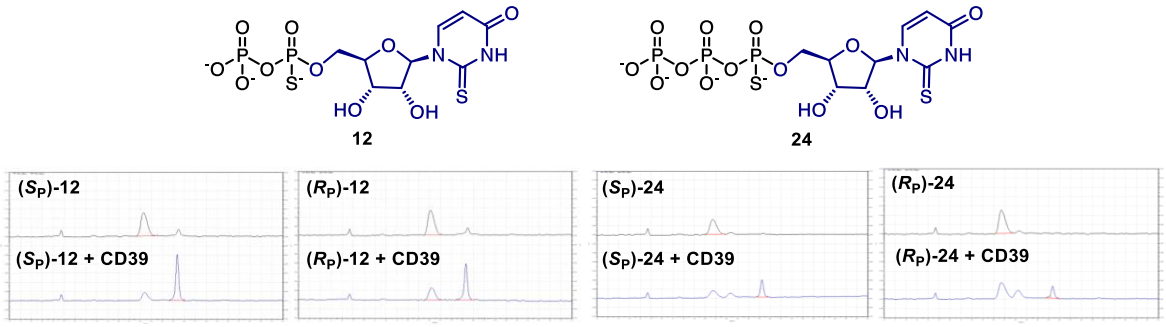


Figure S34. Electropherogram showing hydrolysis of 2-thioUDP and 2-thioUTP analogs by CD39. 20 µM of either compound or substrate (UTP and UDP) were incubated without (blue) or with CD39 (black) for 30 min. Two independent experiments were performed each in duplicate.

All of the investigated nucleotide thioisosteres are substrates of CD39.

Adenine α-thiophosphates are hydrolyzed by the ectonucleotidase CD39. After 30 min of incubation, the conversion to the thio-AMP was as follows: **(*S*P)-32** (12%), **(*R*P)-32** (20%) **(*S*P)-20** (32%), **(*R*P)-32** (39%). The degradation of ATP and ADP by CD39 was similar under the same conditions.

In contrast, β-thio-ADP (**S69**) is not hydrolyzed by CD39 **(***the small additional peak stems from an impurity since it is also present in the absence of the enzyme*). Only after 3h of incubation at 37°C, some degradation can be observed (~35%).

The four 2-thiouridine derivates are also cleaved by the ectoncleotidase CD39 similarly as the corresponding adenine α-thiophosphates. Both 2-thioUDP analogs produced 62% of the corresponding UMP analog within 30 min, similar to the conversion rate of UDP. The 2-thioUTP analogs as well as UTP showed a lower conversion rates to the corresponding UMP analog or UMP (10-30%).

12. Activity of Nucleotide Thioisosteres at P2Y and P2X Receptors

12.1. Human P2Y receptors

12.1.1 Human $P2Y_{13}$ receptor

Compounds **20**, **32** and **S69** were tested in a β-arrestin recruitment assay. All ADP and ATP thioisosteres behaved as $P2Y_{13}$ receptor agonists (see Table S12 and Fig. S35), showing **stereoselective** behavior. The most potent compound was **(R_P)-32** displaying EC_{50} value below 100 nM.

β-Arrestin recruitment assay

The β-arrestin recruitment assay system PathHunter® developed by DiscoverX (Fremont, CA, USA) detects GPCR activation following ligand stimulation. The assay is based on enzyme fragment complementation of β-galactosidase. The assay is performed using a cell line expressing an enzyme acceptor (EA) which is fused to the β-arrestin. The second part of the enzyme (ProLink/PL) is fused to the C-terminus of the GPCR of interest. EA and PL are inactive as single fragments. When a ligand binds and activates the GPCR of interest, the β-arrestin-2 protein is recruited to the GPCR inducing receptor internalization. The recruitment of β-arrestin leads to the complementation of the β-galactosidase. The active enzyme can then catalyze the hydrolysis of a suitable substrate and generate chemiluminescence. The measured chemiluminescence correlates with receptor activation. The recruitment assay was performed using engineered CHO cell lines stably expressing the β-arrestin protein linked to the EA fragment, and the GPCR of interest fused to the ProLink-tag.

One day before the assay, the cells (20000 cells/well), were seeded into 96 well-plates (NunclonTM F96 MicroWellTM, Thermo Fischer Scientific, Schwerte, Germany) in 90 µl of F12 medium supplemented with 10% FCS, 100 units·mL^{-1} penicillin G and 100 µg·mL^{-1} streptomycin and cultivated overnight. On the day of the assay, the medium was exchanged for 90 µl of F12 with 100 units·mL^{-1} penicillin G, 100 µg·mL^{-1} streptomycin, and the cells were incubated for at least 2 h at 37 °C with 5% CO_2. For agonist tests, 10 µl of diluted compounds were added. Unsupplemented Optimem medium and F12 medium was used as negative the $P2Y_{13}$ receptor. For antagonist tests, 5 µl of diluted compound was added and incubated for 30 min. Thereafter, 5 µl of the standard agonist at its EC_{80} concentration was added and the mixture was incubated for 90 min. Then, the PathHunter®detection reagent (DiscoverX, Fremont, CA, USA) was added to the cells (50 µL/well) and the suspension was incubated for 1 h at room temperature in the dark. Finally, chemiluminescence was detected by using a multimode microplate reader (Topcount NXTTM, Packard or Mithras LB 940, Berthold Technologies, Bad Wildbad, Germany).

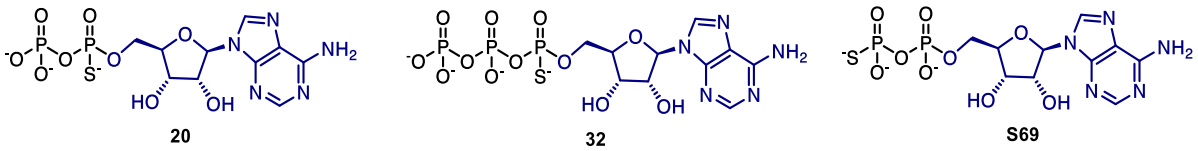


Table S12. Affinity at the human $P2Y_{13}$ in a β-arrestin recruitment assay, showing stereoselective behavior.

Compound	EC_{50} ± SEM (nM)[a]	E_{max} (%)[a]
ADP	33.9 ± 2.1	100
(R_P)-20	122 ± 81	87
(S_P)-20	363 ± 176	86
(R_P)-32	61.6 ± 29.0	81
(S_P)-32	10890 ± 7747	93
S69	98.9 ± 8.0	92

Potencies were determined using engineered human $P2Y_{13}$-expressing β-arrestin-CHO cells. Initial screening was performed at 20 μM. [a]Normalized to 1 μM of ADP (corresponds to maximum effect).

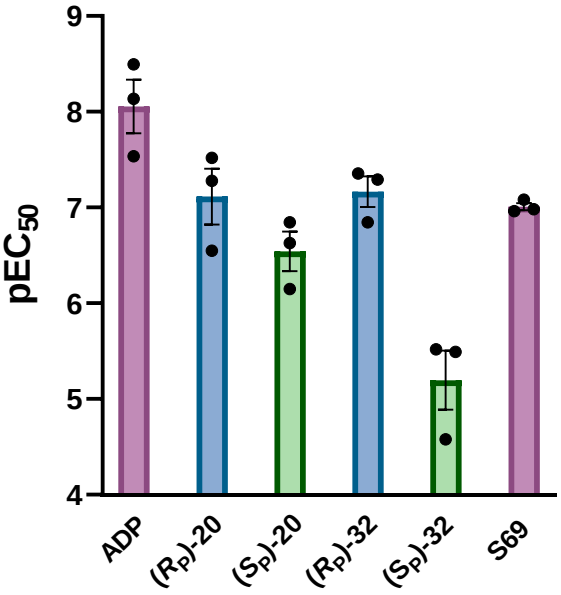


Figure S35. Relative affinity at the human $P2Y_{13}$ receptor expressed in terms of pEC_{50} (y-scale values adjusted for maximal clarity). The results are presented as mean value ± SEM from three independent experiments (n = 3). Black dots represent results of each independent experiment.

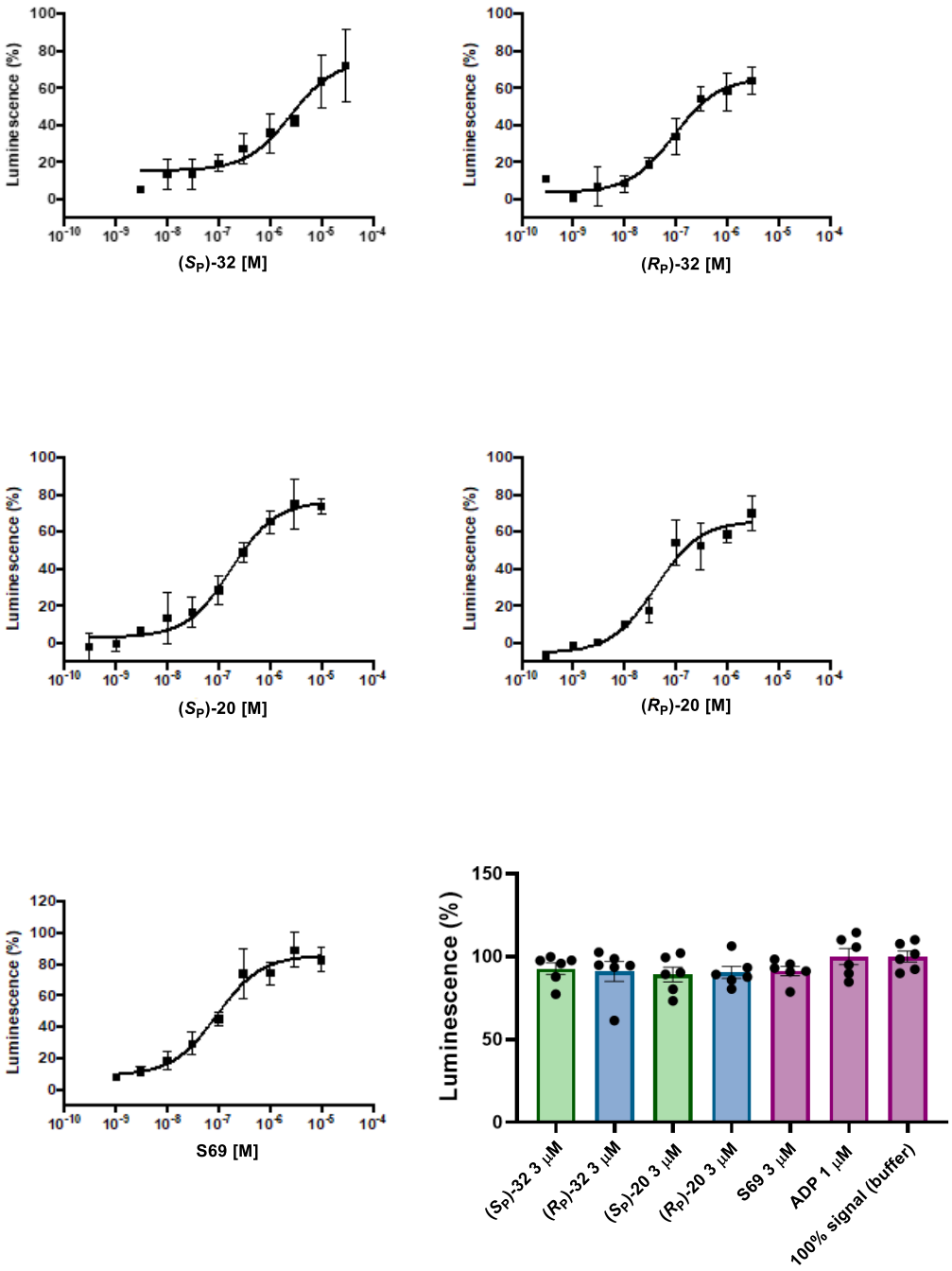


Figure S36. Concentration-dependent activation of the $P2Y_{13}$ receptor by ADP and ATP thioisosteres. Three independent assays were performed in duplicates. The data is represented as mean values ± SEM. The column diagram shows that the compounds do not interfere with the test system (no artifacts). The results on graph and dot diagram are presented as mean value ± SEM from six independent experiments (n = 6). Black dots represent results of each experiment.

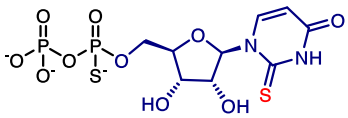
12.1.2. Human $P2Y_6$ receptor

<u>Functional assay at the $hP2Y_6R$ expressed in 1321N1 astrocytoma cells</u>

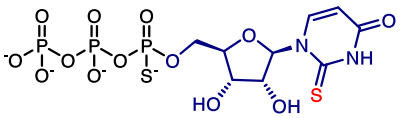
Compounds **12**, **13**, **24** and **25** were tested as agonists of human $P2Y_6$ receptor. Agonist EC_{50} values reflect activation, by a range of agonist concentrations to induce $P2Y_6R$-dependent Ca^{2+} mobilization in 1321N1

human astrocytoma cells stably expressing the human $P2Y_6R$ (*gift of Prof. T.K. Harden, Univ. of North Carolina, Chapel Hill, NC, USA).* EC_{50} values were determined using a High-Throughput FLIPR Tetra Cellular Screening System with Calcium 6 Assay Kits (Molecular Devices, San Jose, CA). Calcium dyes were dissolved in 10 mL of HBSS Loading buffer (10X Hank's Balanced Salt solution plus 20 mM HEPES buffer, pH 7.4) and probenecid for a final concentration of 5 mM probenecid in dye. Aliquots of dye without probenecid could be stored in -20 °C for up to 5 days without loss of activity. 1321N1-$P2Y_6$ cells were plated in a black 96-well flat bottom plate and grown at 37 °C and 5% $CO_{2(g)}$ in Gibco Dulbecco's modified Eagle's medium/nutrient mixture (DMEM) supplemented with 10% fetal bovine serum, 100 units·mL^{-1} penicillin, 100 µg·mL^{-1} streptomycin, and 0.500 mg·mL^{-1} selective antibiotic G418 sulfate. Cells were grown for 24 h to ~90% confluency prior to assay. Media was then removed, and cells were treated with 30 µL of 5 mM probenecid and dye solution for 45 min. Varying concentrations of agonists in HBSS loading buffer ranging from 100 µM to 1 nM was used for calcium mobilization on a separate agonist plate, along with 10 µM UDP as a standard.

The EC_{50} values were determined using a four-parameter logistic equation and the PRISM GraphPad software package (GraphPad, San Diego, CA). The results are presented as mean ± standard error and are the average of three different experiments, unless noted, with each molecule.



12



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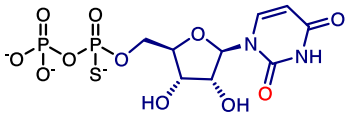

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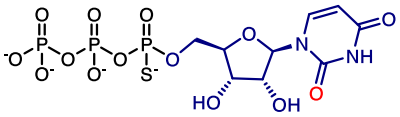


25

Table S13. Affinity at the human $P2Y_6$ receptor [calcium release in whole (transfected) 1321N astrocytoma cells], n = 3.

Compound	EC_{50} ± SEM (nM)
UDP	132 ± 26[a]
2-thioUDP[b]	317 ± 120
(R_P)-12	> 50000 (42%)[c]
(S_P)-12	206 ± 110
(R_P)-13	45100 ± 21900
(S_P)-13	379 ± 160
UTP	> 10000[d]
2-thioUTP	6500 ± 1000
(R_P)-24	> 50000 (14%)[c]
(S_P)-24	2540 ± 471
(R_P)-25	> 50000 (49%)[c]
(S_P)-25	2110 ± 514

[a]Ref. 42. [b]EC_{50} = 447 ± 100 nM (PLC assay), ref. 43. [c]Percent of inhibition or activation at 100 µM. [d]Activation at 10 mM was < 50%, ref. 44.

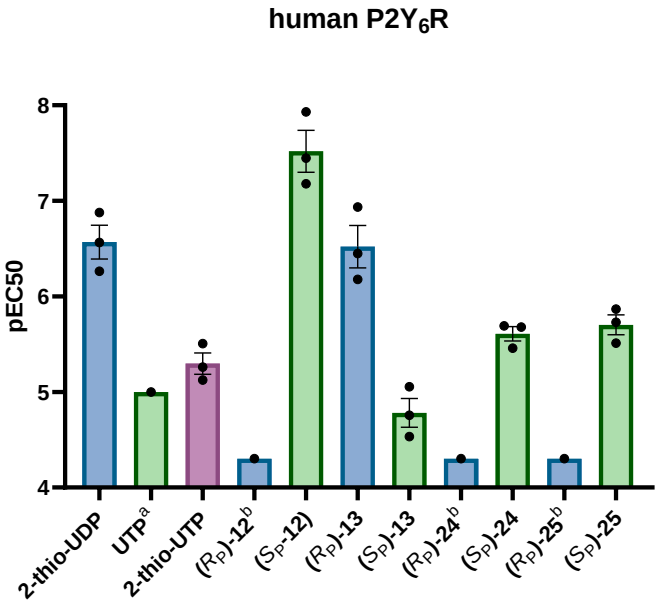


Figure S37. Relative affinity at the human $P2Y_6$ receptor expressed in terms of pEC_{50} (y-scale values adjusted for maximal clarity). The results are presented as mean value ± SEM from three independent experiments (n = 3). Black dots represent results of each independent experiment. [a]EC_{50} > 10000 nM, (n = 1) exact value not determined. [b]EC_{50} > 50000 nM, (n = 1) exact value not determined.

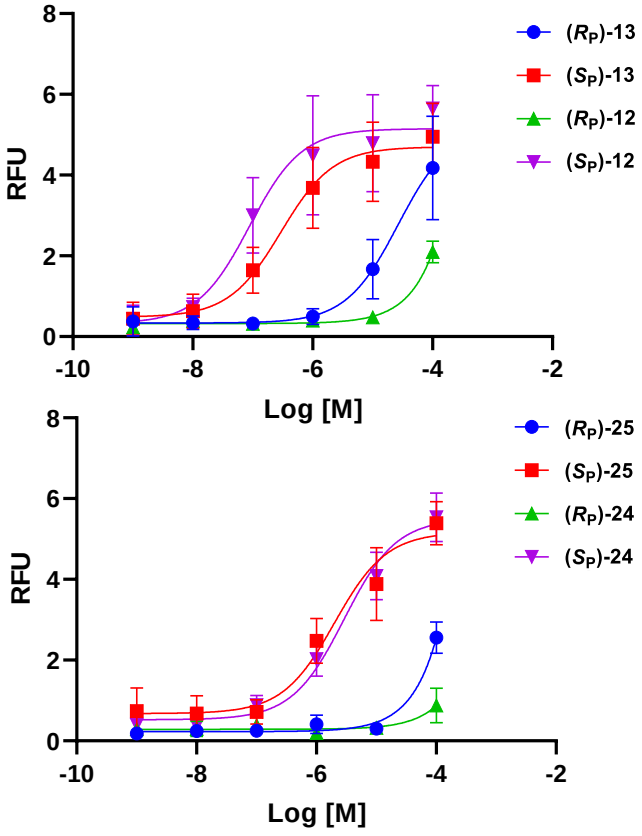
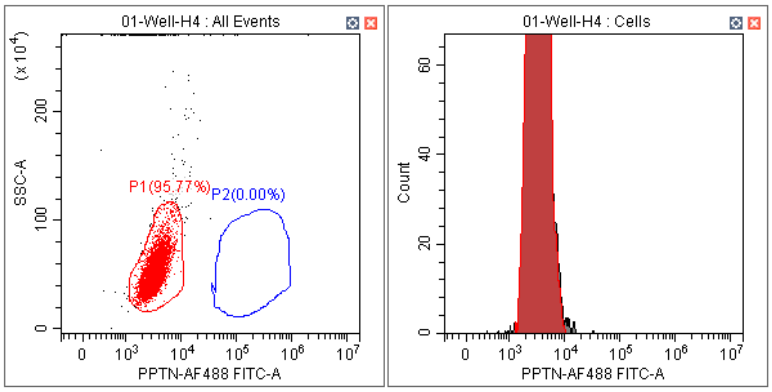


Figure S38. Concentration-dependent activation of the human $P2Y_6$ receptor by UDP and UTP thioisosteres (y-axis in fluorescence units). The results are presented as mean value ± SEM from three independent experiments (n = 3).

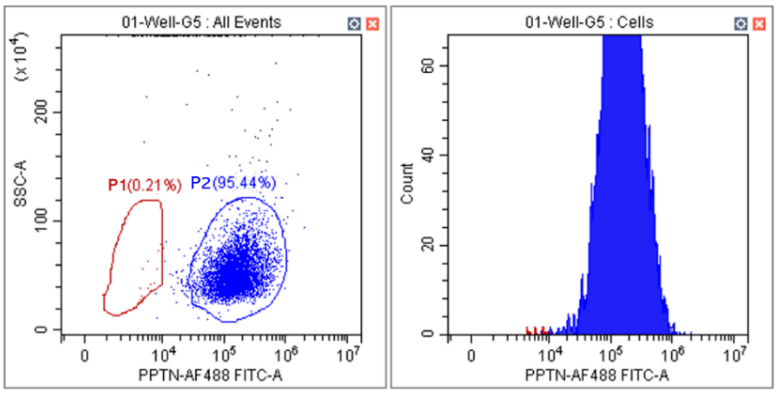
Dot plot analysis of CHO cells stably expressing the hP2Y14R undergoing competitive binding flow cytometry assay

Each plot represents CHO-hP2Y14 cells treated with either a UDP or UTP derivative (near its respective IC_{50} concentration) and fluorescent antagonist MRS4174 (5000 events were taken). MRS4174 is a PPTN derivative containing AlexaFluor-488. The plot shown represents approximately 50% of cells at maximum fluorescence, indicated by the cells in the P2 or maximum fluorescence gate. 50% of cells at maximum fluorescence suggests that the concentration used is near IC_{50} value. Minimum and maximum fluorescence gates are determined by testing cells with no antagonist present and with and without MRS4174.

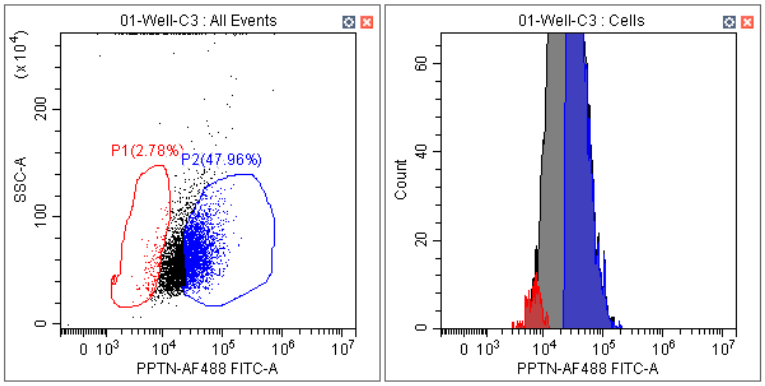
Minimum Fluorescence



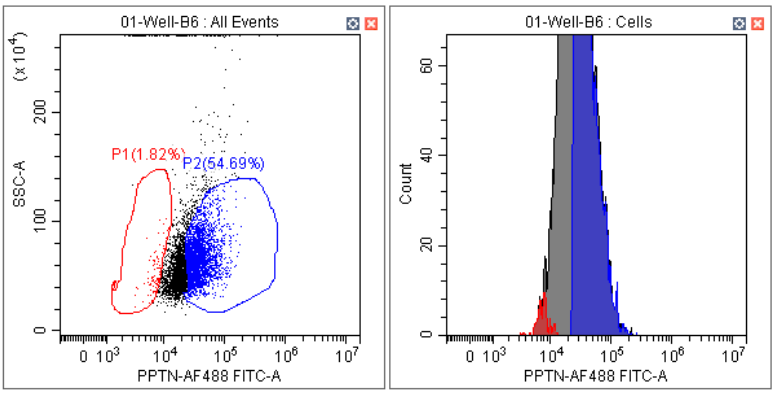
Maximum Fluorescence



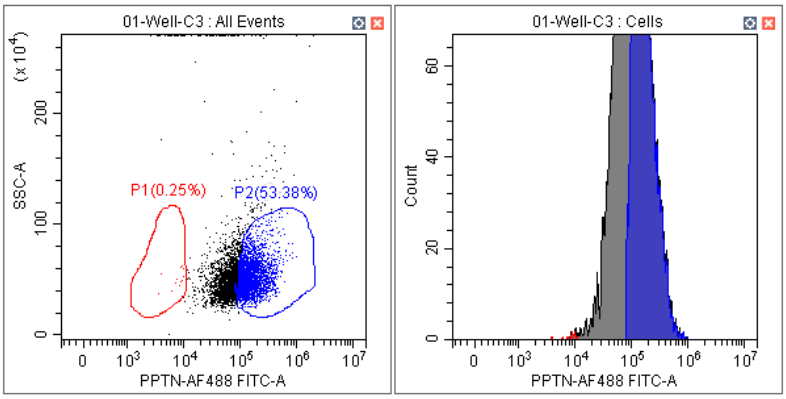
Compound (R_P)-12 near IC_{50} (100 nM)



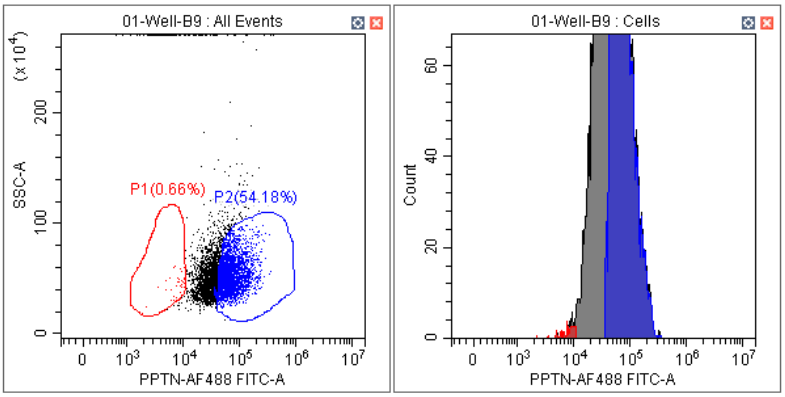
Compound (S_P)-12 near IC_{50} (100 nM)



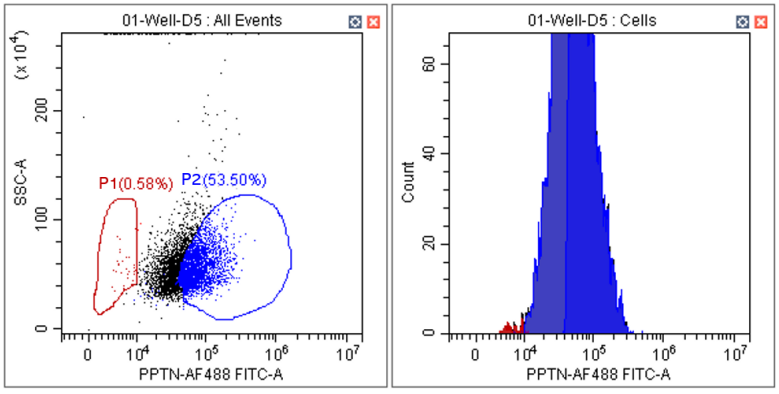
Compound (R_P)-13 near IC_{50} (1 μM)



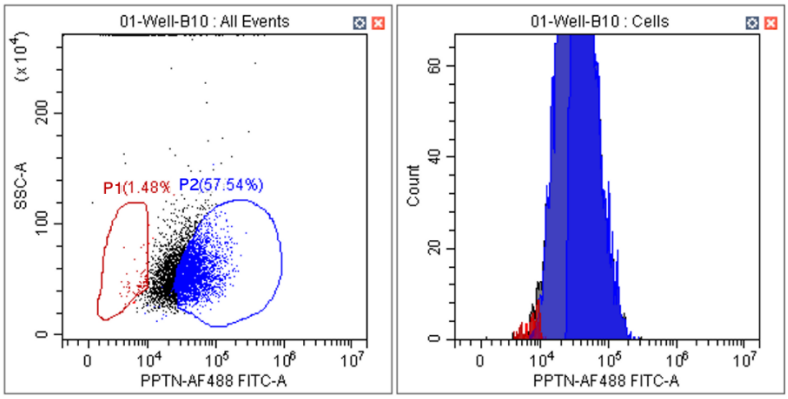
Compound (S_P)-13 near IC_{50} (1 μM)



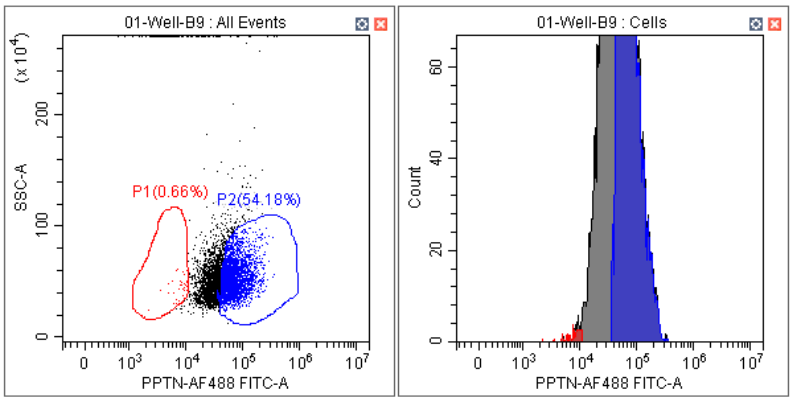
Compound (R_P)-24 near IC_{50} (10 nM)



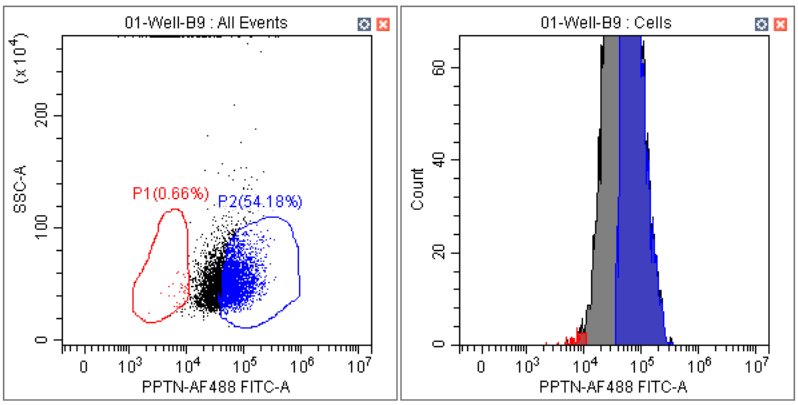
Compound (S_P)-24 near IC_{50} (100 nM)



Compound (R_P)-25 near IC_{50} (1 µM)



Compound (S_P)-25 near IC_{50} (1 µM)



SSC/FSC plots show that only one relevant cell population exists. Autogate technology was used to draw a polygon around the relevant cell population.

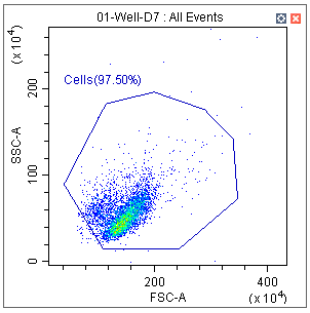
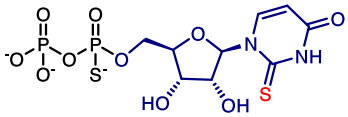


Figure S39. Example depicting gating strategy used for all flow cytometry experiments.

12.1.3. Human $P2Y_{14}$ receptor

Fluorescent binding assay at the $hP2Y_{14}R$ expressed in CHO cells

Compounds **12**, **13**, **24** and **25** were tested as agonists of human $P2Y_{14}$ receptor. IC_{50} values reflect binding inhibition at the human $P2Y_{14}$ receptor stably expressed in CHO cells. CHO-$hP2Y_{14}R$ cells (*gift of Prof. T.K. Harden, Univ. of North Carolina, Chapel Hill, NC, USA*) were plated in a flat bottom 96-well plate and grown at 37 °C and 5% $CO_{2(g)}$ in Gibco Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F12, 1:1) supplemented with 10% fetal bovine serum, 100 units·mL^{-1} penicillin, 100 µg·mL^{-1} streptomycin, and 0.500 mg·mL^{-1} selective antibiotic G418 sulfate. Cells were grown for 24 h to ~90% confluency prior to assay. A 10-fold serial dilution of each ligand was prepared in the appropriate complete cell medium to generate solutions of unlabeled ligands with concentrations ranging from 100 µM to 1 nM. CHO-$hP2Y_{14}R$ cells were first incubated with test antagonist solutions for 30 min in an incubator at 37 °C and 5% $CO_{2(g)}$ followed by another 30 min of incubation with 20 nM fluorescent antagonist MRS4174 under the same conditions. After the incubation periods, cell medium was removed, and the cells were washed three times with sterile 1X Dulbecco's phosphate-buffered saline (DPBS) minus Ca^{2+}/Mg^{2+}. Cells were then detached from the plate using Corning Cellstripper and resuspended in sterile 1X DPBS minus Ca^{2+}/Mg^{2+} for flow cytometry analysis. All competitive binding assays were performed on a BD FACSCalibur flow cytometer in conjunction with the software programs BD Bioscience PlateManager and CellQuest. The gathered data were analyzed to obtain IC_{50} values using GraphPad Prism.



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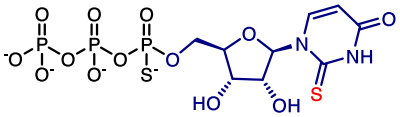

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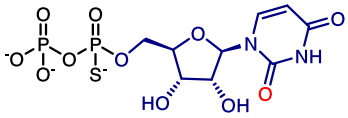

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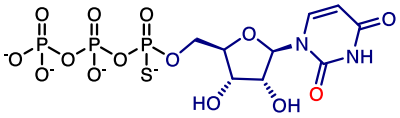


25

Table S14. Affinity at the human $P2Y_{14}$ receptor [(fluorescent binding assay in whole (transfected) CHO cells], n = 3.

Compound	IC_{50} ± SEM (nM)
UDP	1690 ± 240
2-thioUDP [a]	617 ± 240
UDP-glucose [b]	7270 ± 1490
(R_P)-12	111 ± 18
(S_P)-12	436 ± 153
(R_P)-13	1140 ± 350
(S_P)-13	3070 ± 1230
UTP	5050 ± 1140
2-thioUTP	1230 ± 170
(R_P)-24	37.4 ± 12.6
(S_P)-24	208 ± 31
(R_P)-25	1050 ± 210
(S_P)-25	4140 ± 350

[a] EC_{50} = 1.92 ± 0.69 nM (cAMP assay), ref. 43. [b] EC_{50} = 400 nM (cAMP assay), ref. 43.

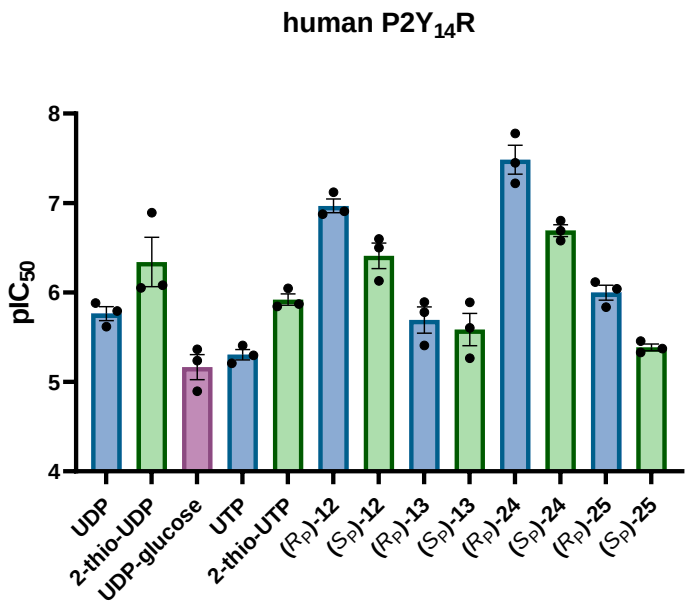


Figure S40. Relative affinity at the human $P2Y_{14}$ receptor expressed in terms of pIC_{50} (y-scale values adjusted for maximal clarity). The results are presented as mean value ± SEM from three independent experiments (n = 3). Black dots represent results of each independent experiment.

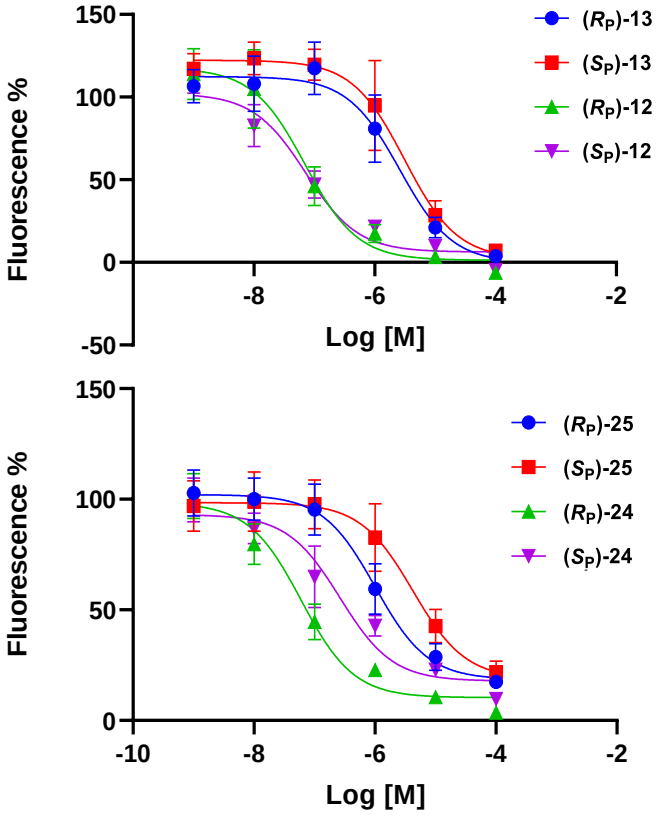


Figure S41. Concentration-dependent activation of the human $P2Y_{14}$ receptor by UDP and UTP thioisosteres (y-axis is normalized). The results are presented as mean value ± SEM from three independent experiments (n = 3).

12.2. Human P2X receptors

Calcium influx assays

To determine receptor function, agonist-mediated increases in cytosolic Ca^{2+} concentrations were measured as previously described[45]. Fluorescent Ca^{2+}-chelating dyes were used as indicators for quantifying intracellular Ca^{2+} concentrations. For astrocytoma cells expressing the human P2X2, or P2X4 receptor, the dye Fluo-4 AM (Thermo Fisher Scientific, Waltham, MA, USA) was used, while for the human P2X1- and the human P2X3-expressing cells the dye Calcium 5 (Molecular Devices, San Jose, CA, USA) was employed. One day before the assay, 45000 cells per well were seeded in a 96-well measurement plate (Assay Plate, 96 Well; black with clear flat bottom; 3340; CORNING, Kennebunk, Maine, USA). For the human P2X3 receptor, 30000 cells per well were used. The plate was incubated over night at 37 °C and 10% CO_2 (5% CO_2 for the cell line expressing the human P2X3 receptor). On the day of the assay, the overnight medium was removed by flipping over the plate, and the dye solution was added to each well. After that, the plate was covered with aluminum foil and incubated for 1 h at room temperature while shaking at 25 x *g*. The cells expressing the human P2X1 and P2X3 receptor, to which the Calcium 5 dye had been added, were incubated for 1 h at 37 °C. For determining the EC_{50} values of the compounds, dilution series of the respective agonists were prepared in transparent reagent plates (Boettger, Bodenmais, Germany). The dilution series were prepared in HBSS buffer. The volume per well in the reagent plate was at least 30 µL. As a control, buffer was added to the wells. After the incubation time, the dye solution was removed from the plate, and 180 µL of buffer were added to each well. The 96-well plates were measured using a fluorescence imaging plate reader NOVOstar (BMG Labtech GmbH, Offenburg, Germany) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The measurement of Ca^{2+} influx started directly after the addition of 20 µL of the compound solution.

12.2.1. Human P2X1 receptor

Compounds **20**, **32** and **S69** were tested in a calcium influx assays on 1321N1 astrocytoma cells stable expressing the human P2X1 receptor (see Table S15 and Fig. S42). In order to characterize fast-sensitizing receptor a chimeric receptor was designed for the P2X1[46]. Dose-response curves of at least three independent experiments performed in duplicates. All results were normalized to the maximal effect induced by ATP (100/10 µM) for P2X1.

At the human P2X1 receptor, all compounds displayed significant receptor activation. The two most potent compounds were **(R_P)-32** and **(R_P)-32**, showing EC_{50} values of similar order as ATP. This indicates that an α-thiophosphate motif is well tolerated. On the other hand, stereoselectivity of the receptor binding was not observed. The ADP analogs were less potent, with compound **S69** showing the lowest potency.

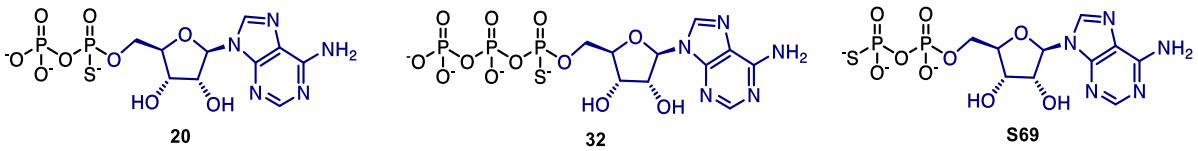


Table S15. Activity at the human P2X1 receptor (calcium influx assay on 1321N1 astrocytoma cells).

Compound	EC_{50} ± SEM (nM)
ATP	16.7 ± 2.6
(R_P)-20	459 ± 114
(S_P)-20	605 ± 147
(R_P)-32	79.6 ± 2.8
(S_P)-32	83.7 ± 7.8
S69	3980 ± 540

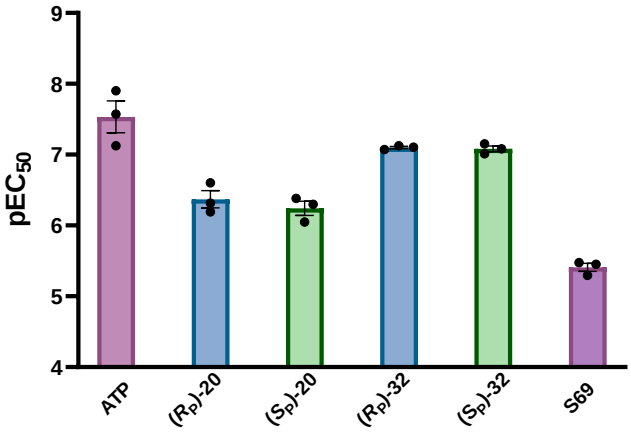


Figure S42. Relative affinity at the human P2X1 receptor expressed in terms of pEC_{50} (y-scale values adjusted for maximal clarity). The results are presented as mean value ± SEM from three independent experiments (n = 3). Black dots represent results of each independent experiment.

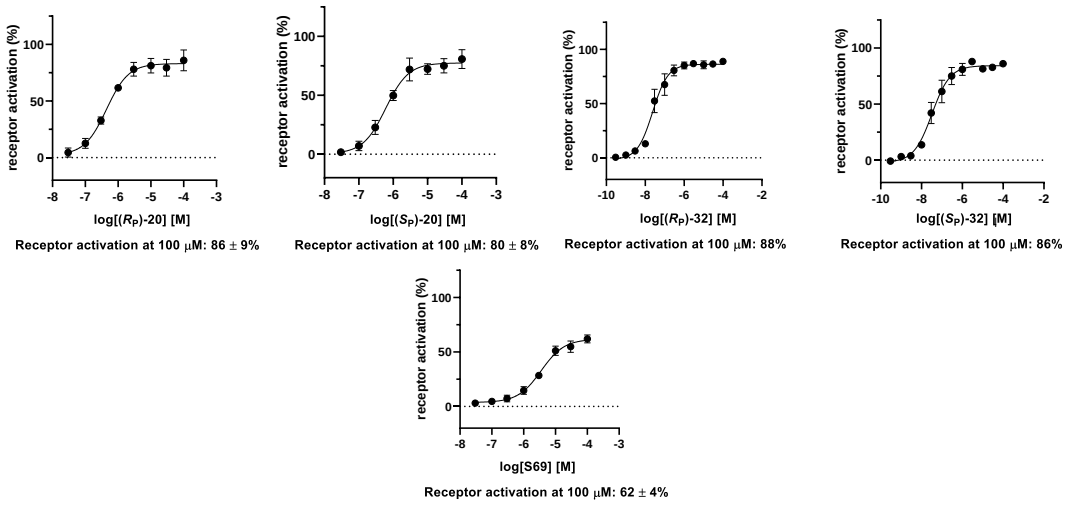


Figure S43. Concentration-dependent activation of the P2X1 receptor by ADP and ATP thioisosteres. Receptor activation values relative to maximum effect induced by ATP. The results are presented as mean value ± SEM from three independent experiments (n = 3).

12.2.2. Human P2X2 receptor

Compounds **20**, **32** and **S69** were tested in a calcium influx assays on 1321N1 astrocytoma cells stable expressing the human P2X2 receptor (see Table S16 and Fig. S44). Dose-response curves of at least three independent experiments performed in duplicates. All results were normalized to the maximal effect induced by ATP (100/10 µM) for P2X2.

The ATP analogs were much more potent than the ADP analogs. They showed similar potency as ATP with low stereoselectivity.

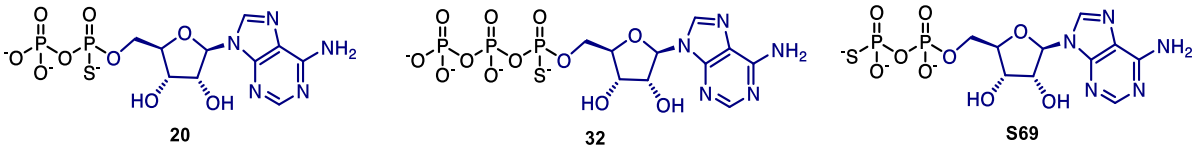


Table S16. Activity at the human P2X2 receptor (calcium influx assay on 1321N1 astrocytoma cells).

Compound	EC_{50} ± SEM (nM)
ATP	847 ± 125
(R_P)-20	21300 ± 4200
(S_P)-20	37000 ± 8170
(R_P)-32	368 ± 103
(S_P)-32	620 ± 156
S69	39100 ± 1200

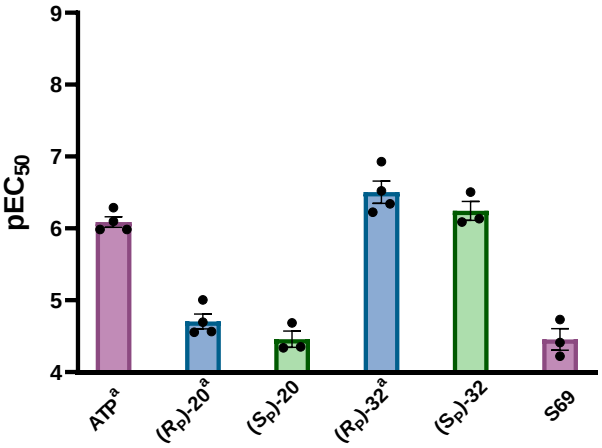


Figure S44. Relative affinity at the human P2X2 receptor expressed in terms of pEC_{50} (y-scale values adjusted for maximal clarity). The results are presented as mean value ± SEM from three independent experiments (n = 3). Black dots represent results of each independent experiment. [a]The results for those experiments are presented as mean value ± SEM from four independent experiments (n = 4).

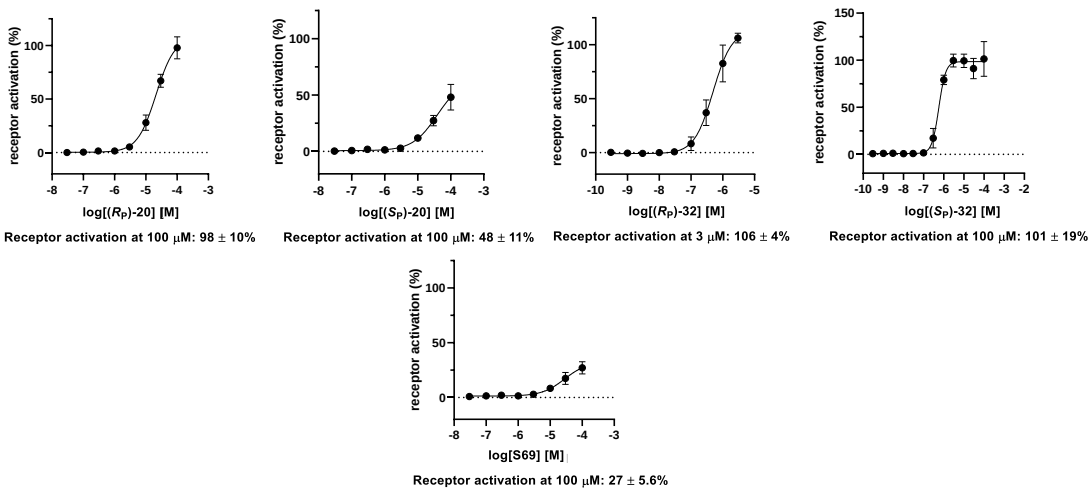


Figure S45. Concentration-dependent activation of the P2X2 receptor by ADP and ATP thioisosteres. Receptor activation values relative to maximum effect induced by ATP. The results are presented as mean value ± SEM from three independent experiments (n = 3).

12.2.3. Human P2X3 receptor

Compounds **20**, **32** and **S69** were tested in a calcium influx assays on 1321N1 astrocytoma cells stable expressing the human P2X3 receptor (see Table S17 and Fig. S45). In order to characterize fast-sensitizing P2X3 receptor the non-desensitizing mutation S15V was inserted[46]. Dose-response curves of at least three independent experiments performed in duplicates. All results were normalized to the maximal effect induced by ATP (100/10 μM) for P2X3.

All compounds activated the P2X3 receptor. The ATP analogs were significantly more potent that the ADP analogs. Interestingly, clear stereoselectivity ($R_P > S_P$) was observed, not only regarding potency, but also with regard to efficacy (maximal effect). Diastereoisomer **(R_P)-32** showed similar potency to ATP.

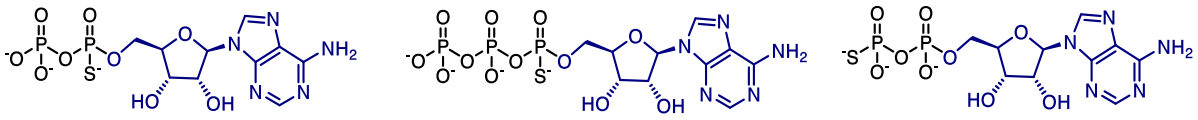


Table S17. Activity at the human P2X3 receptor (calcium influx assay on 1321N1 astrocytoma cells).

Compound	EC_{50} ± SEM (nM)
ATP	21.6 ± 5.8
(R_P)-20	793 ± 17
(S_P)-20	6240 ± 116
(R_P)-32	22.7 ± 7.6
(S_P)-32	112 ± 2
S69	15200 ± 4000

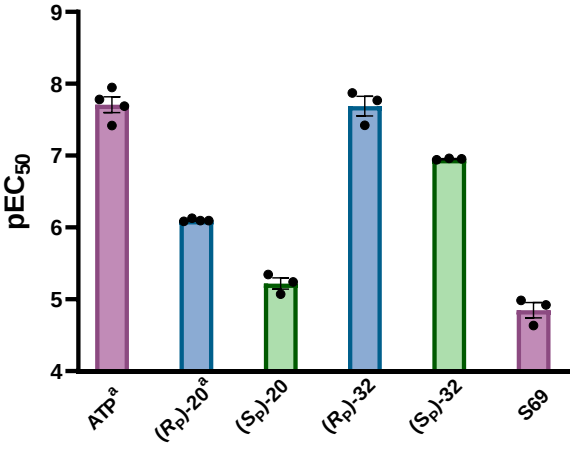


Figure S46. Relative affinity at the human P2X3 receptor expressed in terms of pEC_{50} (y-scale values adjusted for maximal clarity). The results are presented as mean value ± SEM from three independent experiments (n = 3). Black dots represent results of each independent experiment. [a]The results for those experiments are presented as mean value ± SEM from four independent experiments (n = 4).

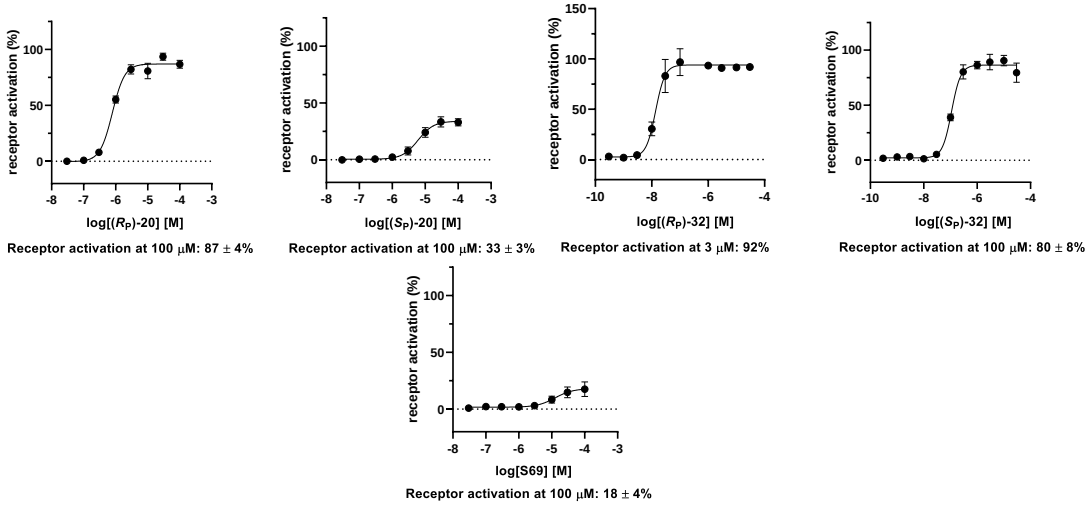


Figure S47. Concentration-dependent activation of the P2X3 receptor by ADP and ATP thioisosteres. Receptor activation values relative to maximum effect induced by ATP. The results are presented as mean value ± SEM from three independent experiments (n = 3).

12.2.4. Human P2X4 receptor

Compounds **20**, **32** and **S69** were tested in a calcium influx assays on 1321N1 astrocytoma cells stable expressing the human P2X4 receptor (see Table S18 and Fig. S48). Dose-response curves of at least three independent experiments performed in duplicates. All results were normalized to the maximal effect induced by ATP (100/10 μM) for P2X4.

The ATP analogs were much more potent than the ADP analogs with stereoselectivity favoring R_P isomer. Moreover diastereoisomer **(R_P)-32** showed similar potency to ATP.

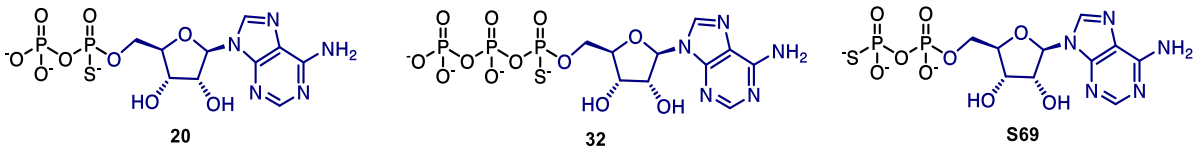


Table S18. Activity at the human P2X4 receptor (calcium influx assay on 1321N1 astrocytoma cells).

Compound	EC_{50} ± SEM (nM)
ATP	207 ± 43
(R_P)-20	4520 ± 510
(S_P)-20	5510 ± 860
(R_P)-32	149 ± 38
(S_P)-32	472 ± 60
S69	10200 ± 1810

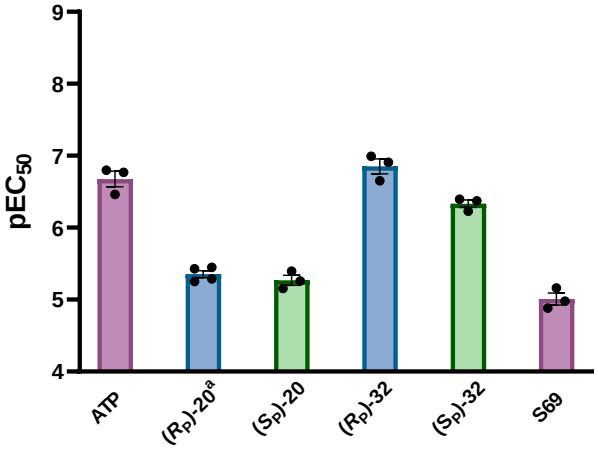


Figure S48. Relative affinity at the human P2X4 receptor expressed in terms of pEC_{50} (y-scale values adjusted for maximal clarity). The results are presented as mean value ± SEM from three independent experiments (n = 3). Black dots represent results of each independent experiment. [a]The results for **(R_P)-20** are presented as mean value ± SEM from four independent experiments (n = 4).

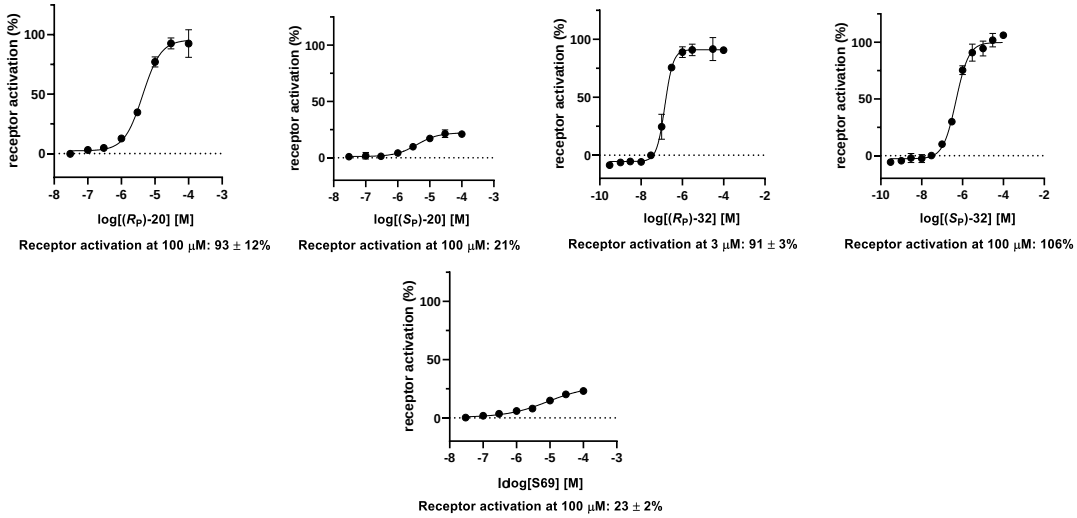


Figure S49. Concentration-dependent activation of the P2X4 receptor by ADP and ATP thioisosteres. Receptor activation values relative to maximum effect induced by ATP. The results are presented as mean value ± SEM from three independent experiments (n = 3).

13. $P2Y_{14}$ Receptor-Ligand Docking Simulation

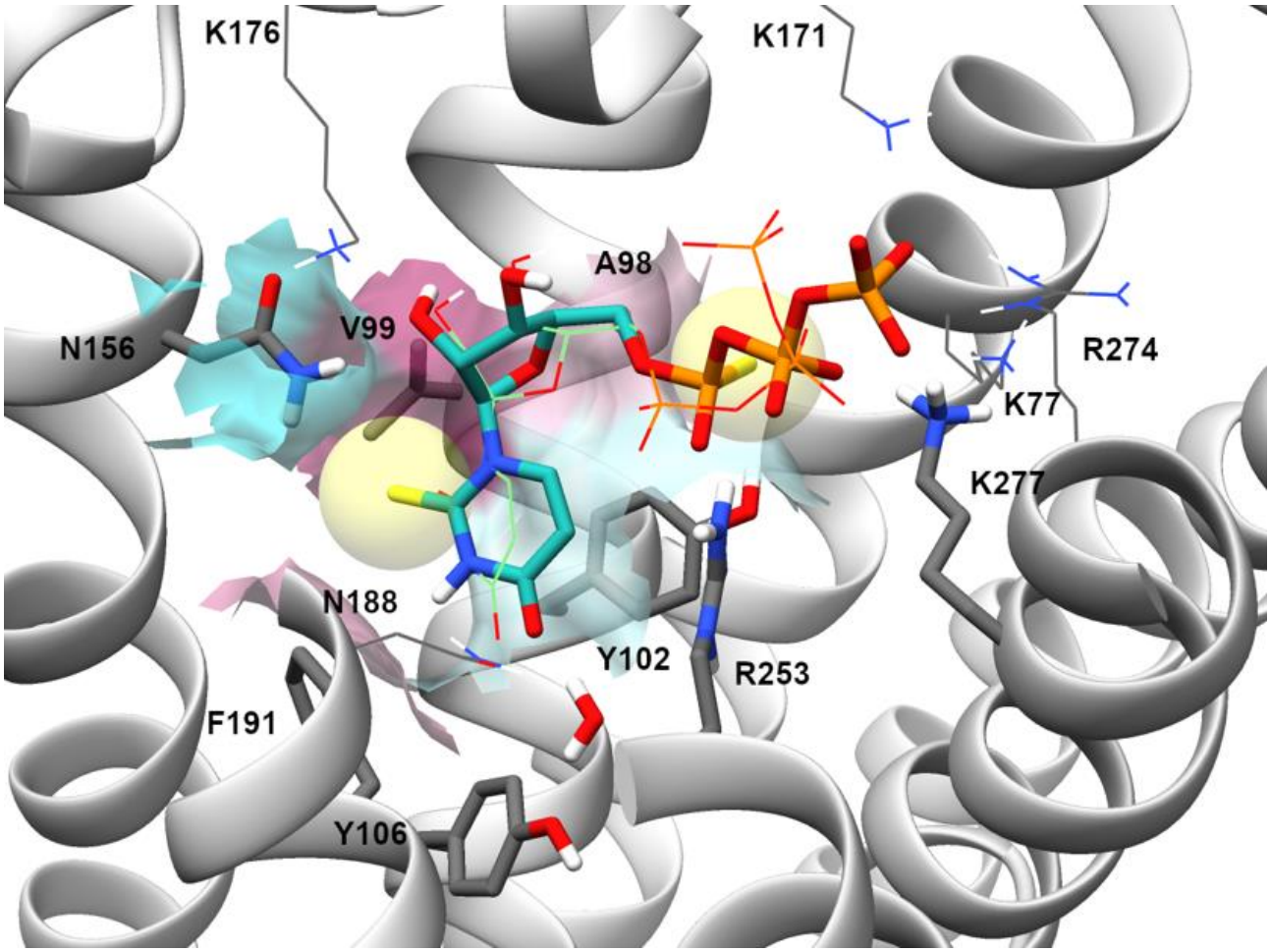


Figure S50. Predicted binding mode of compound **(R_P)-24** (cyan sticks) at a human $P2Y_{14}$ receptor model (last frame from 30 ns post-docking MD simulation). The receptor is rendered with a grey ribbon, residues in proximity of the ligand with wires, and those in contact with uridine base sulfur (S^2) and thiophosphate sulfur (Sα) with sticks. Surface around sulfur is colored according to the hydrophobicity scale of Kyte and Doolittle, [47] with hydrophilic to hydrophobic areas ranging from cyan to magenta. The tips of TM5, TM6 and TM7 are not shown for clarity. The initial UTP docking pose is reported in green wires. The image was generated with the UCSF Chimera software[48].

Results

A binding mode of compound **(R_P)-24** at the human $P2Y_{14}$ receptor model was predicted by molecular dynamics refinement of a pose built in place of the docking pose of UTP. The high affinity ligand **(R_P)-24**, similarly to the low affinity UTP, is predicted to make a π-π stacking between the pyrimidine base and Tyr-$102^{3.33}$ on transmembrane helix (TM) 3, with the ribose moiety facing extracellular loop 2 forming hydrogen bonds with Lys-176^{EL2} and Asn-$156^{4.60}$. Ionic hydrogen bonds are predicted between Lys-171^{EL2}, Arg-$253^{6.55}$, Arg-$274^{7.32}$, Lys-$277^{7.35}$ and the β- and γ-phosphate residues.

Uridine base sulfur (S^2) is located in a mostly hydrophobic pocket, defined by Val-$99^{3.30}$, Tyr-$102^{3.33}$, Phe-$191^{5.43}$, Asn-$156^{4.60}$. Thiophosphate sulfur (Sα) is surrounded within 4.5 Å by Ala-$98^{3.29}$, Tyr-$102^{3.33}$, Arg-$253^{6.55}$ and Lys-$277^{7.35}$ that give the pocket both hydrophobic and electrostatic character, which was previously associated with sulfur binding pockets for phosphorothioate-modified nucleic acids[49,50]. The improved binding affinity of **(R_P)-24** as compared to UTP might be due to a hydrophobic effect, with a desolvation advantage for *S* over *O*.

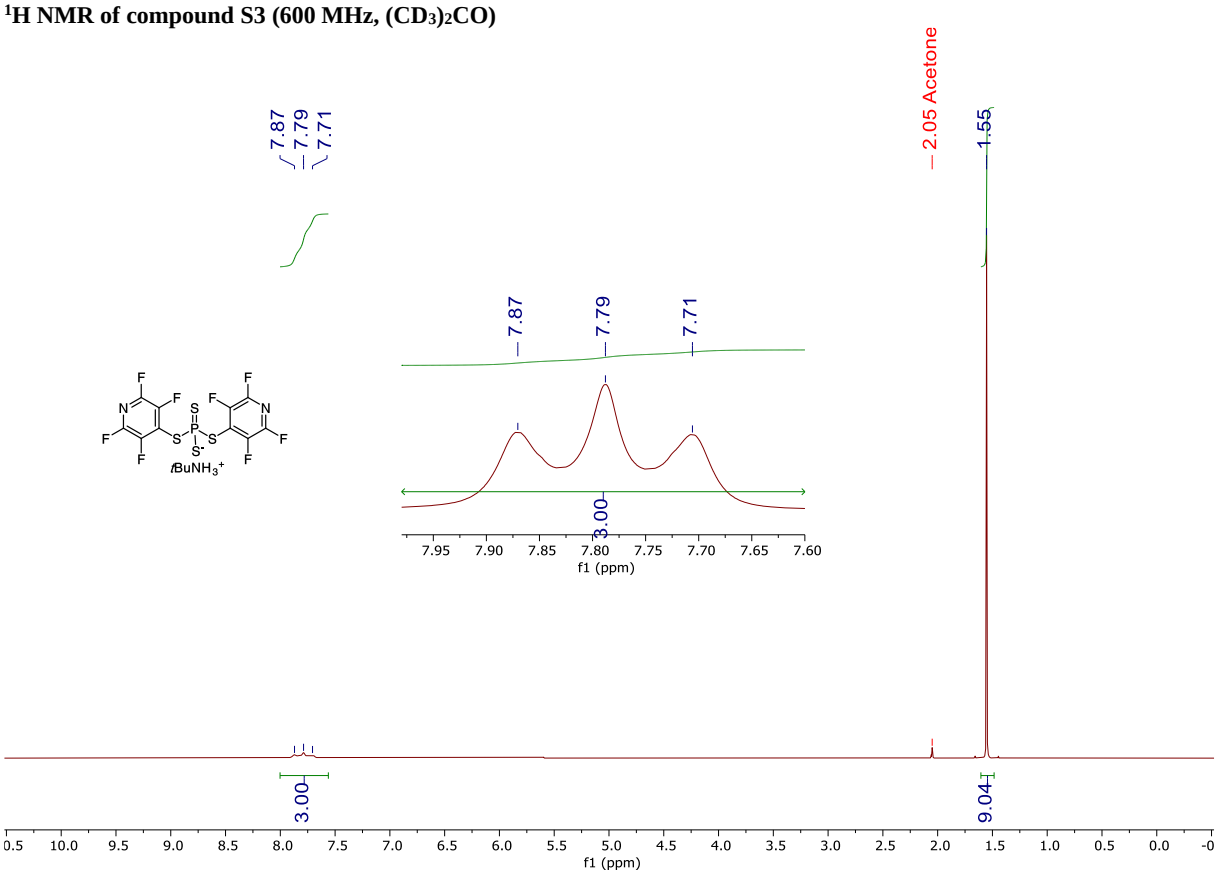
Methods

A model for the human $P2Y_{14}$ receptor was built using a 2MeSATP-bound human $P2Y_{12}$ receptor structure (PDB ID: 4PY0) as a template for homology modeling[51]. The Prime (Schrödinger Release 2021-2: Prime; Schrödinger, LLC: New York, NY, 2021) knowledge-based homology modeling tool of the Schrödinger suite (Schrödinger Release 2021-2: Maestro; Schrödinger, LLC: New York, NY, 2021) was employed, and the protein was prepared with the Protein Preparation Wizard tool of the same suite[52]. UTP was docked to the $P2Y_{14}$ receptor orthosteric binding site (box with 20 Å inner and 30 Å outer box, centered on the center of mass of the 2MeSATP bound to the template) with Glide XP[53-55]. **(R_P)-24** was built in place on the UTP selected pose (visual inspection).

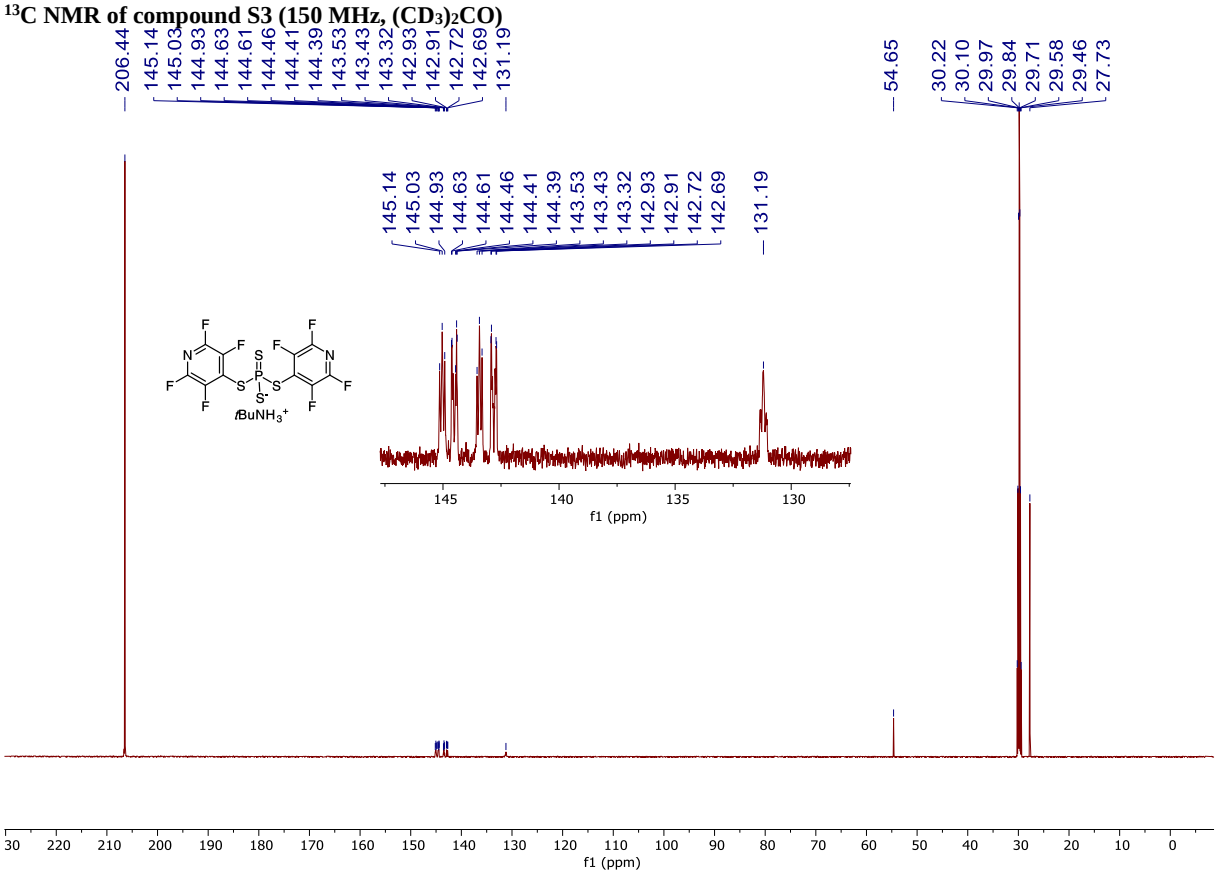
The resulting complexes ($P2Y_{14}$R-UTP and $P2Y_{14}$R-**(R_P)-24**) were oriented using the OPM (Orientation of Proteins in Membranes)[56] structure and inserted in a 90Å X 90Å 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) lipid bilayer generated with the VMD Membrane Plugin,[57] removing phospholipids within 1 Å from the protein. Simulation boxes were built with the HTMD tool,[58] using TIP3P water molecules and neutralizing the system with NaCl at 0.154 concentration. CHARMM36 force field[59,60] was employed, and parameters were assigned to the ligands by analogy to nucleotides and modified nucleotides[61-64]. Three independent molecular dynamics (MD) equilibration and productive runs were collected for each system, using Acemd3 as MD engine[65]. The equilibration protocol involved 40 ns in the NPT ensemble, with restraints on ligands and protein atoms (1 $kcal \cdot mol^{-1} \cdot Å^{-2}$ for ligand heavy atoms and protein Cα atoms, and 0.5 $kcal \cdot mol^{-1} \cdot Å^{-2}$ for other protein atoms) which were linearly reduced in the first 20 ns of the simulation and then removed. The productive runs were collected in the NVT ensemble (30 ns). Temperature was kept at 310 K (thermostat damping constant of 1 ps^{-1} during equilibration and 0.1 ps^{-1} during production), and pressure at 1 atm (in the equilibration phase). A switching distance of 7.5 Å and a 9 Å cutoff were employed for van der Waals interactions, and the particle-mesh Ewald (PME) method was adopted for long-range electrostatic interactions. The simulations were run with a timestep of 4 fs saved with a stride of 20 ps (100 ps for equilibration). Simulations were run on Nvidia Tesla V100 GPUs on the NIH HPC Biowulf cluster (http://hpc.nih.gov). Replicates were analyzed in terms of ligand heavy atoms RMSD (after receptor TM alignment) and ligand-receptor contacts using VMD.[57] **(R_P)-24** replicate with lowest average RMSD (1.78 Å as compared to 1.94 Å and 2.06 Å) was selected and the last frame extracted for discussion.

14. NMR spectra

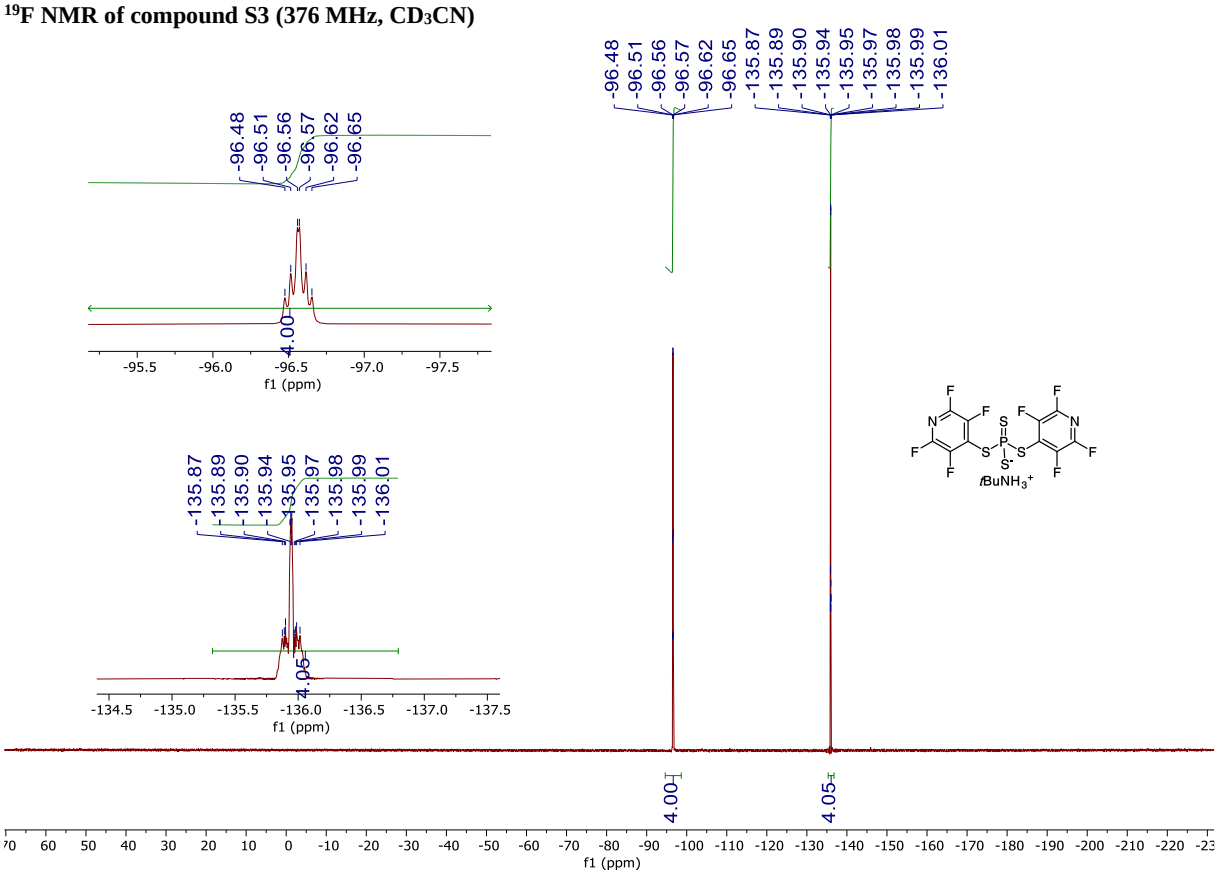
^{1}H NMR of compound S3 (600 MHz, $(CD_3)_2CO$)



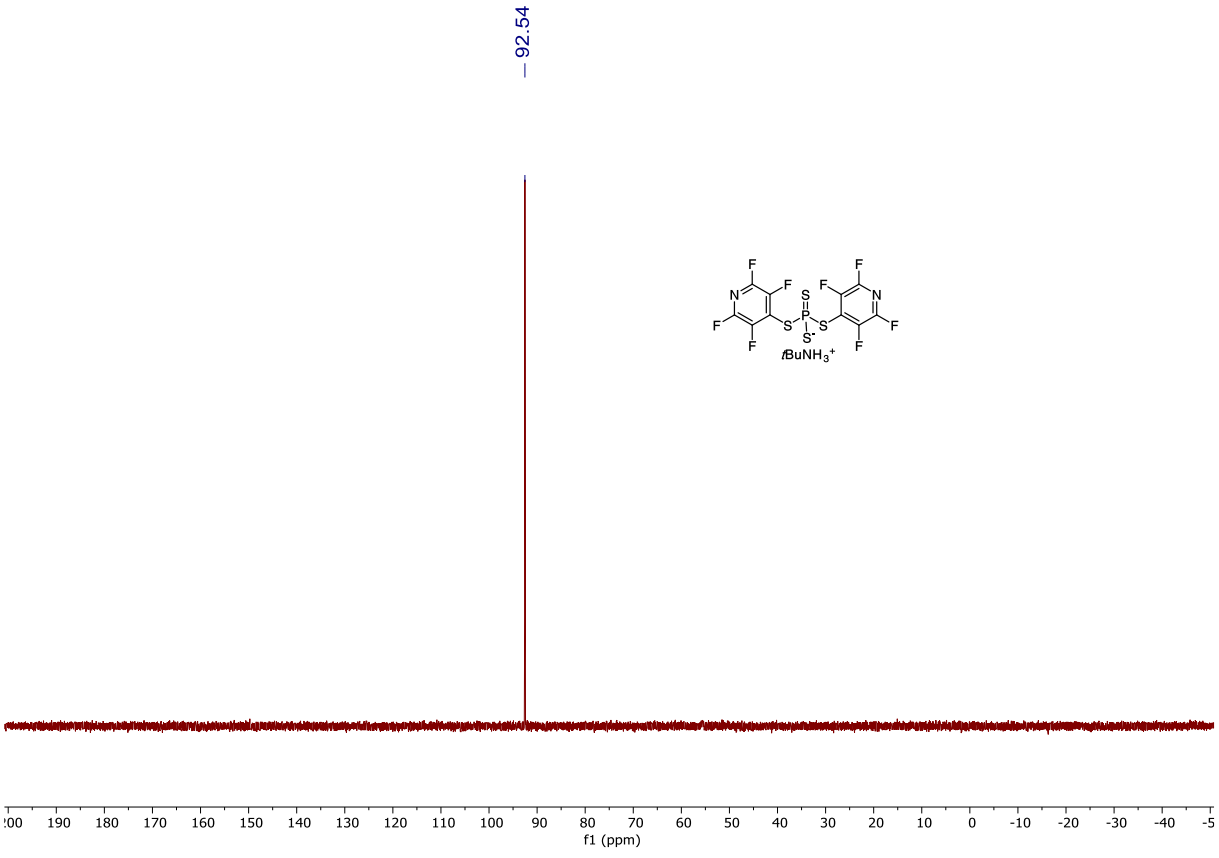
^{13}C NMR of compound S3 (150 MHz, $(CD_3)_2CO$)



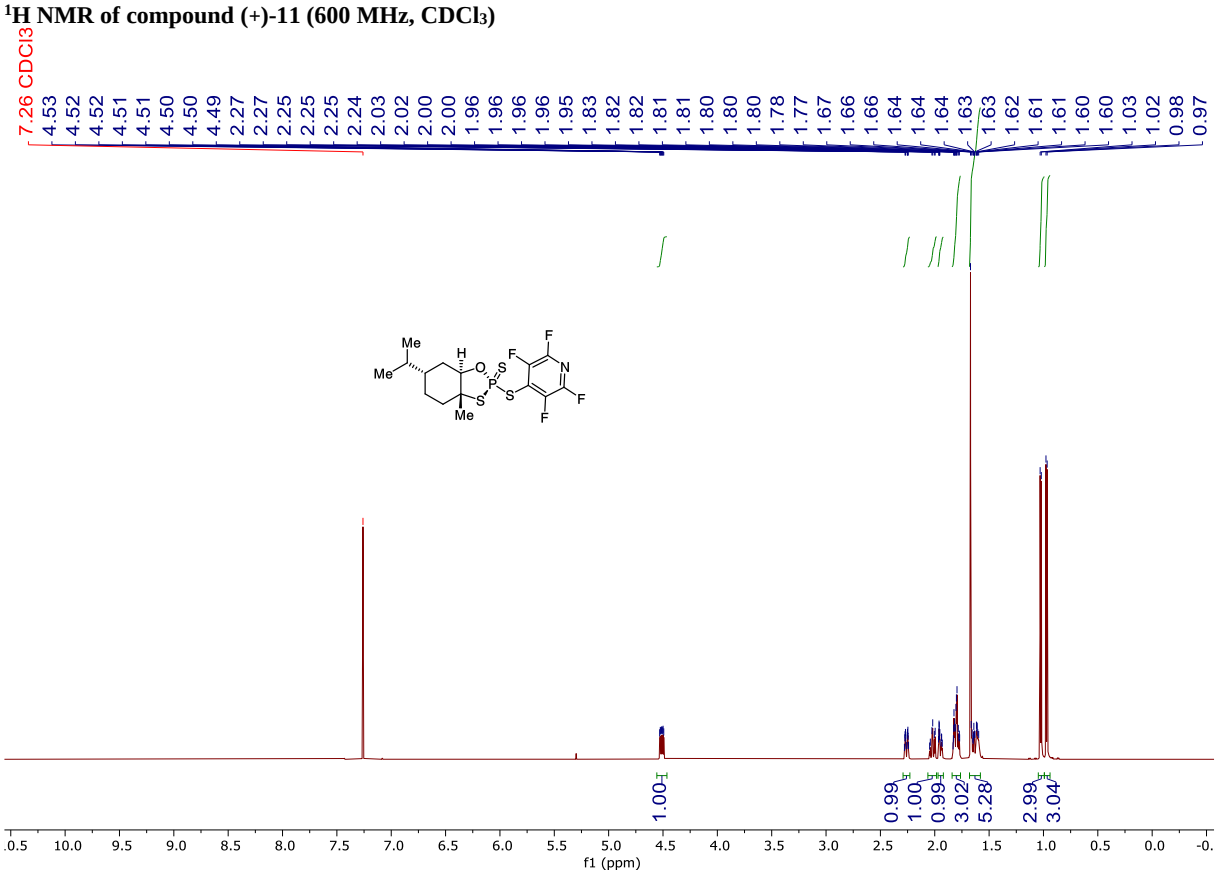
^{19}F NMR of compound S3 (376 MHz, CD_3CN)



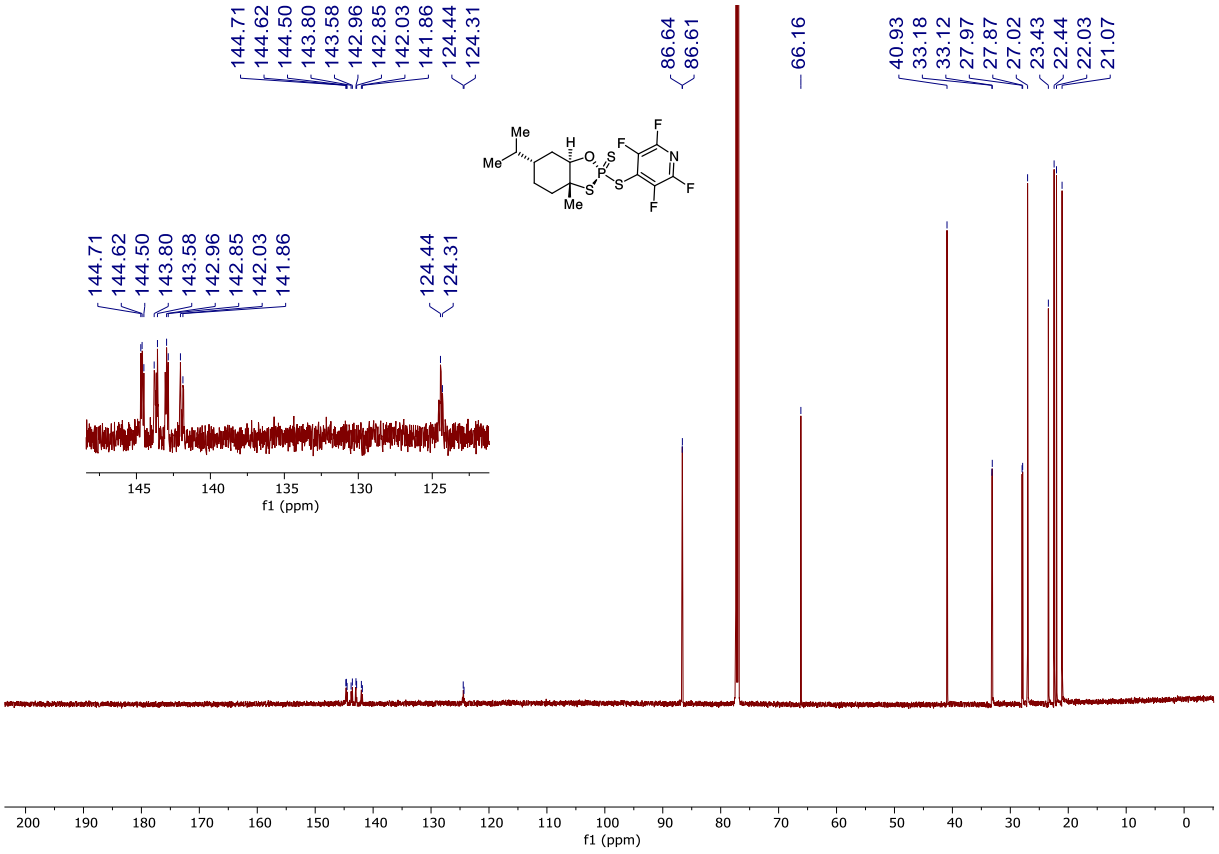
^{31}P NMR of compound S3 (162 MHz, CD_3CN)



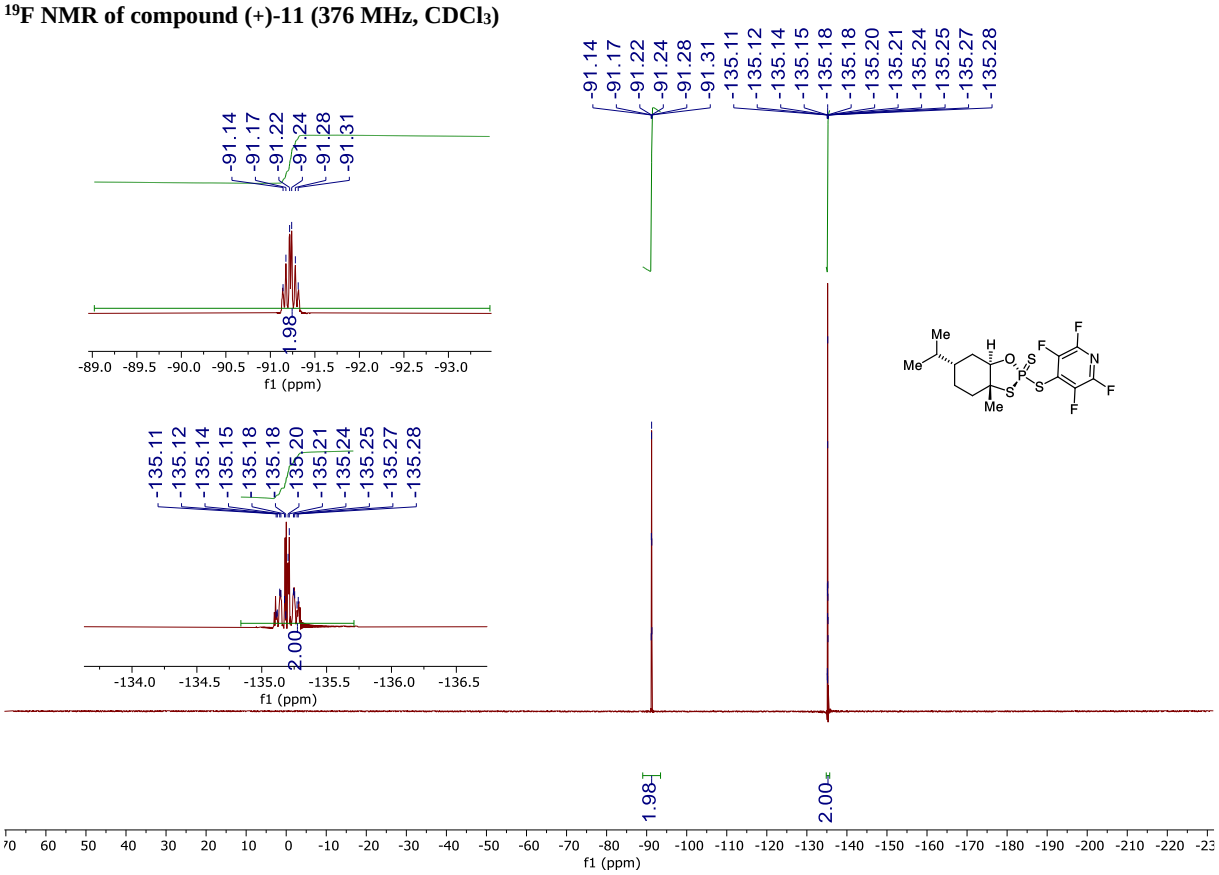
^{1}H NMR of compound (+)-11 (600 MHz, $CDCl_3$)



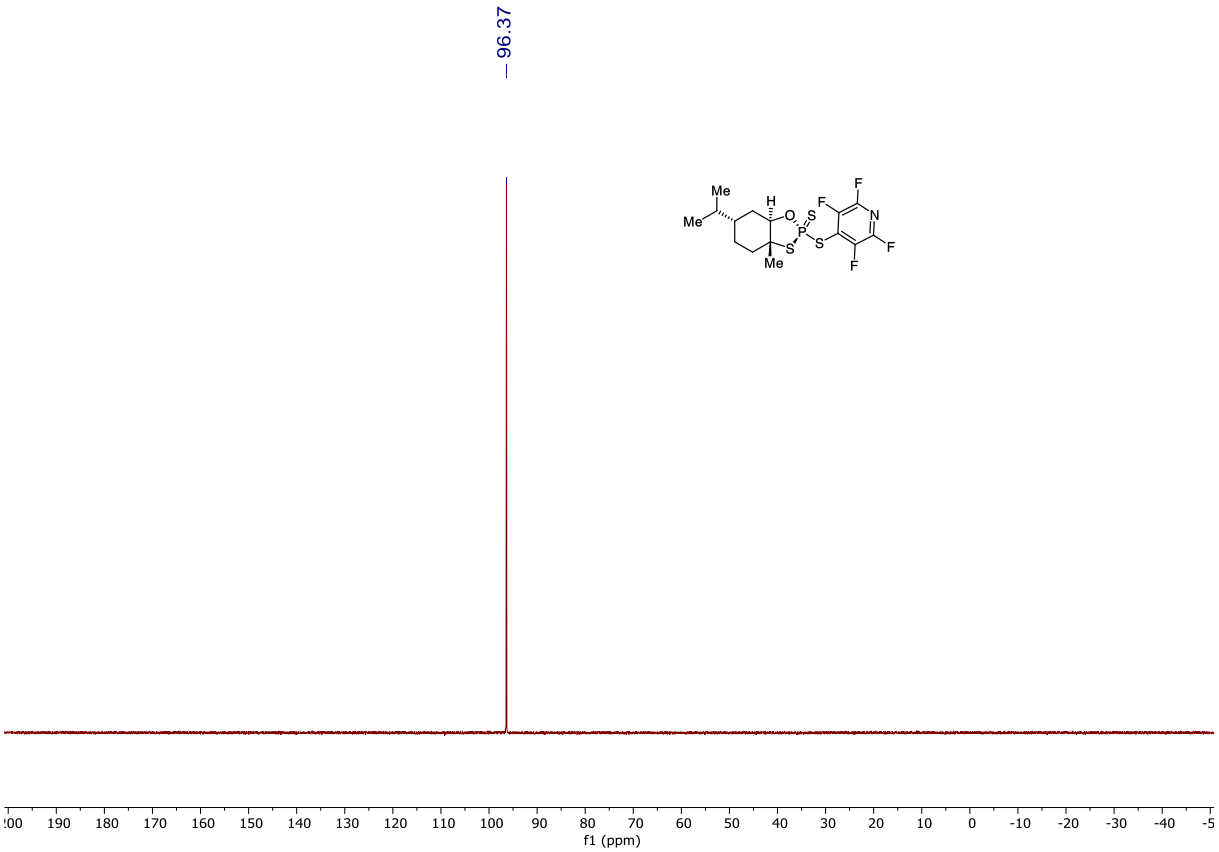
^{13}C NMR of compound (+)-11 (150 MHz, $CDCl_3$)



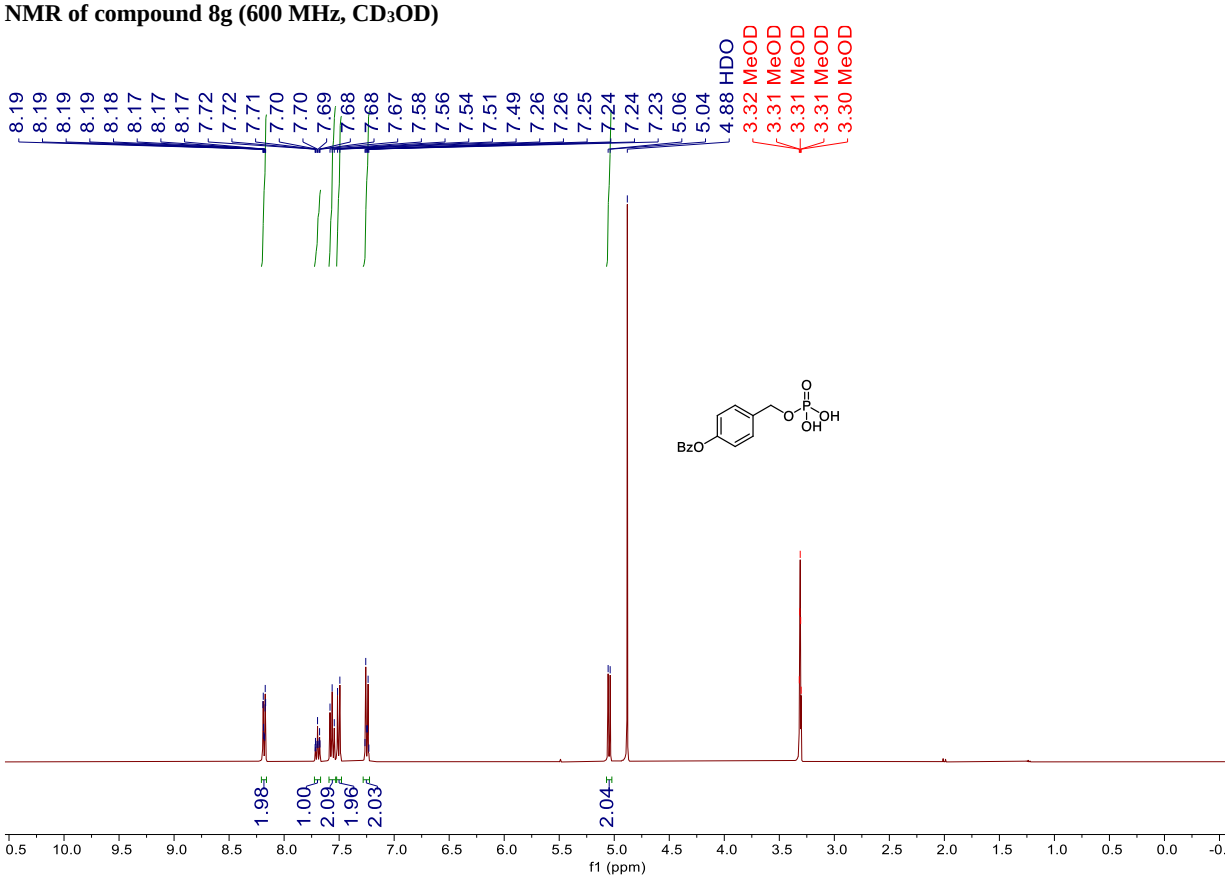
^{19}F NMR of compound (+)-11 (376 MHz, $CDCl_3$)



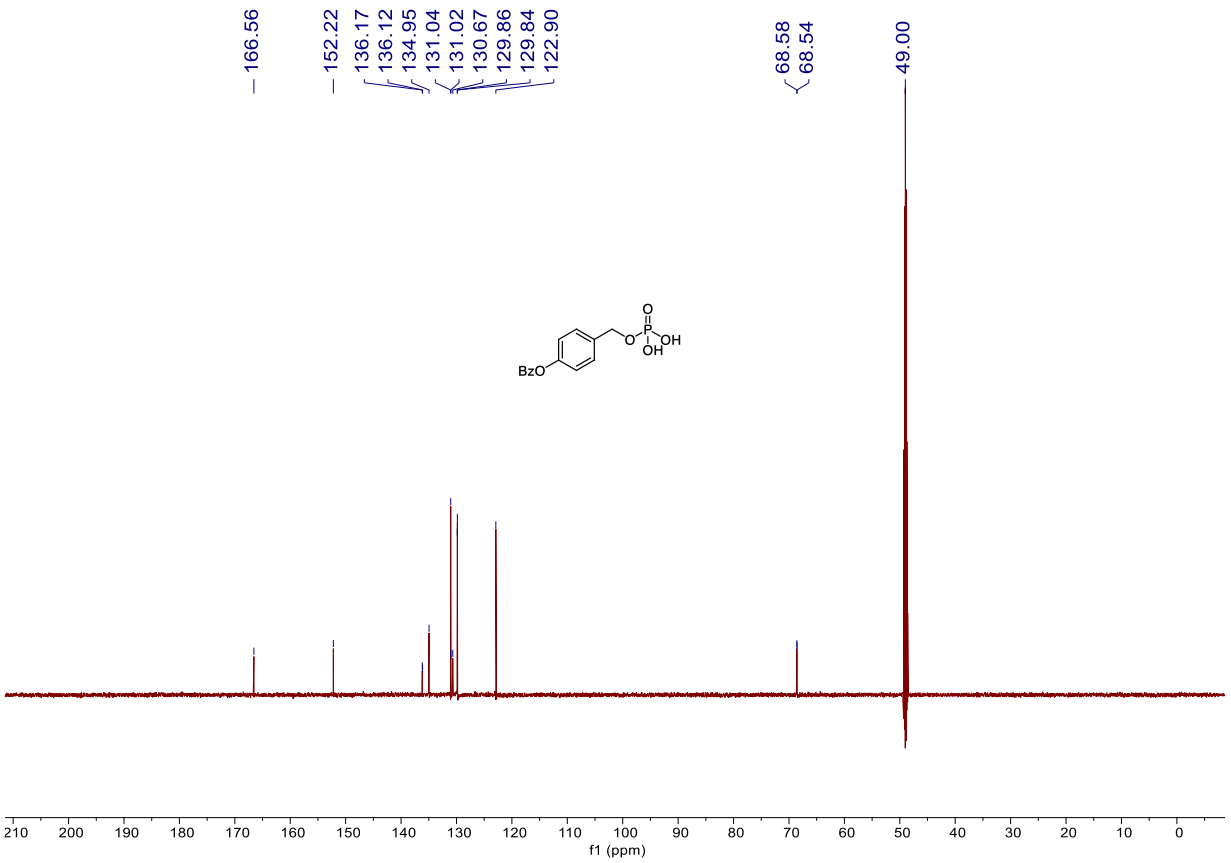
^{31}P NMR of compound (+)-11 (376 MHz, $CDCl_3$)



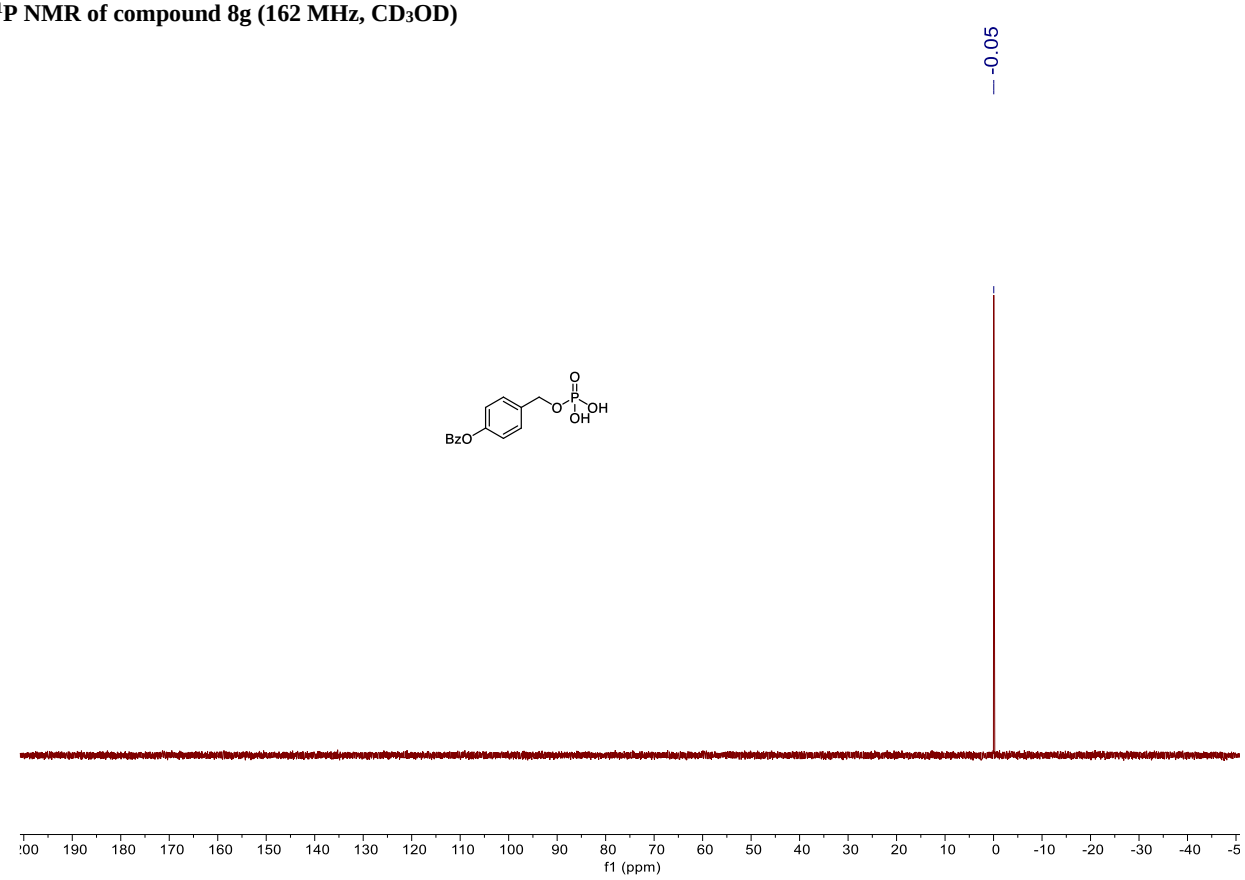
^{1}H NMR of compound 8g (600 MHz, CD_3OD)



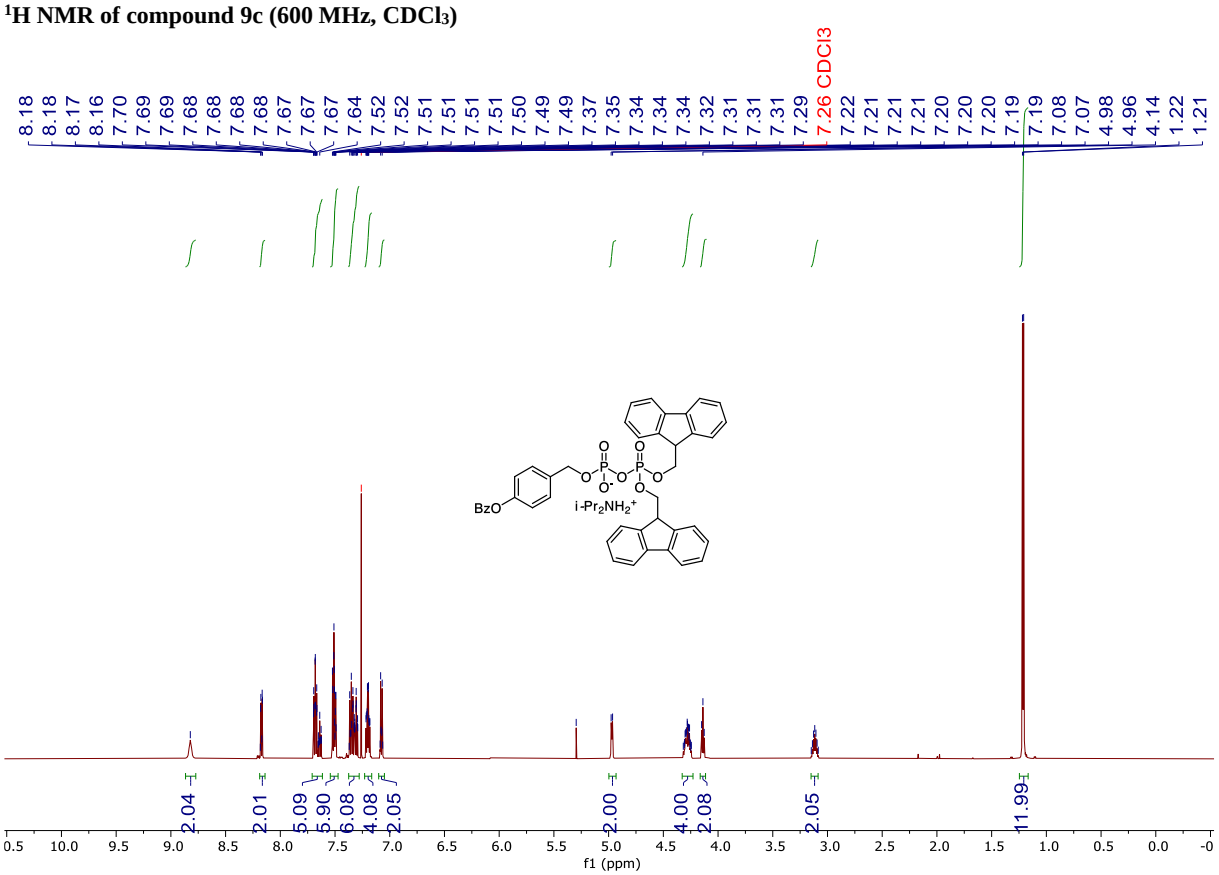
^{13}C NMR of compound 8g (150 MHz, CD_3OD)



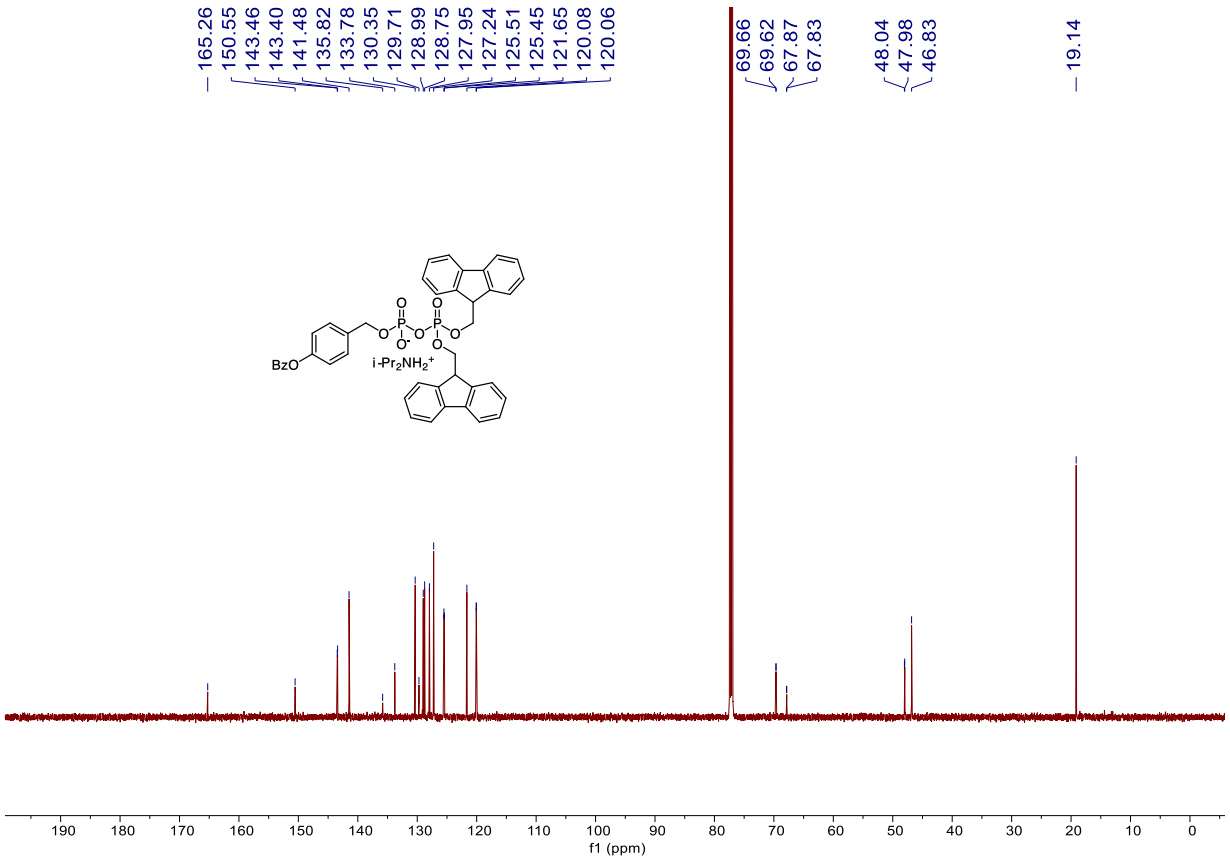
^{31}P NMR of compound 8g (162 MHz, CD_3OD)



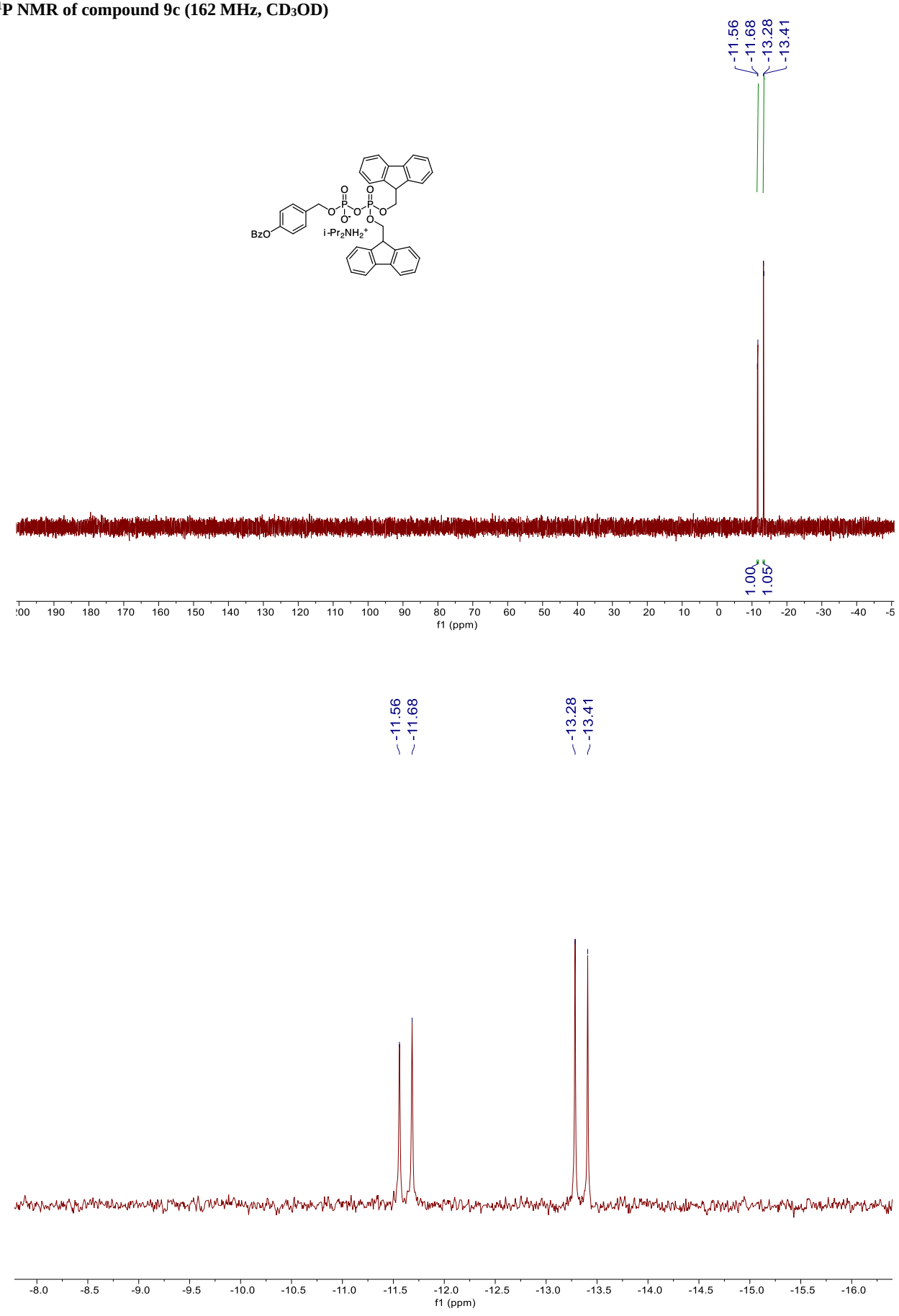
^{1}H NMR of compound 9c (600 MHz, $CDCl_3$)



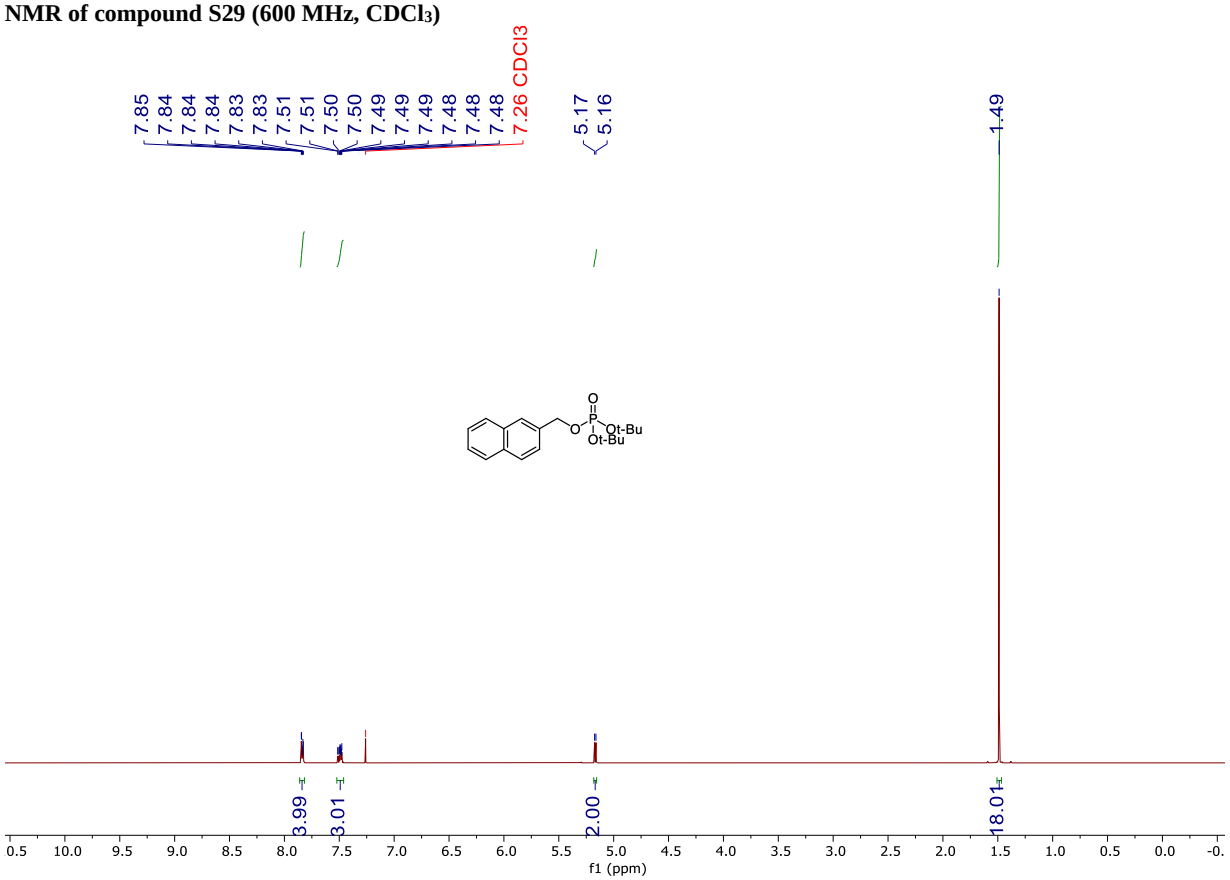
^{13}C NMR of compound 9c (150 MHz, $CDCl_3$)



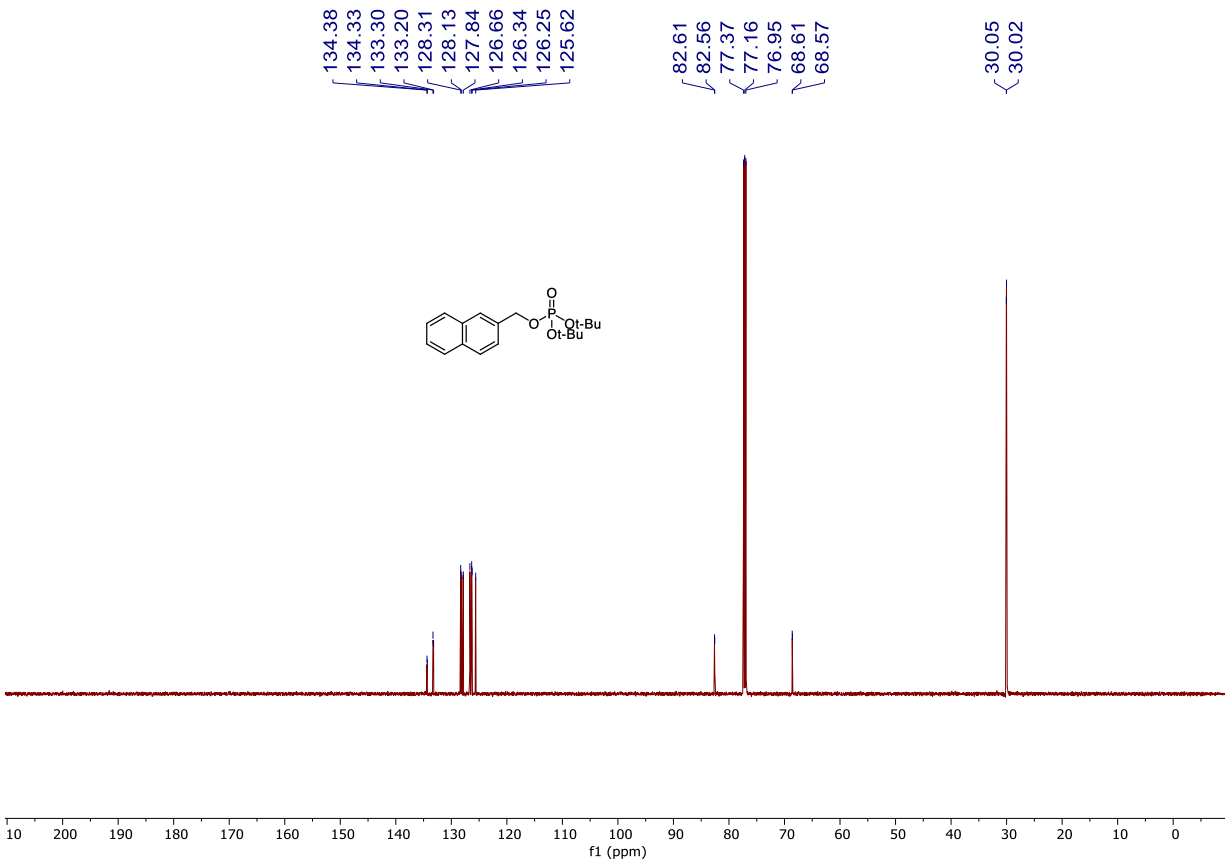
^{31}P NMR of compound 9c (162 MHz, CD_3OD)



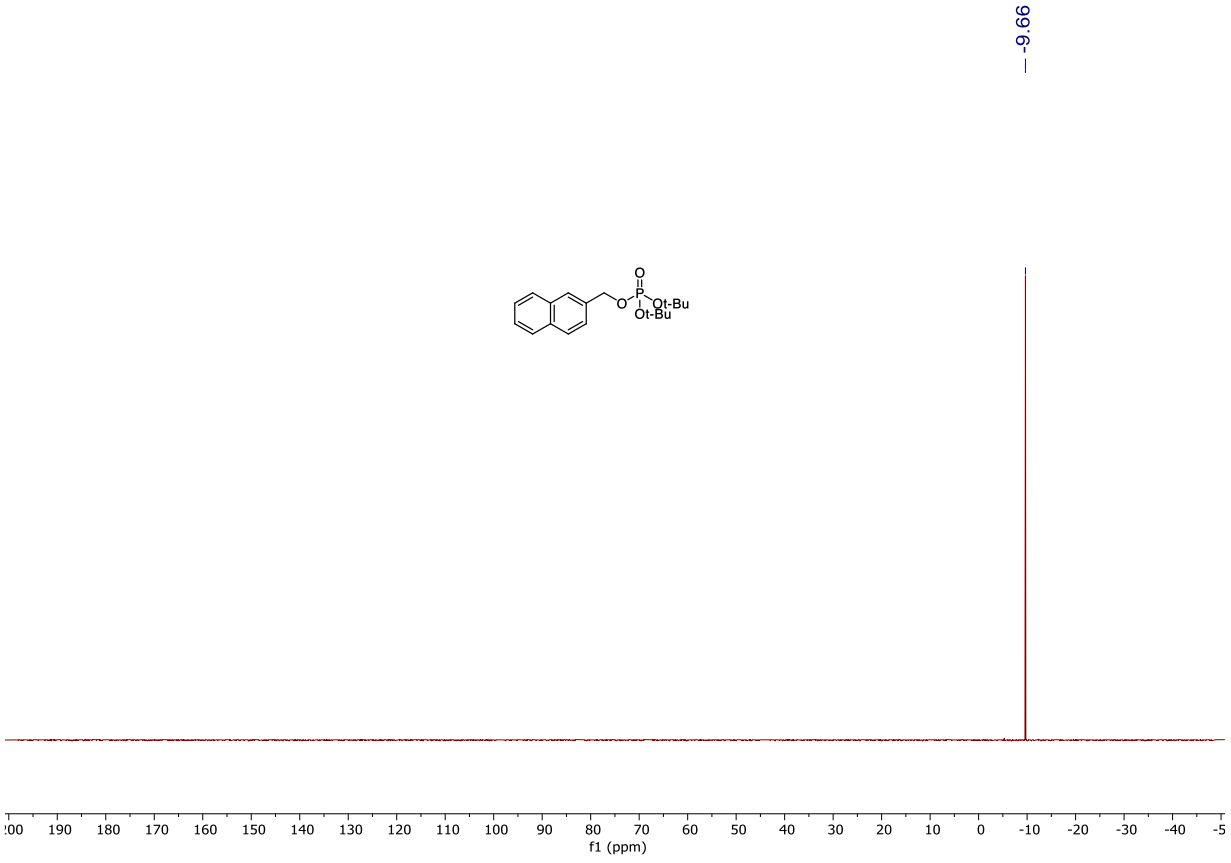
^{1}H NMR of compound S29 (600 MHz, $CDCl_3$)



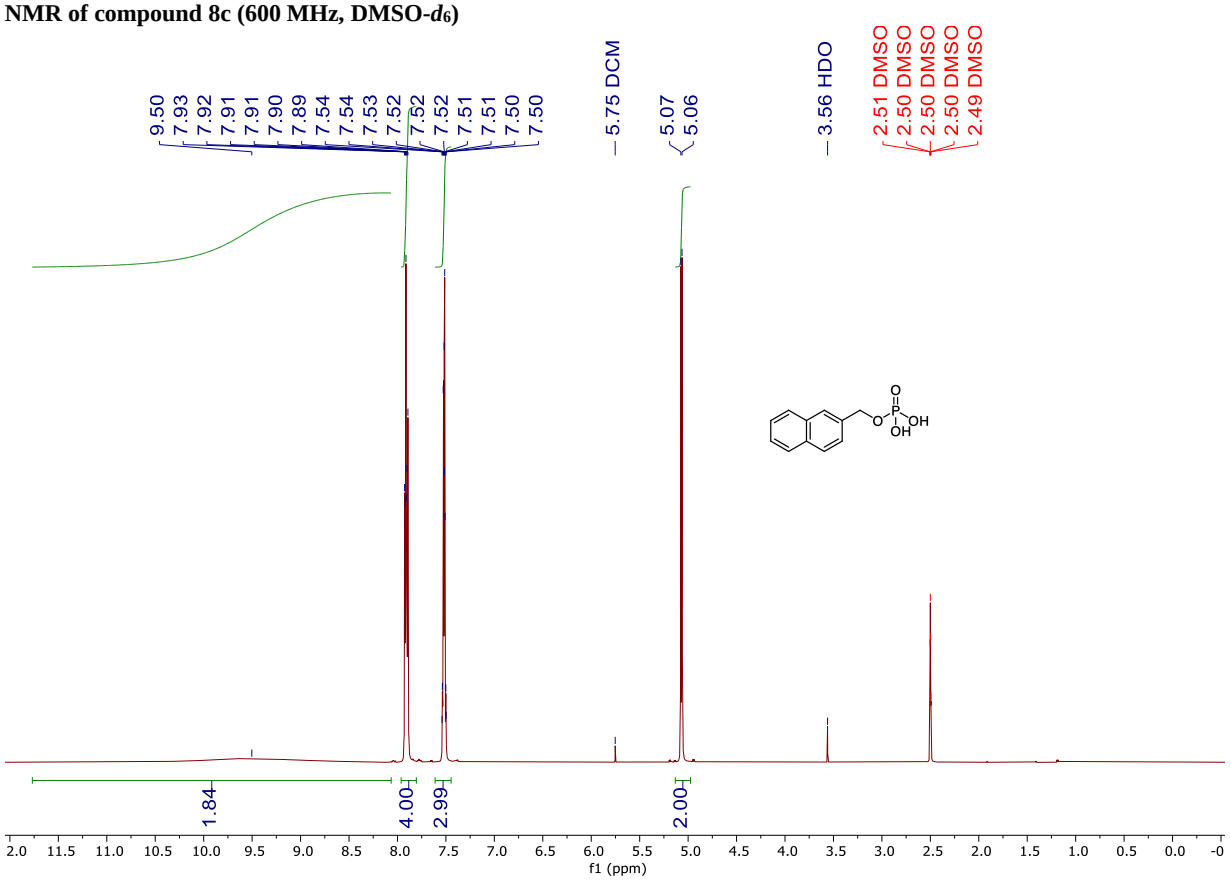
^{13}C NMR of compound S29 (150 MHz, $CDCl_3$)



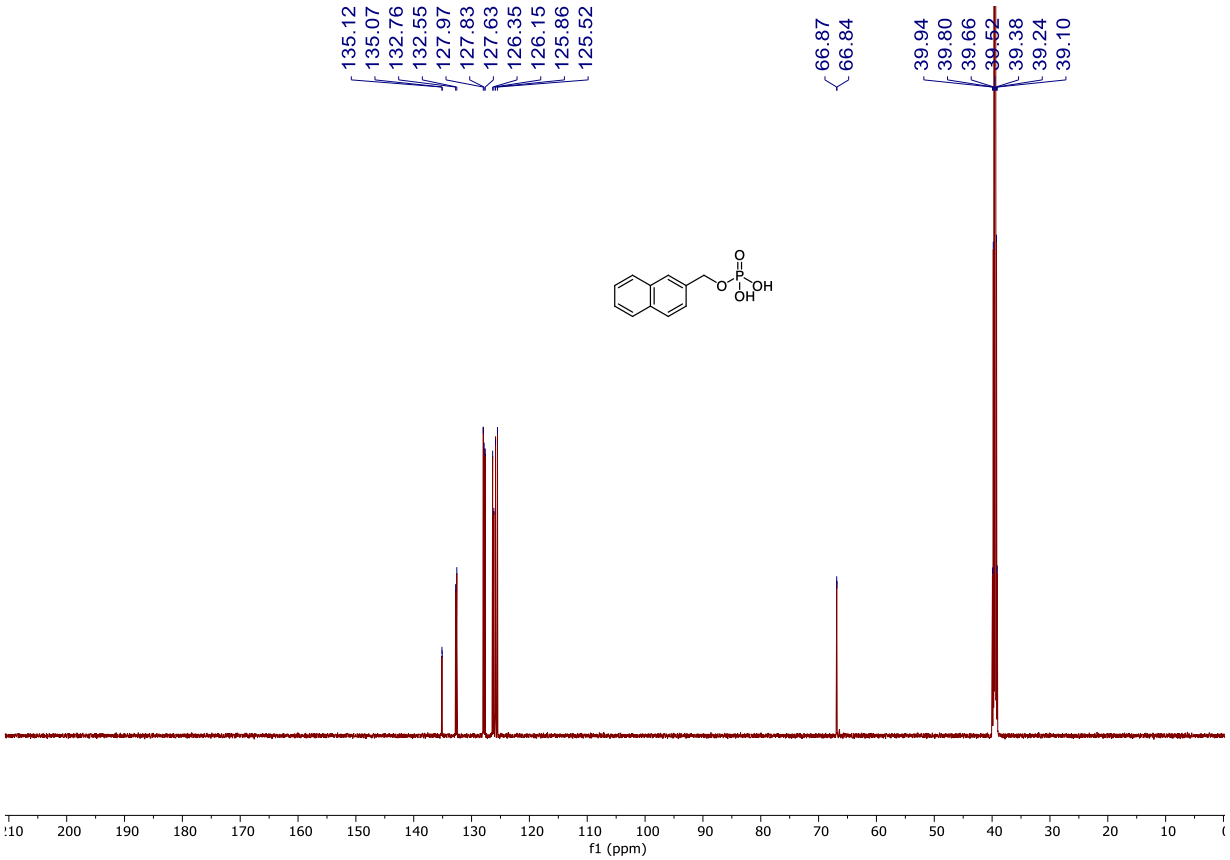
^{31}P NMR of compound S29 (162 MHz, $CDCl_3$)



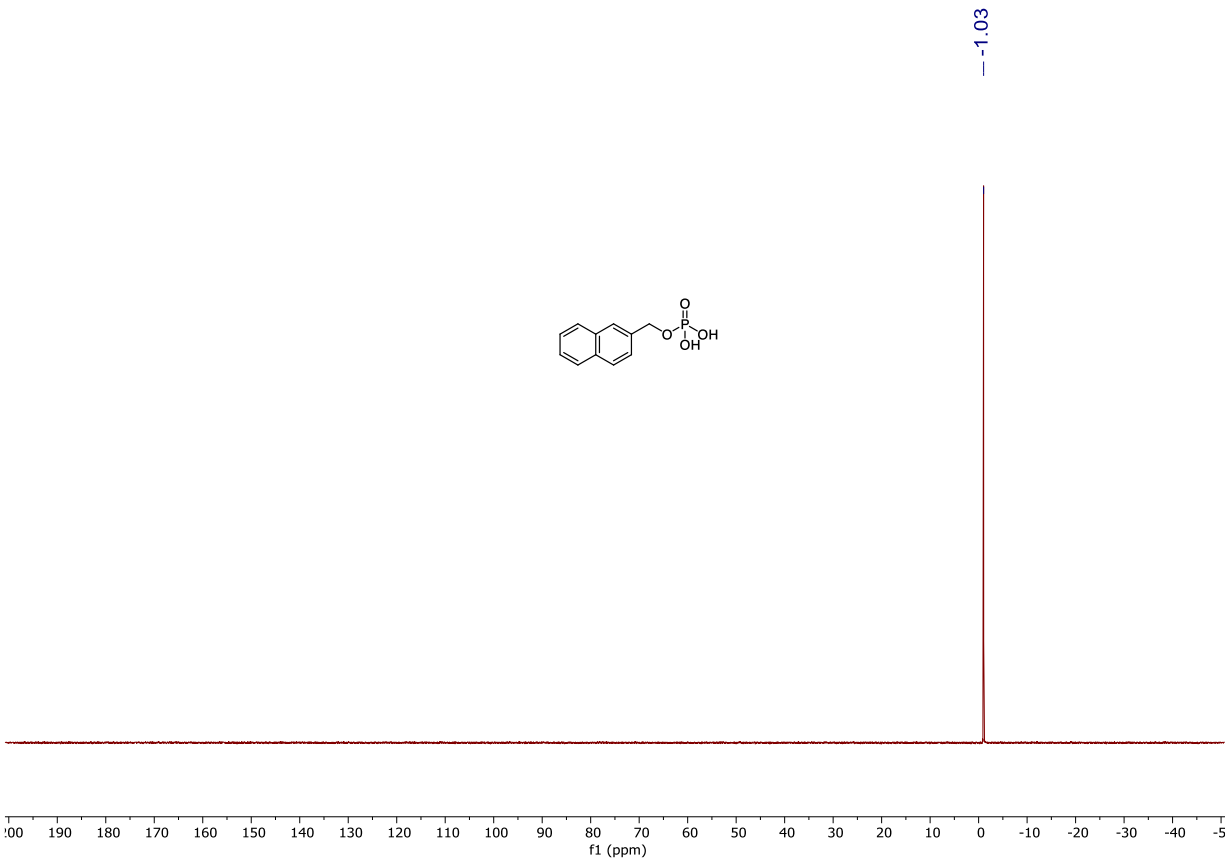
^{1}H NMR of compound 8c (600 MHz, DMSO-d_6)



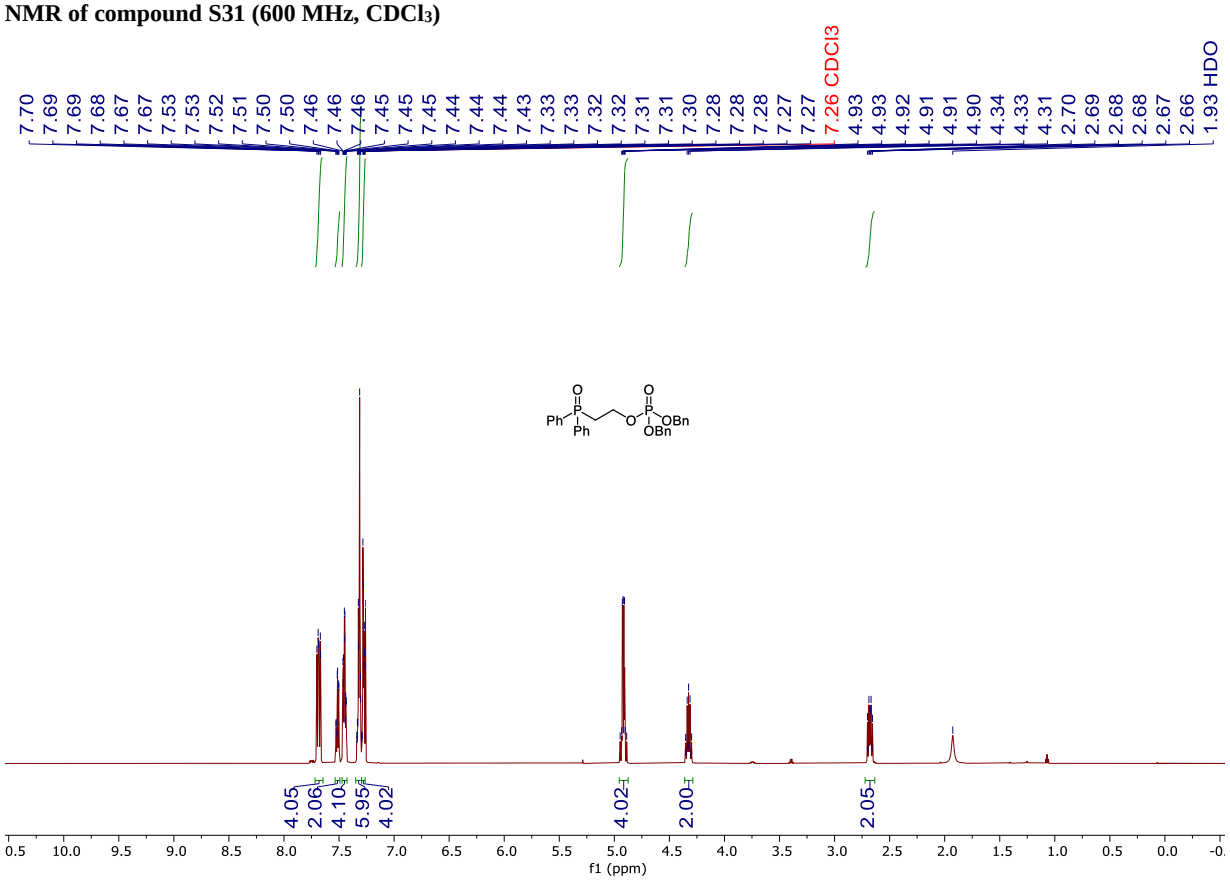
^{13}C NMR of compound 8c (150 MHz, DMSO-d_6)



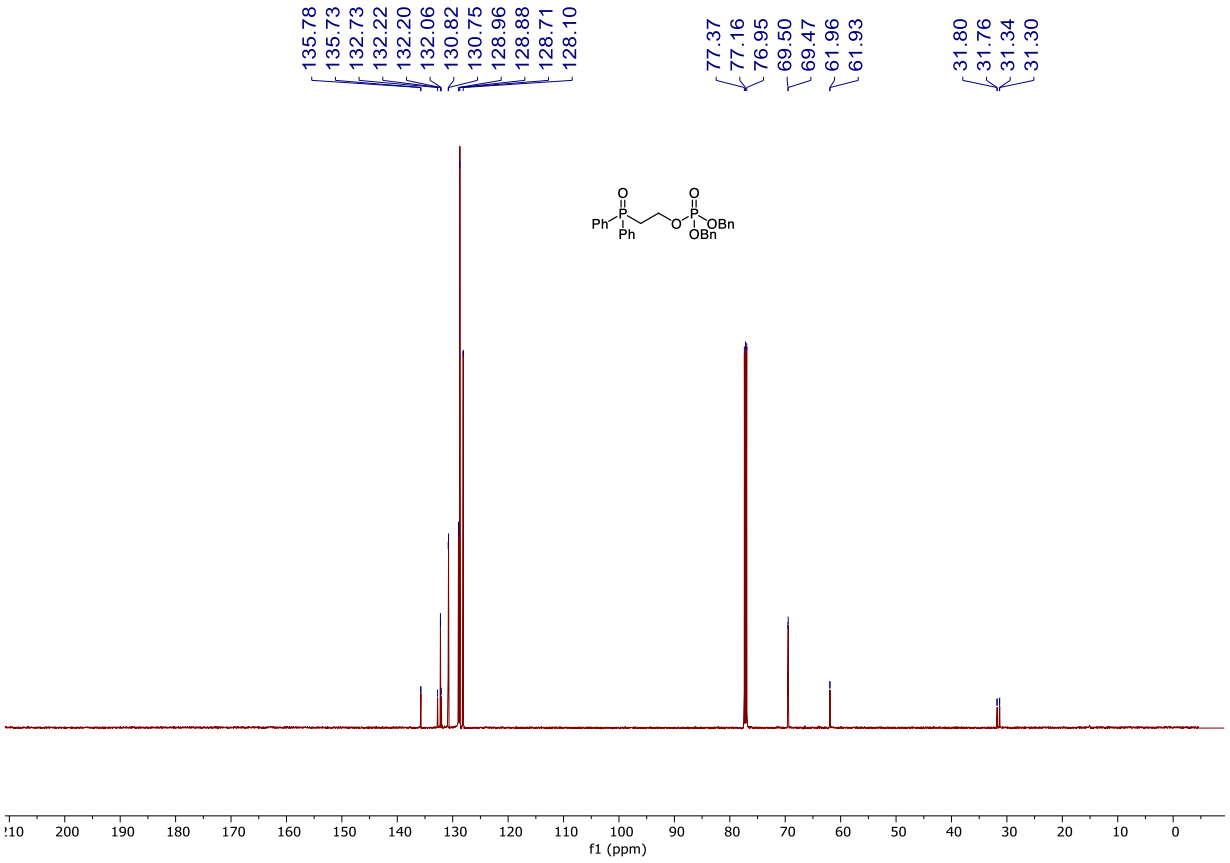
^{31}P NMR of compound 8c (162 MHz, DMSO-d_6)



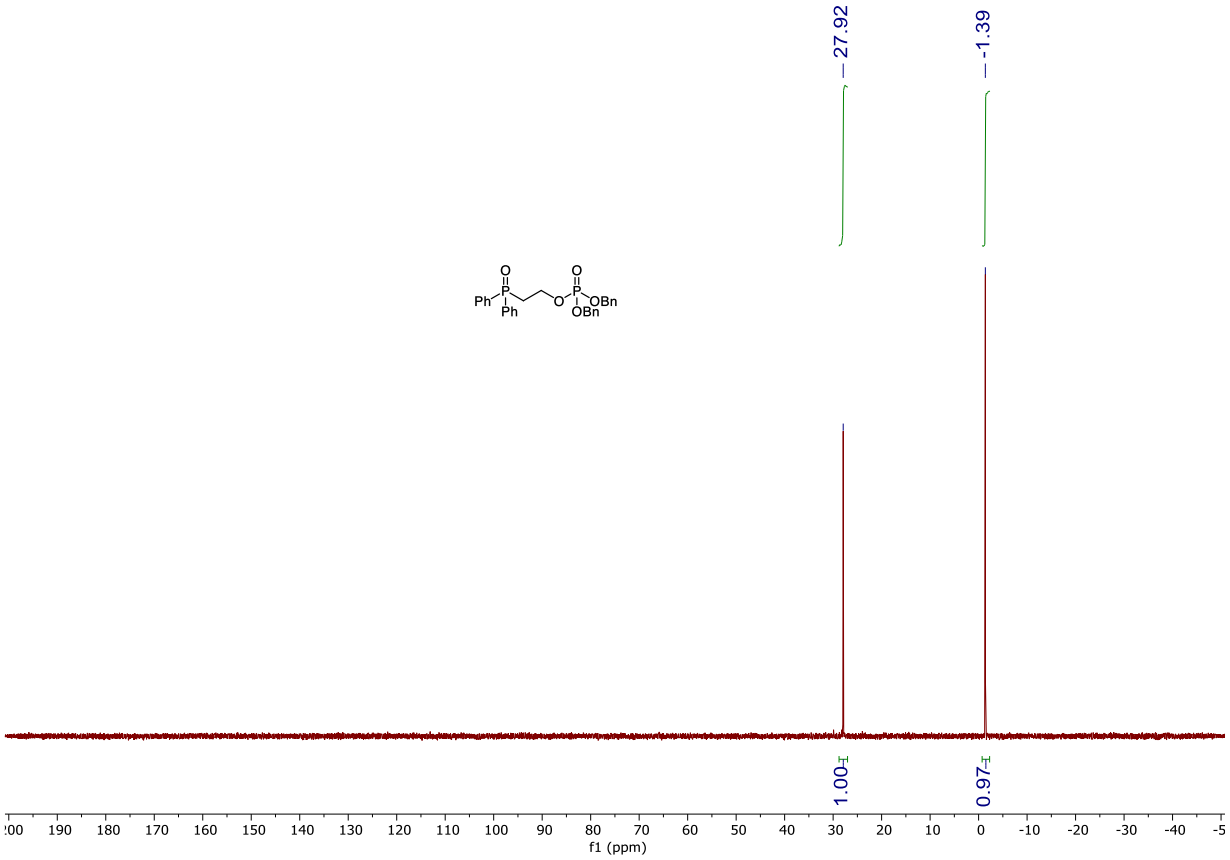
^{1}H NMR of compound S31 (600 MHz, $CDCl_3$)



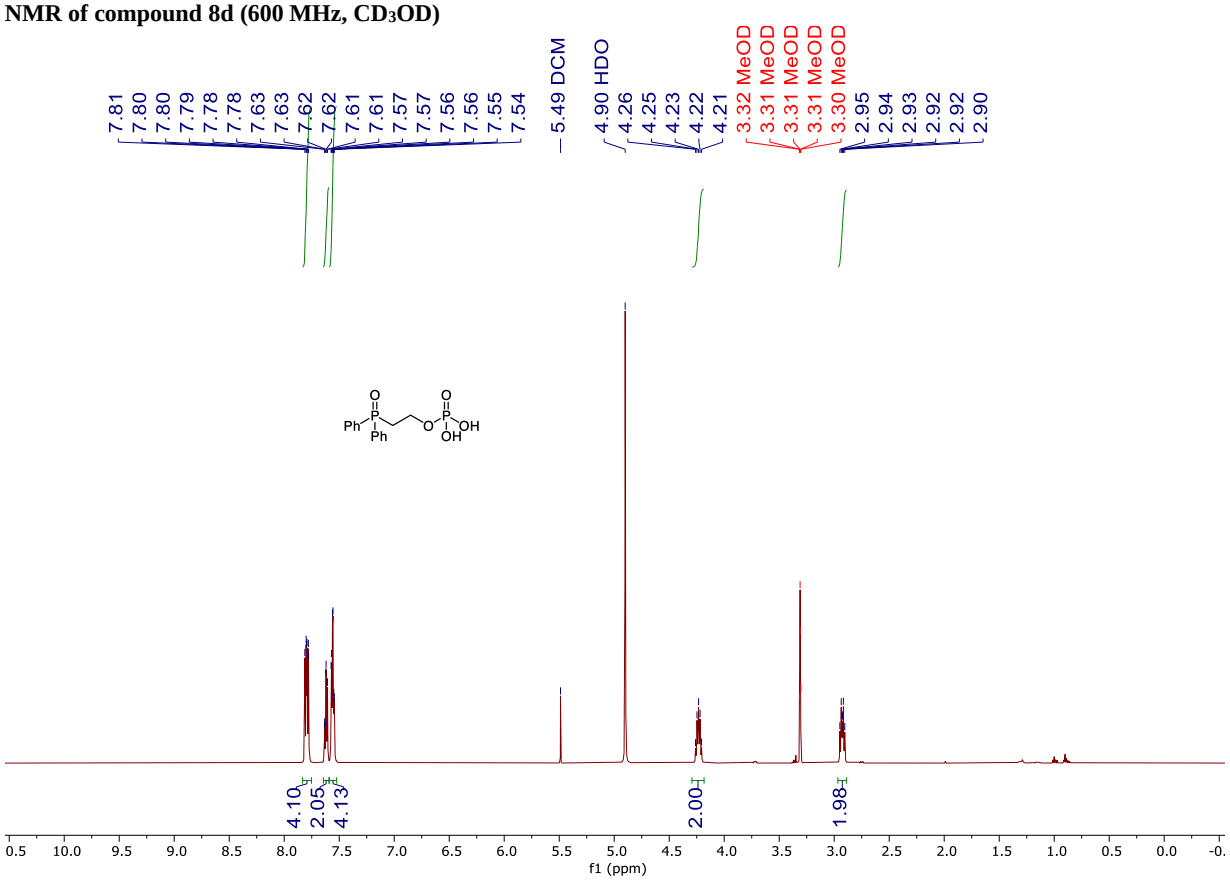
^{13}C NMR of compound S31 (150 MHz, $CDCl_3$)



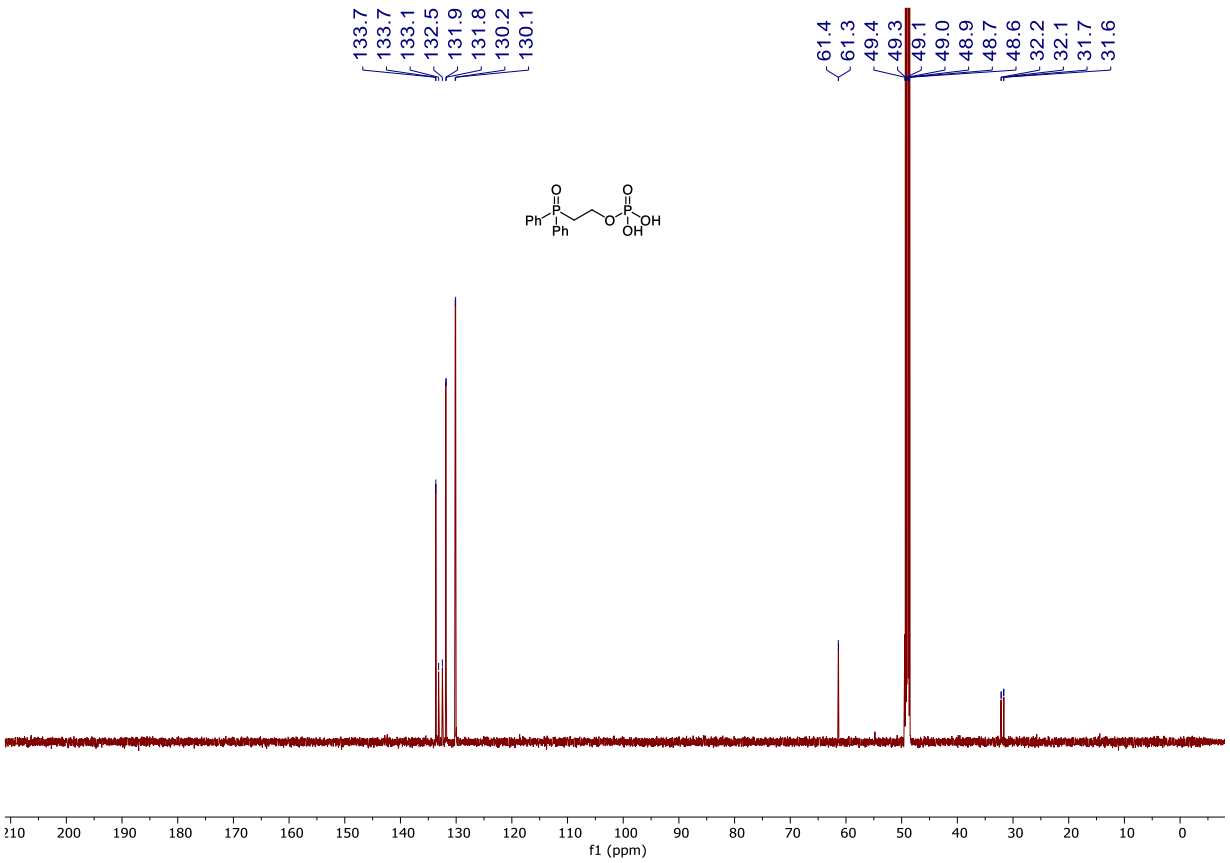
^{31}P NMR of compound S31 (162 MHz, $CDCl_3$)



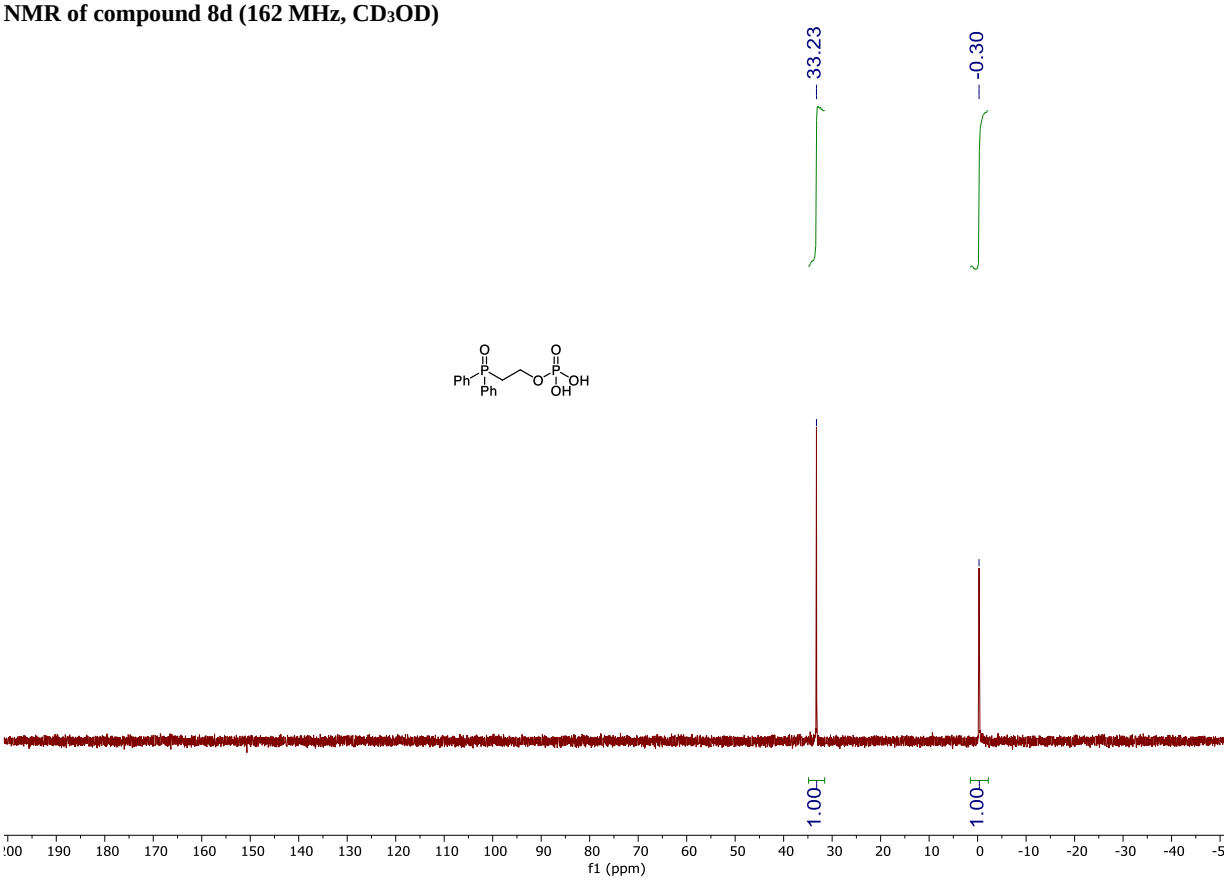
^{1}H NMR of compound 8d (600 MHz, CD_3OD)



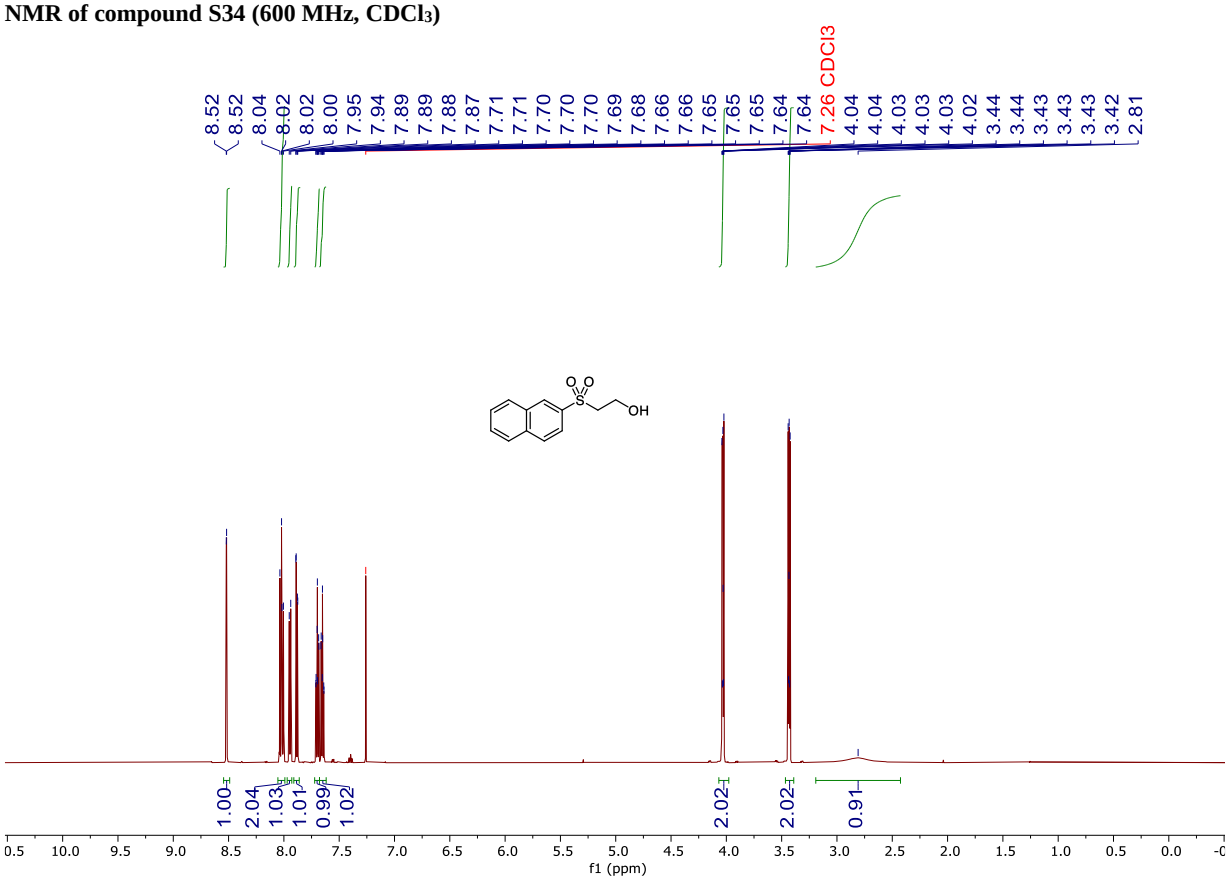
^{13}C NMR of compound 8d (150 MHz, CD_3OD)



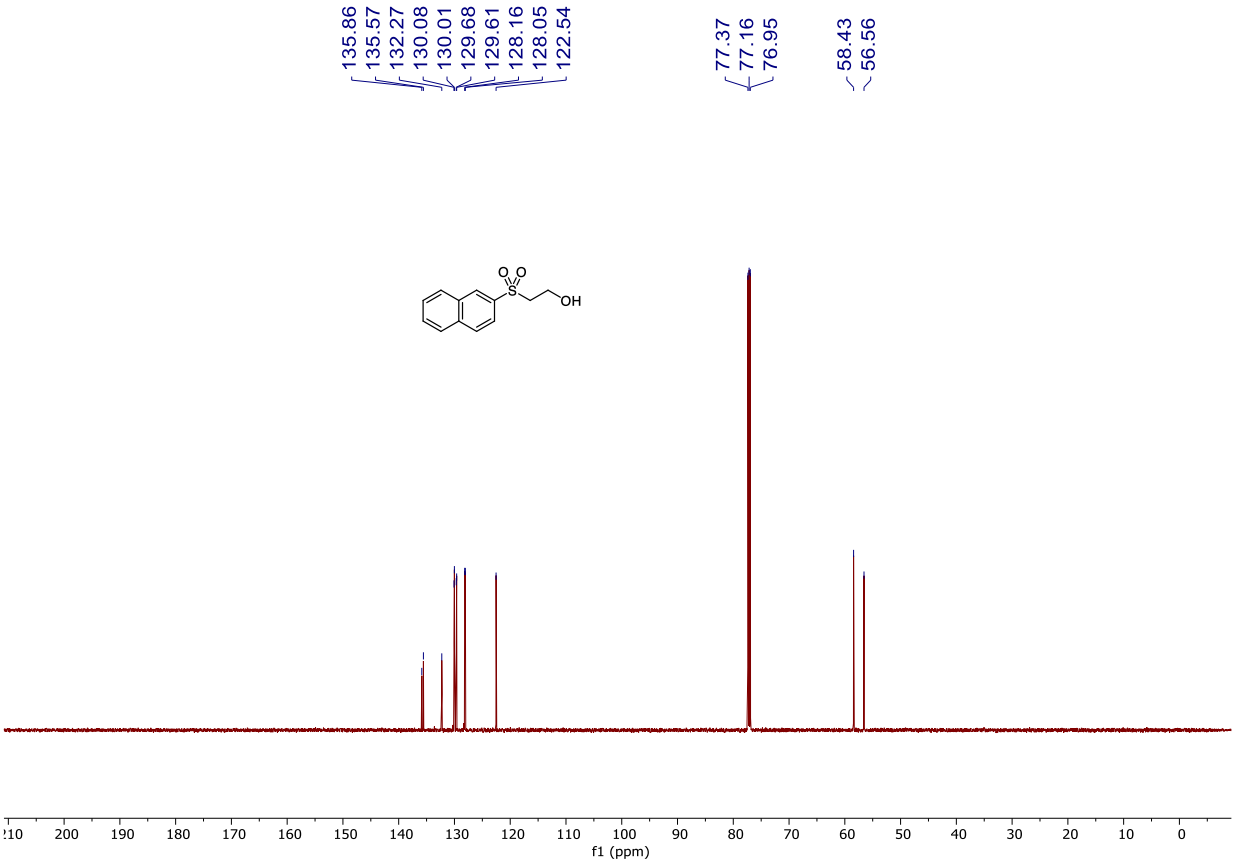
^{31}P NMR of compound 8d (162 MHz, CD_3OD)



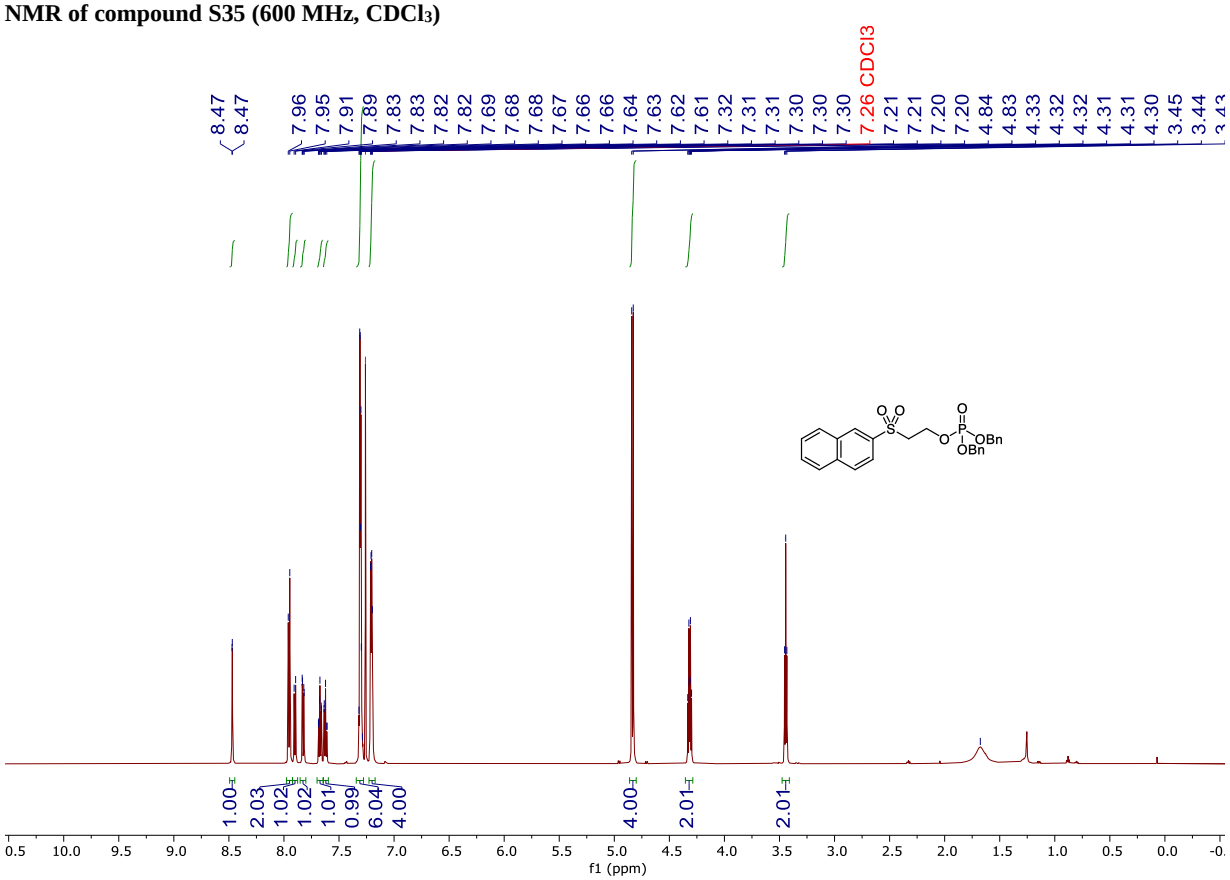
^{1}H NMR of compound S34 (600 MHz, $CDCl_3$)



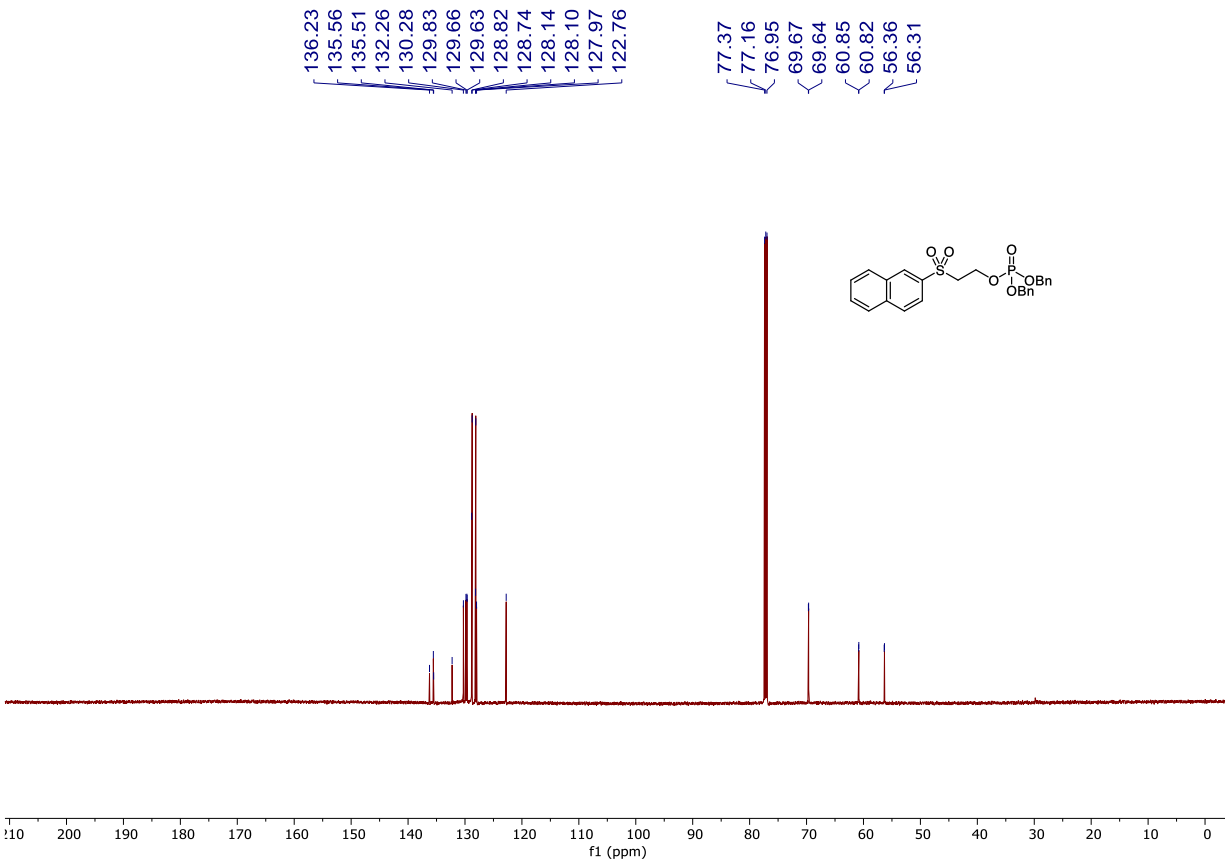
^{13}C NMR of compound S34 (150 MHz, $CDCl_3$)



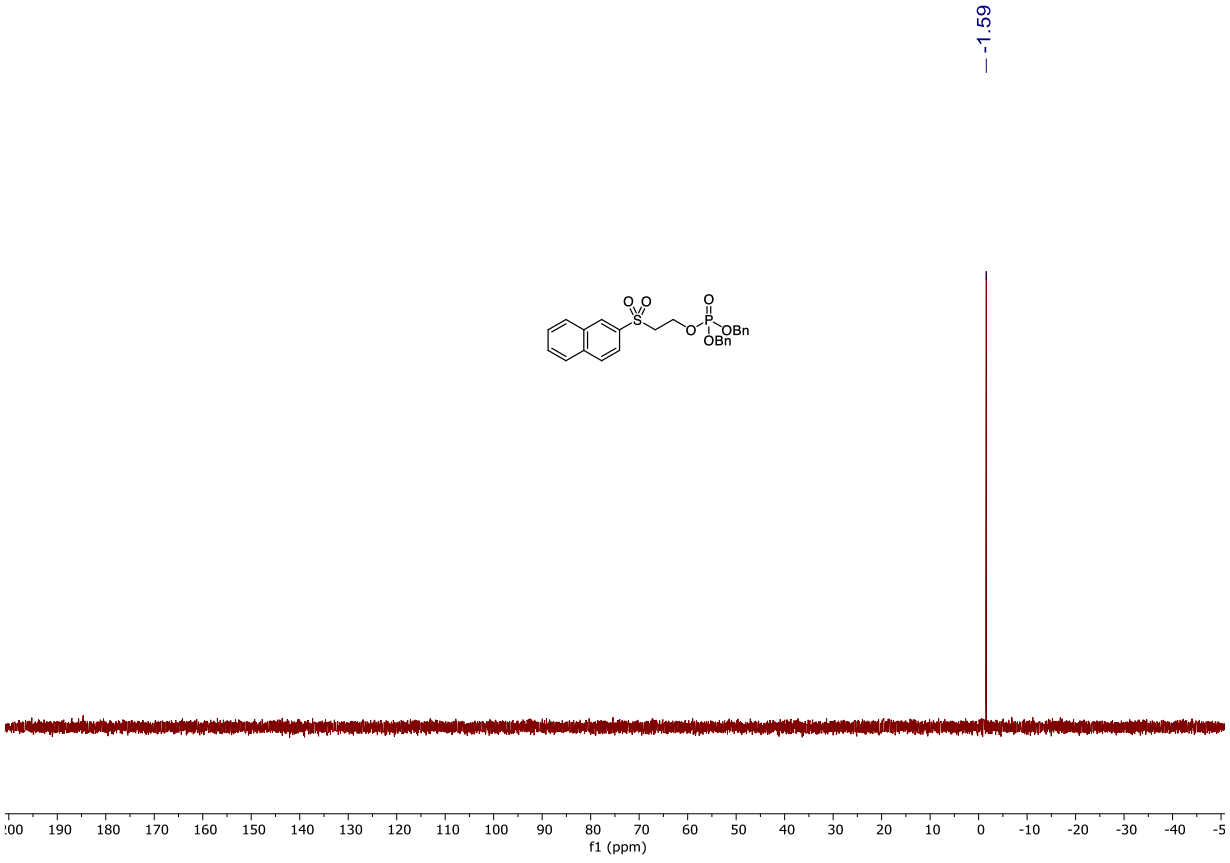
^{1}H NMR of compound S35 (600 MHz, $CDCl_3$)



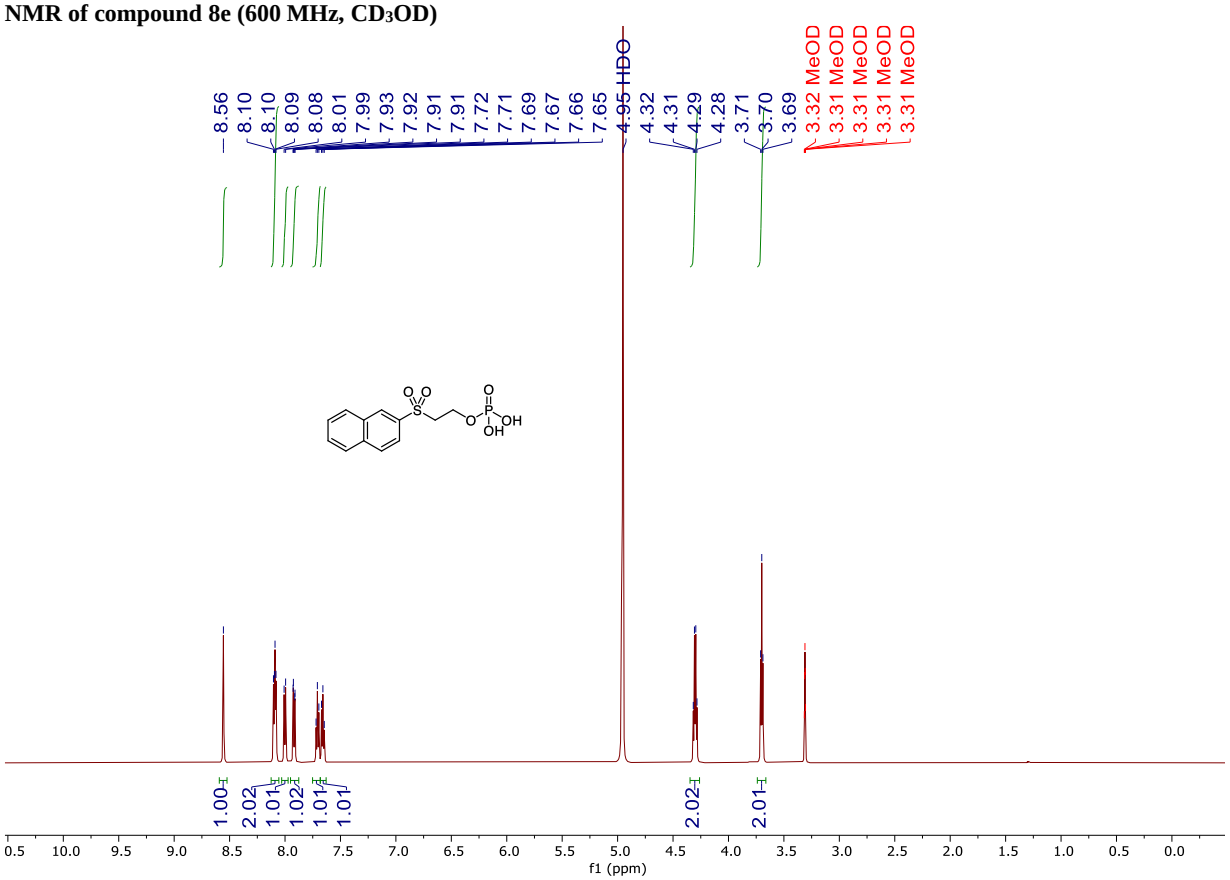
^{13}C NMR of compound S35 (150 MHz, $CDCl_3$)



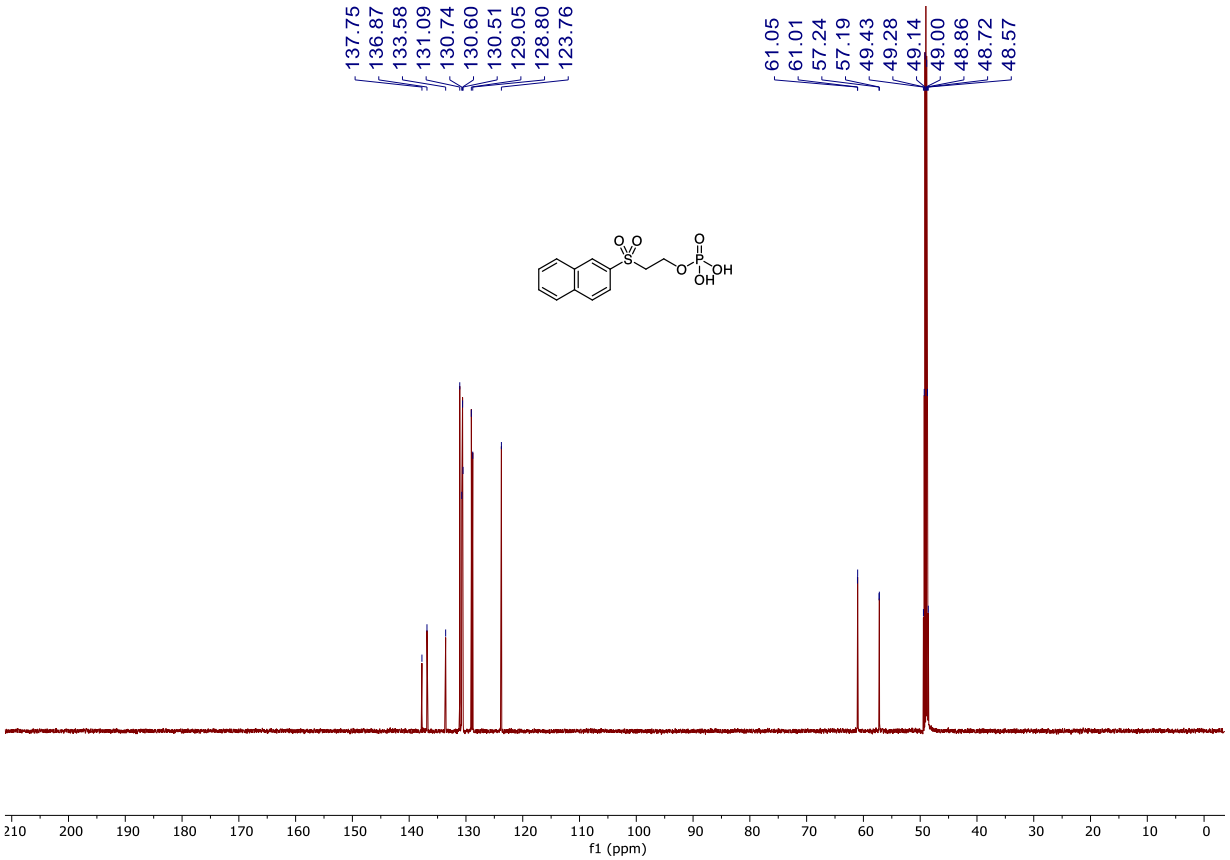
^{31}P NMR of compound S35 (162 MHz, $CDCl_3$)



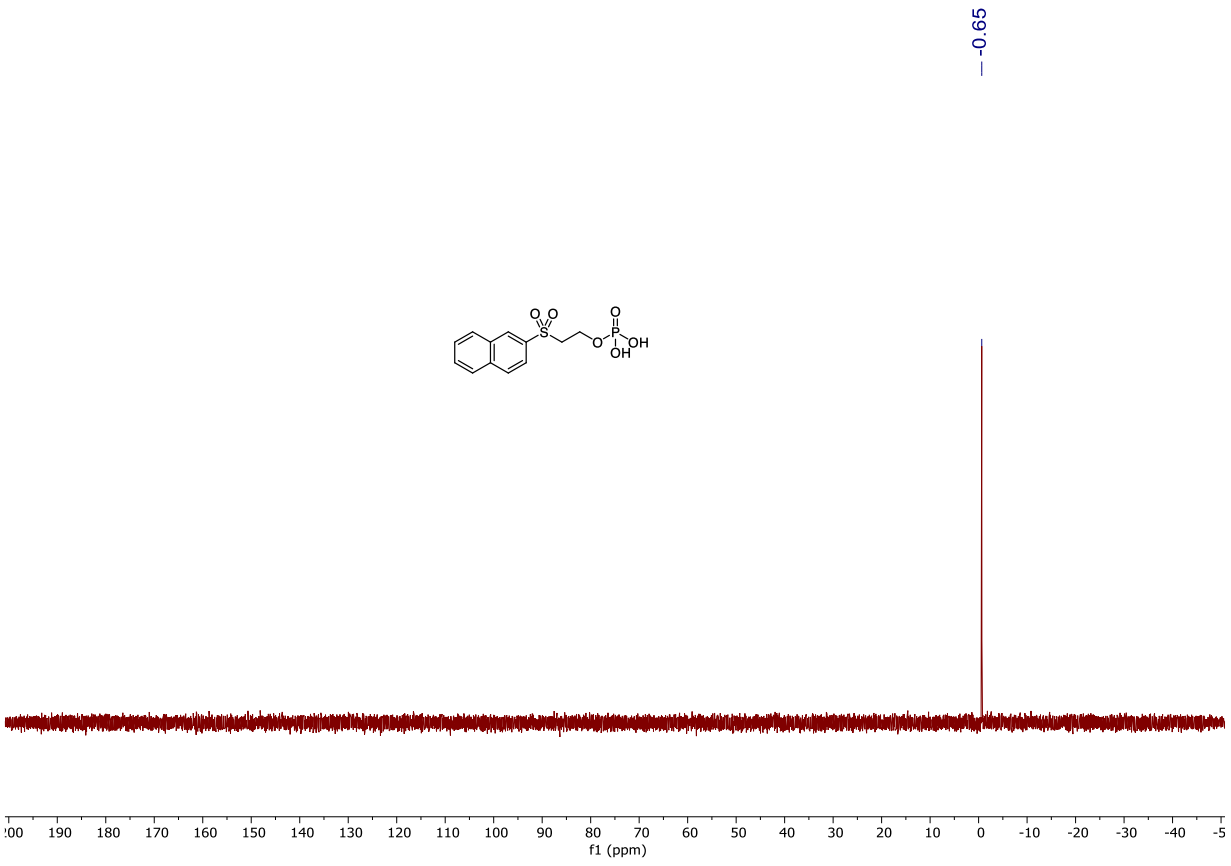
^{1}H NMR of compound 8e (600 MHz, CD_3OD)



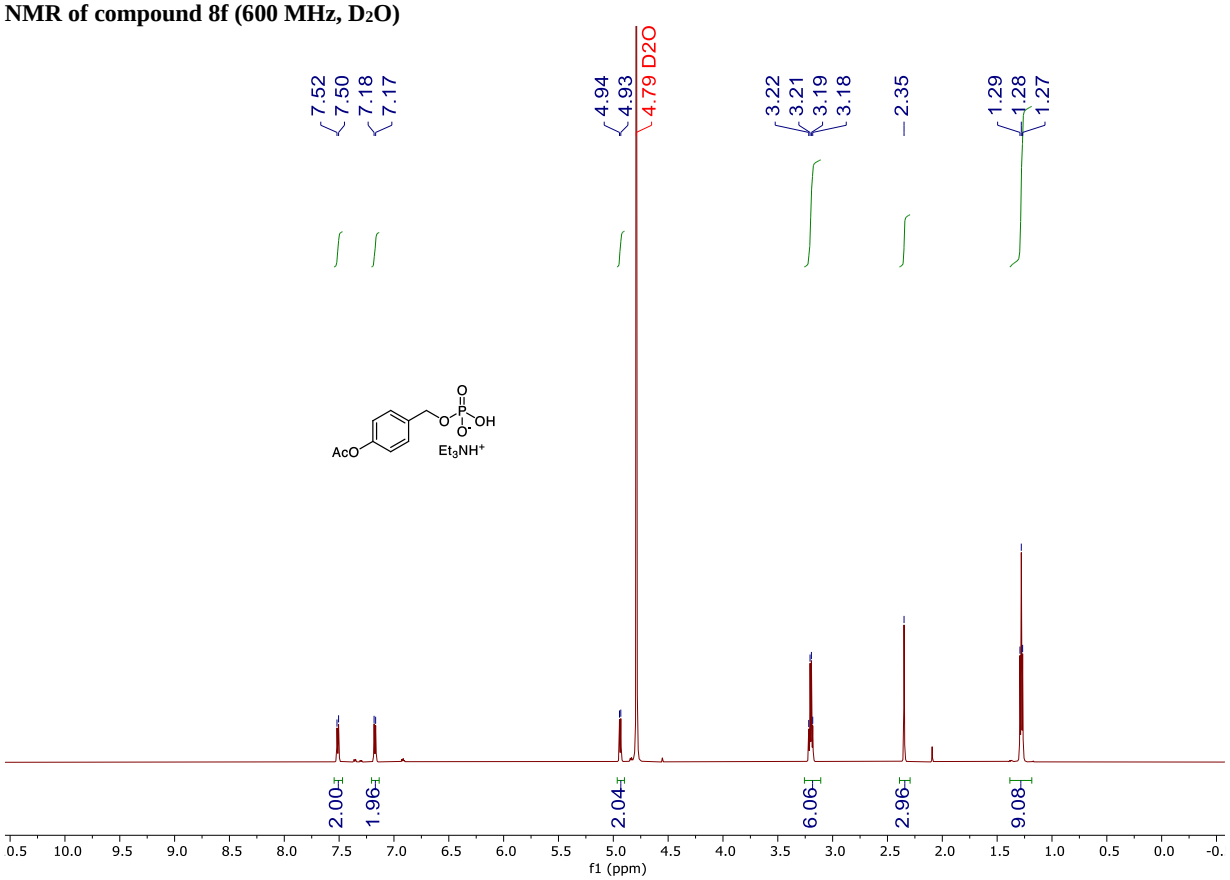
^{13}C NMR of compound 8e (150 MHz, CD_3OD)



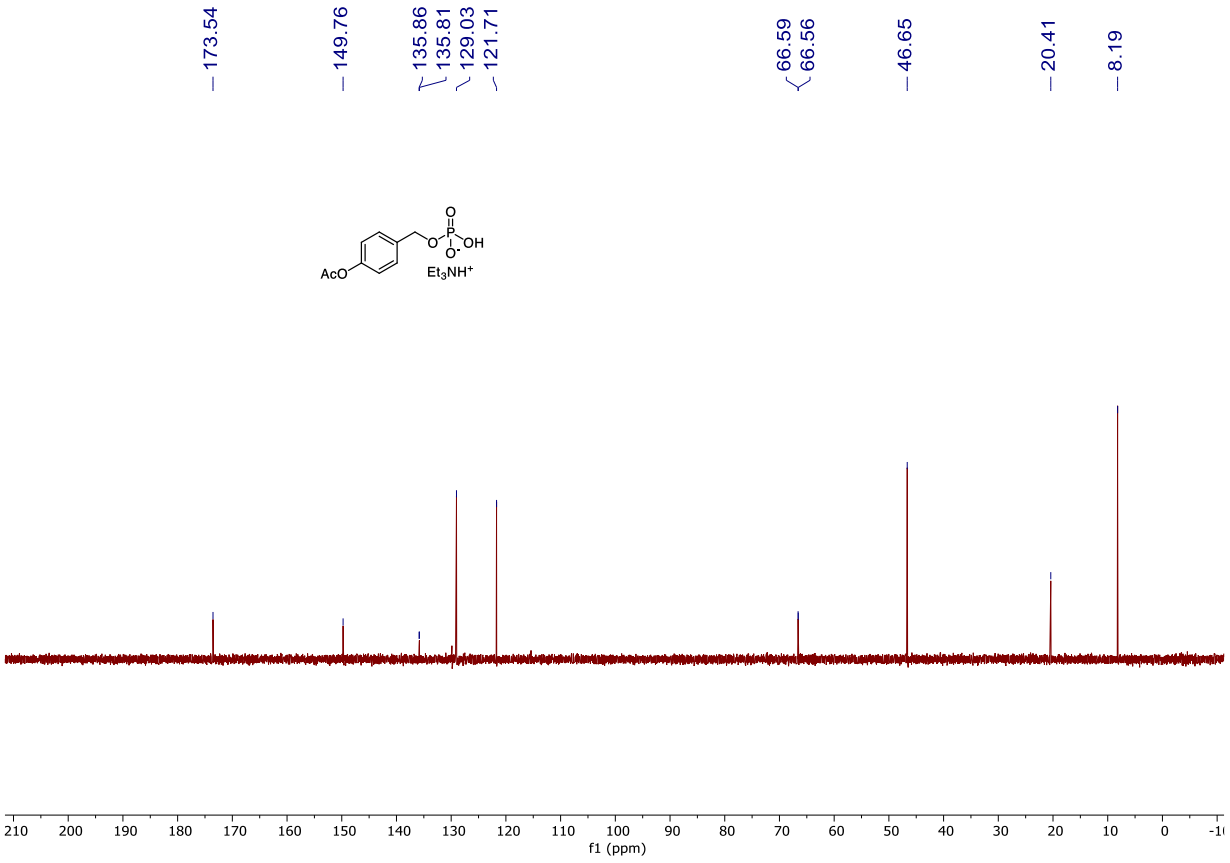
^{31}P NMR of compound 8e (162 MHz, CD_3OD)



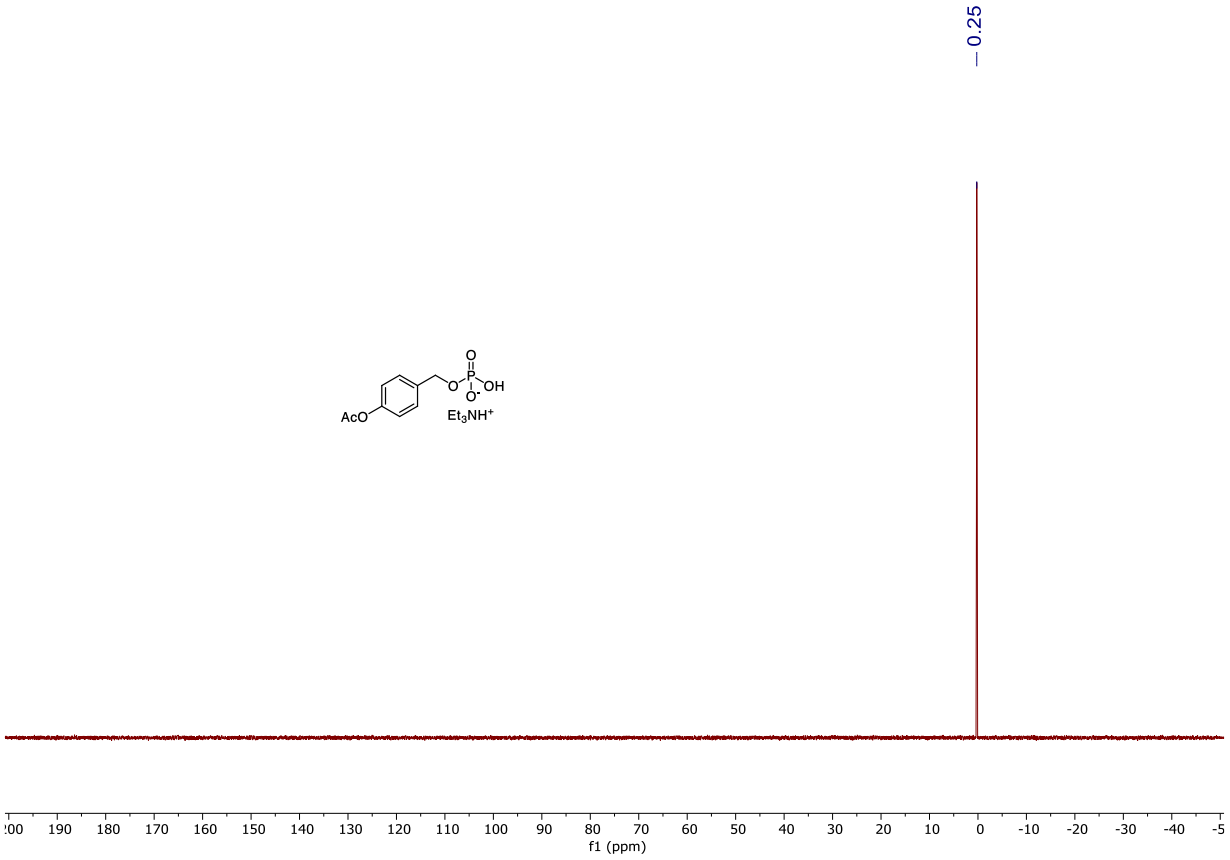
^{1}H NMR of compound 8f (600 MHz, D_2O)



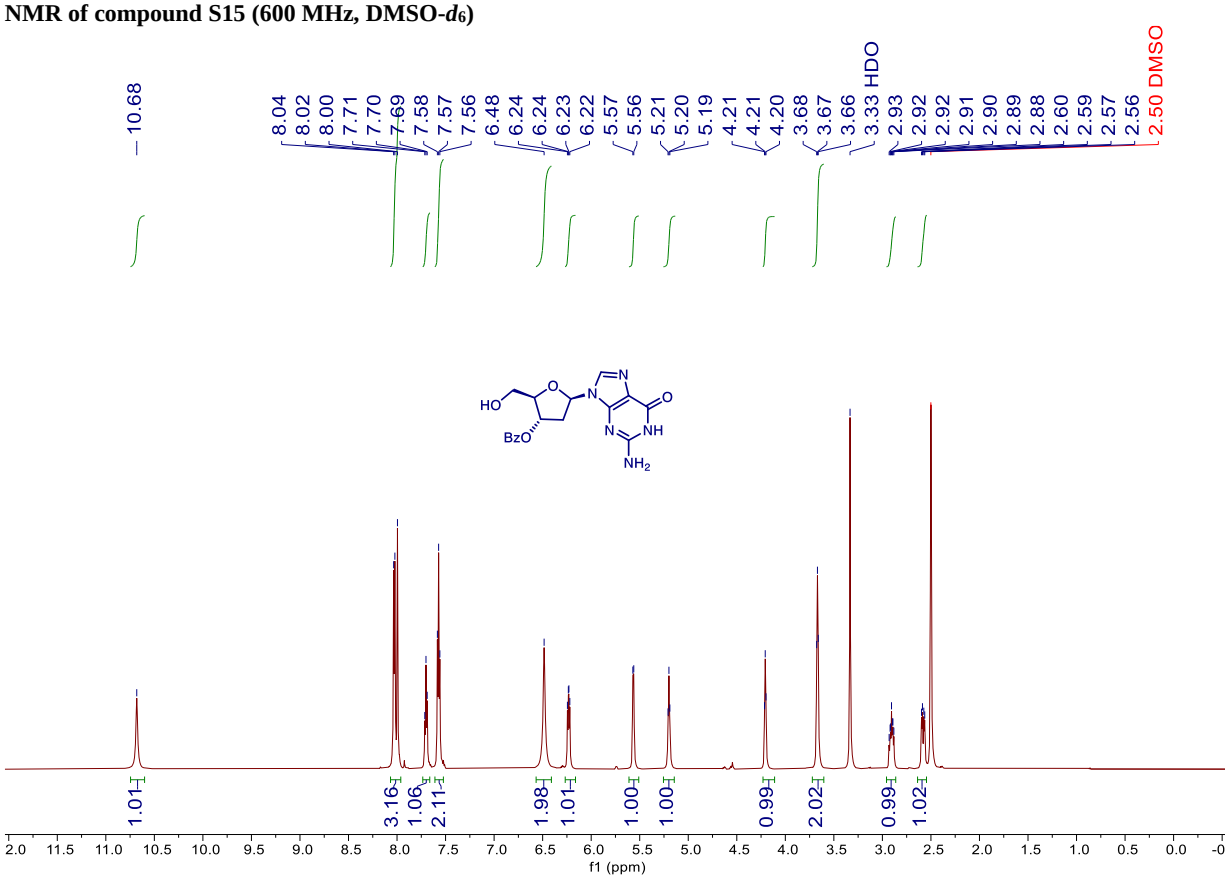
^{13}C NMR of compound 8f (150 MHz, D_2O)



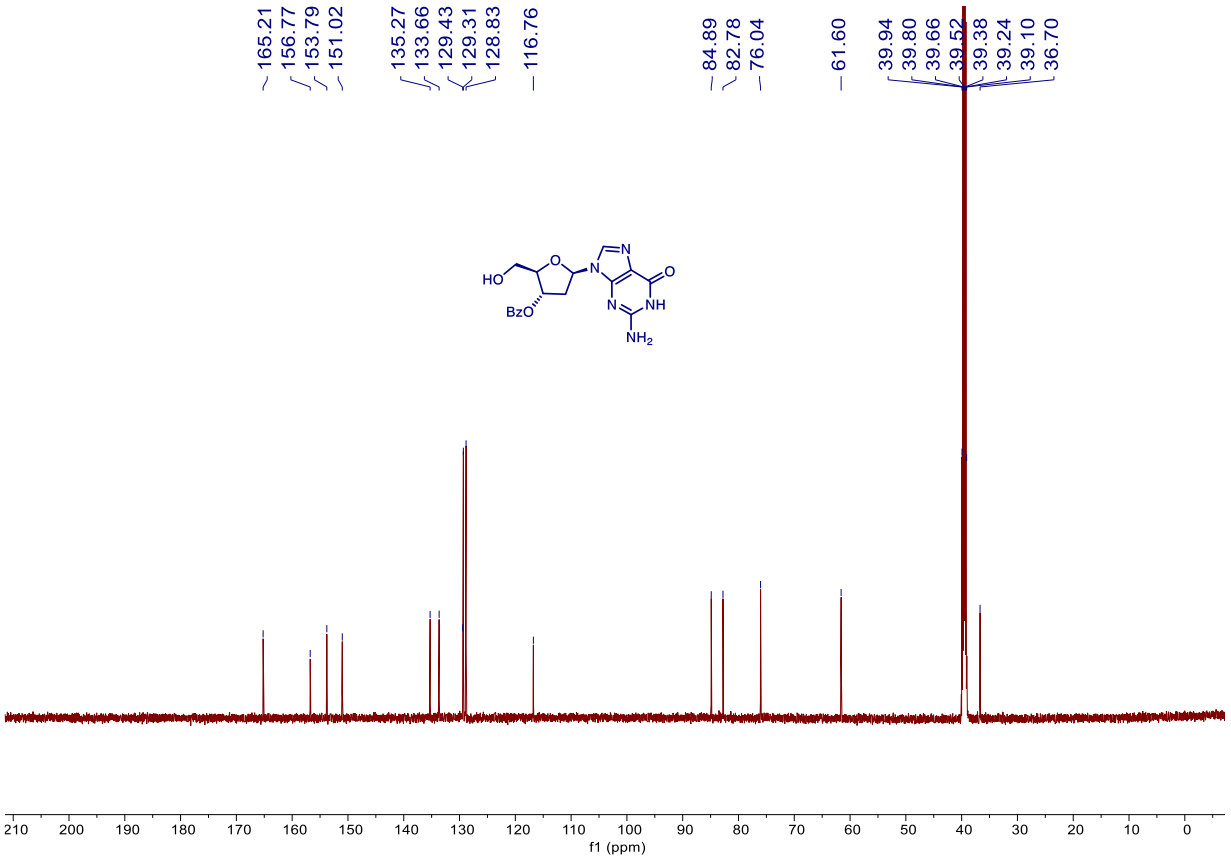
^{31}P NMR of compound 8f (162 MHz, D_2O)



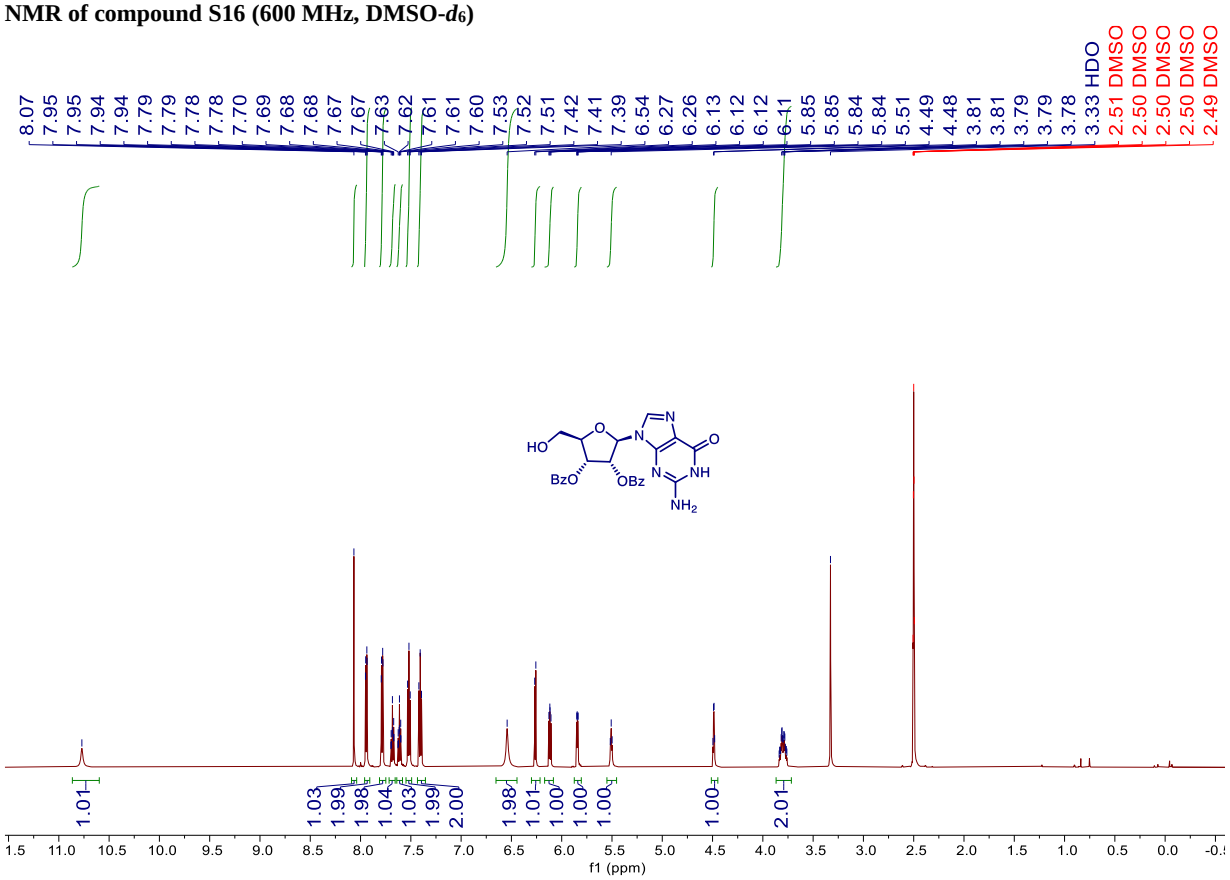
^{1}H NMR of compound S15 (600 MHz, DMSO-*d*$_6$)



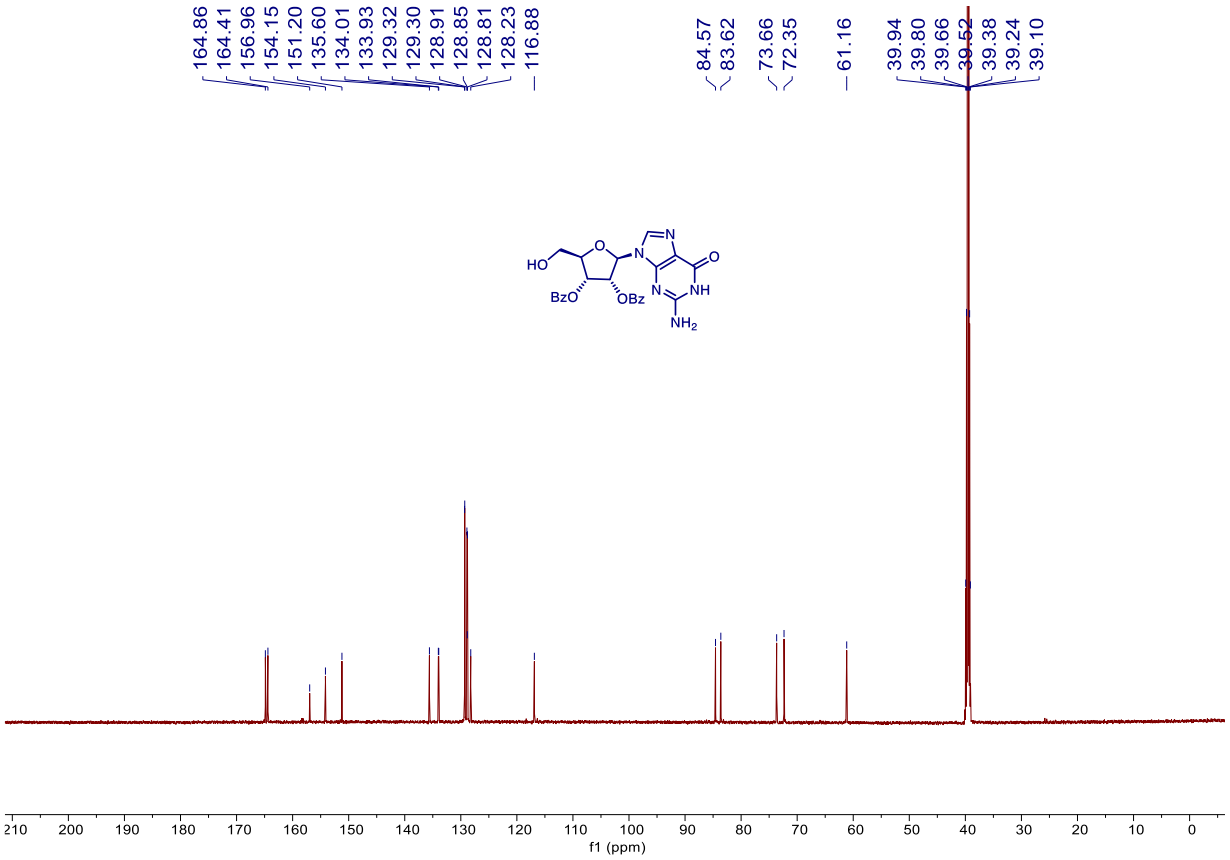
^{13}C NMR of compound S15 (150 MHz, DMSO-*d*$_6$)



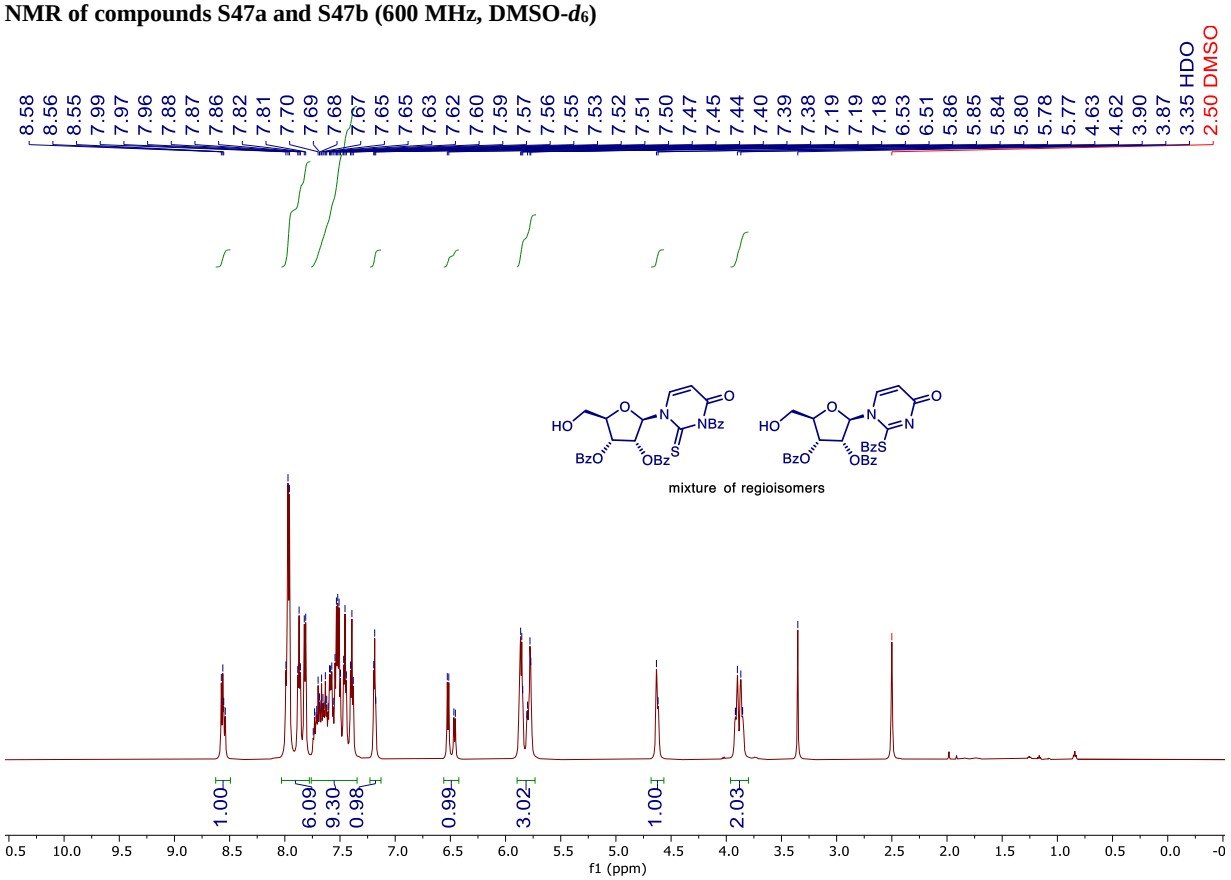
^{1}H NMR of compound S16 (600 MHz, DMSO-d_6)



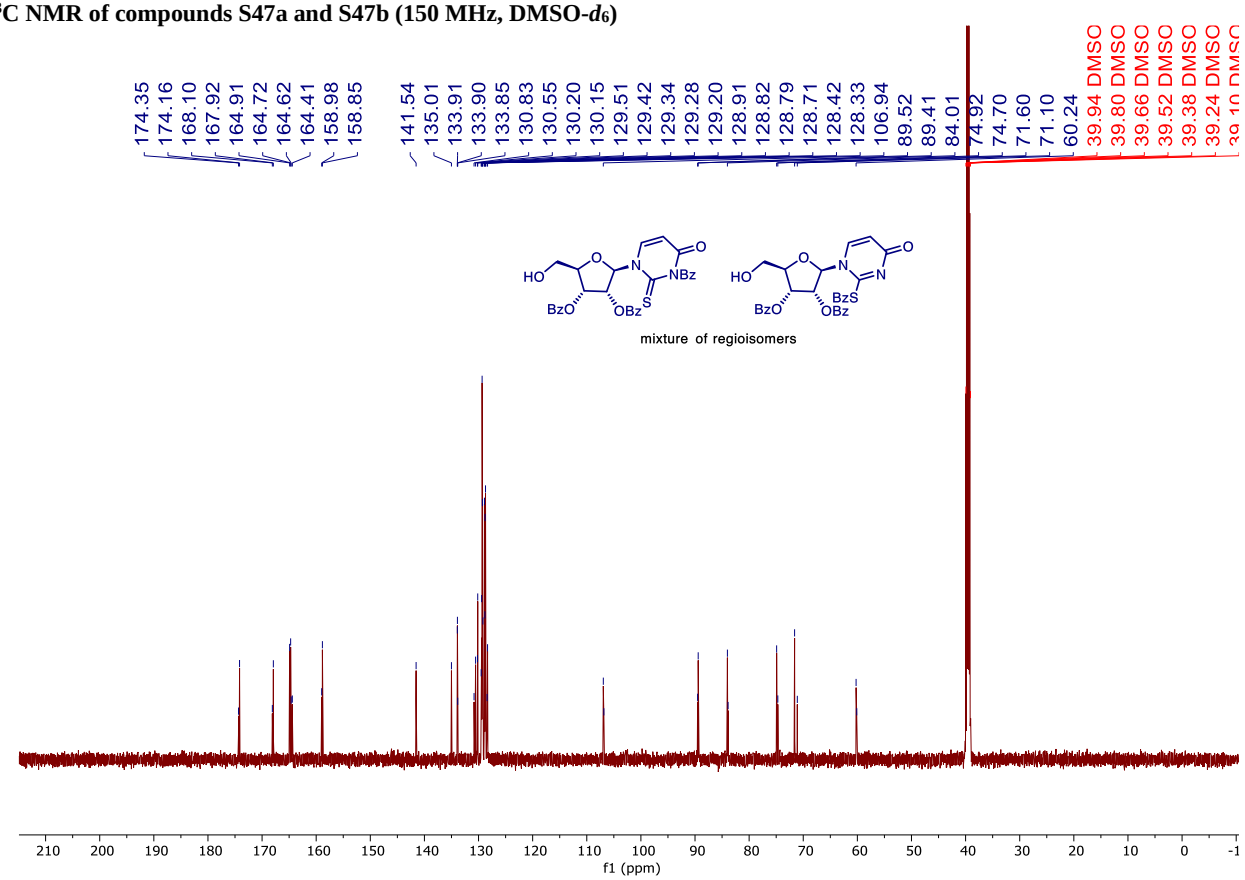
^{13}C NMR of compound S16 (150 MHz, DMSO-d_6)



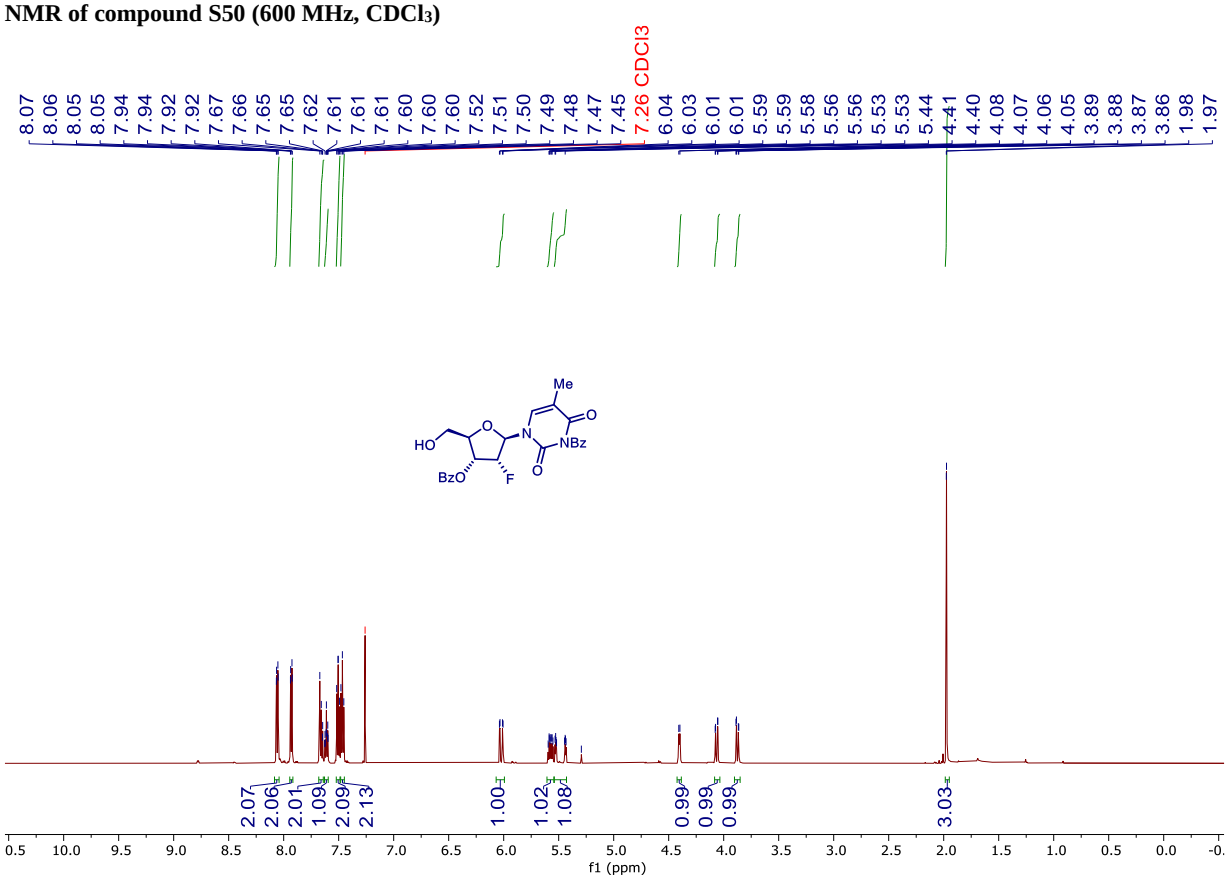
^{1}H NMR of compounds S47a and S47b (600 MHz, DMSO-d_6)



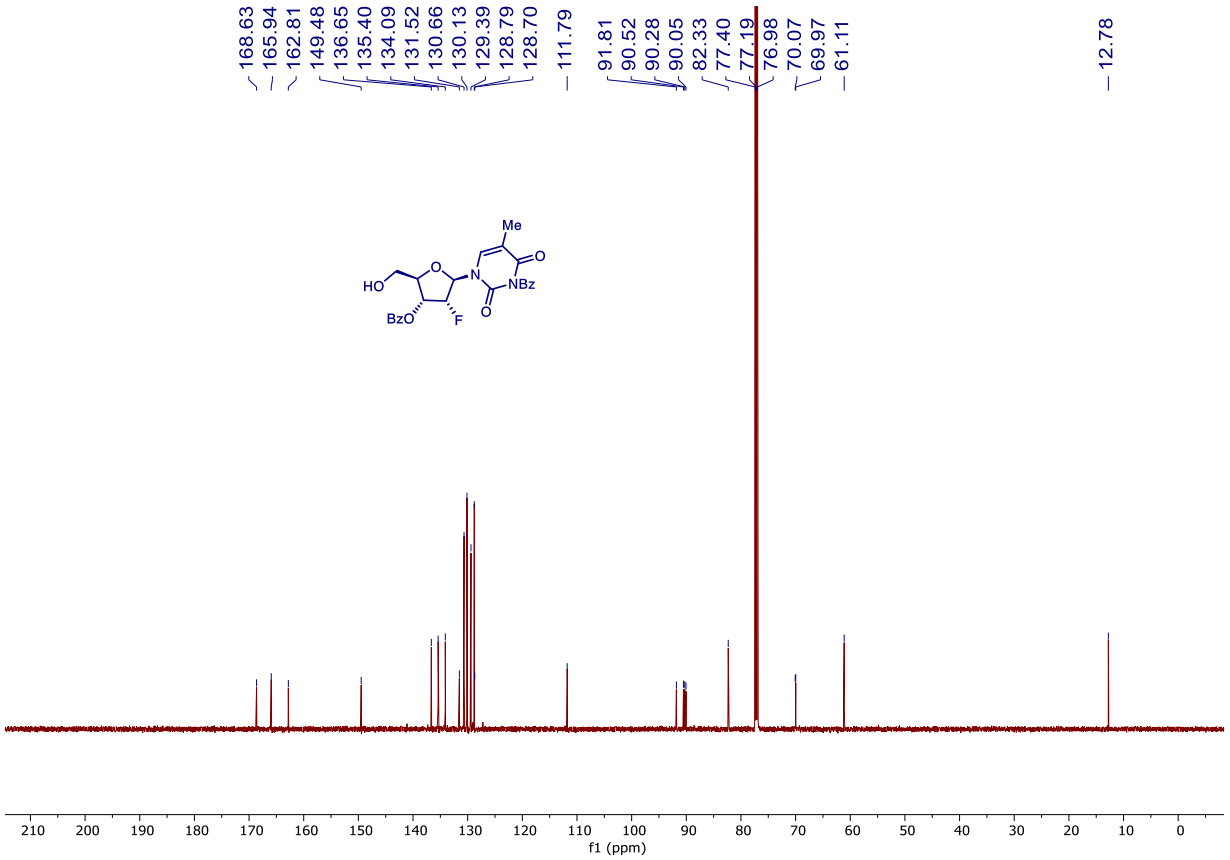
^{13}C NMR of compounds S47a and S47b (150 MHz, DMSO-d_6)



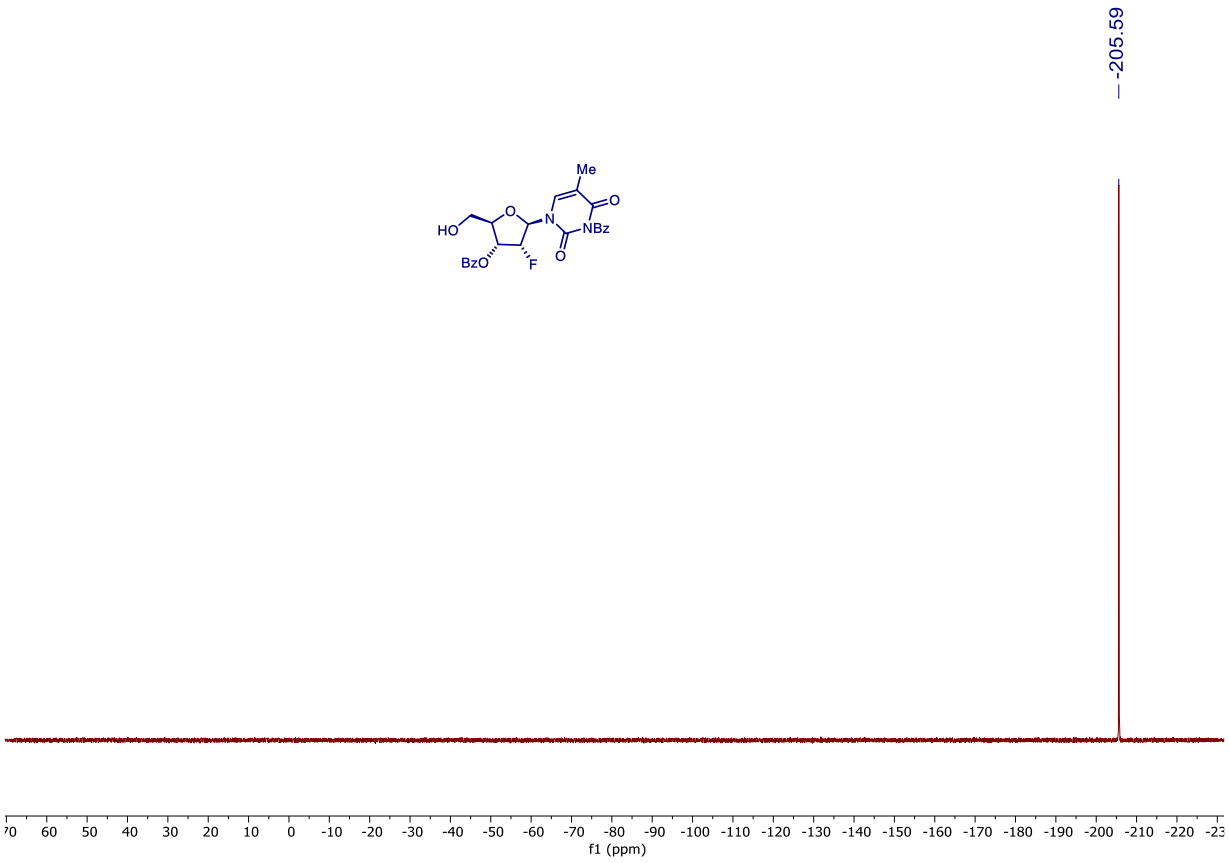
^{1}H NMR of compound S50 (600 MHz, $CDCl_3$)



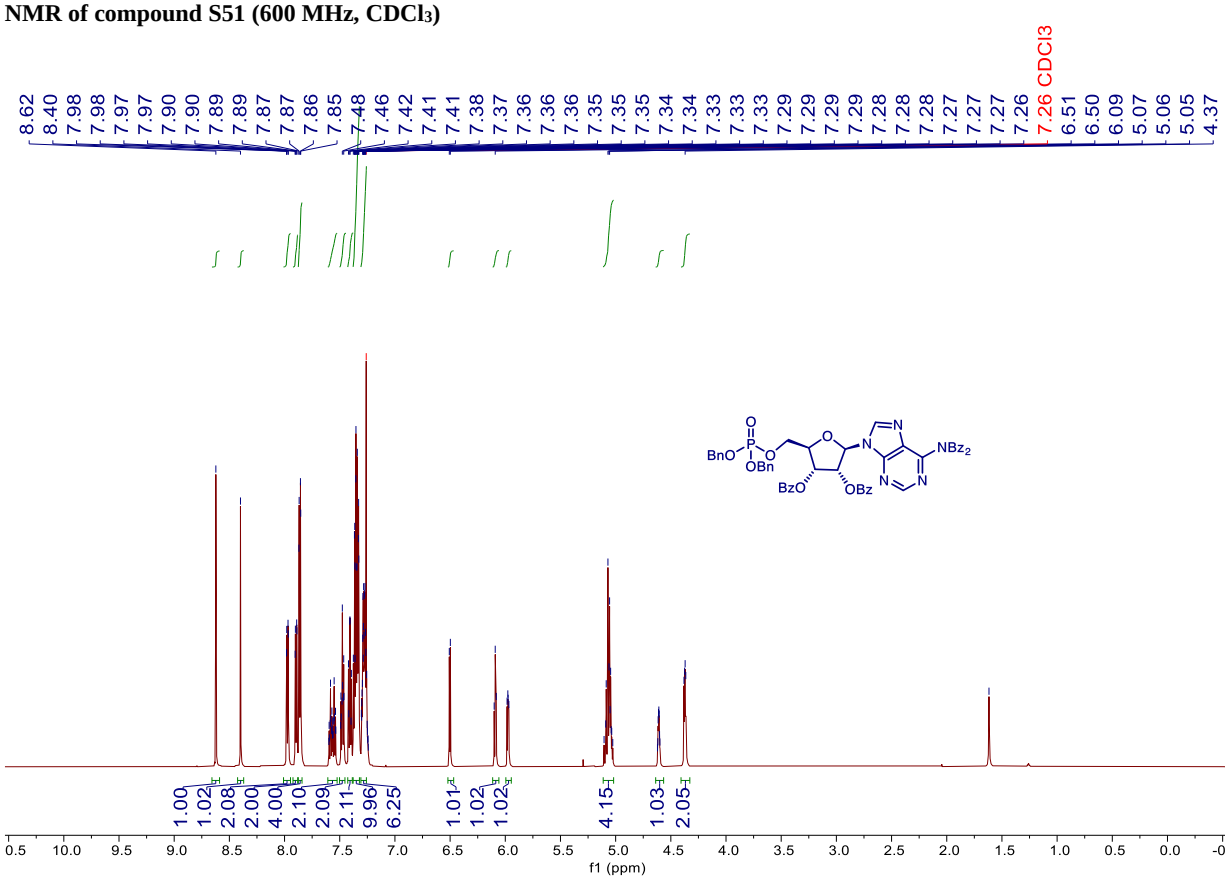
^{13}C NMR of compound S50 (150 MHz, $CDCl_3$)



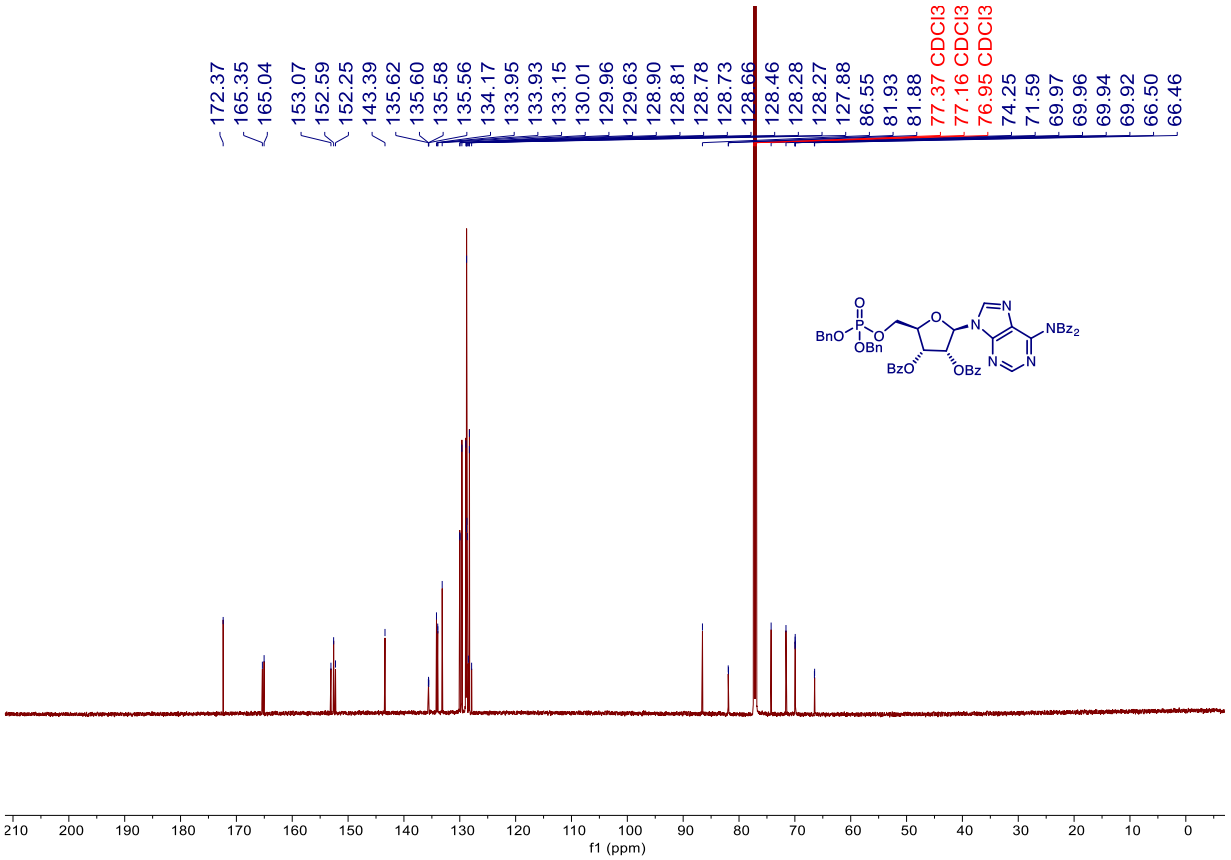
^{19}F NMR of compound S50 (376 MHz, $CDCl_3$)



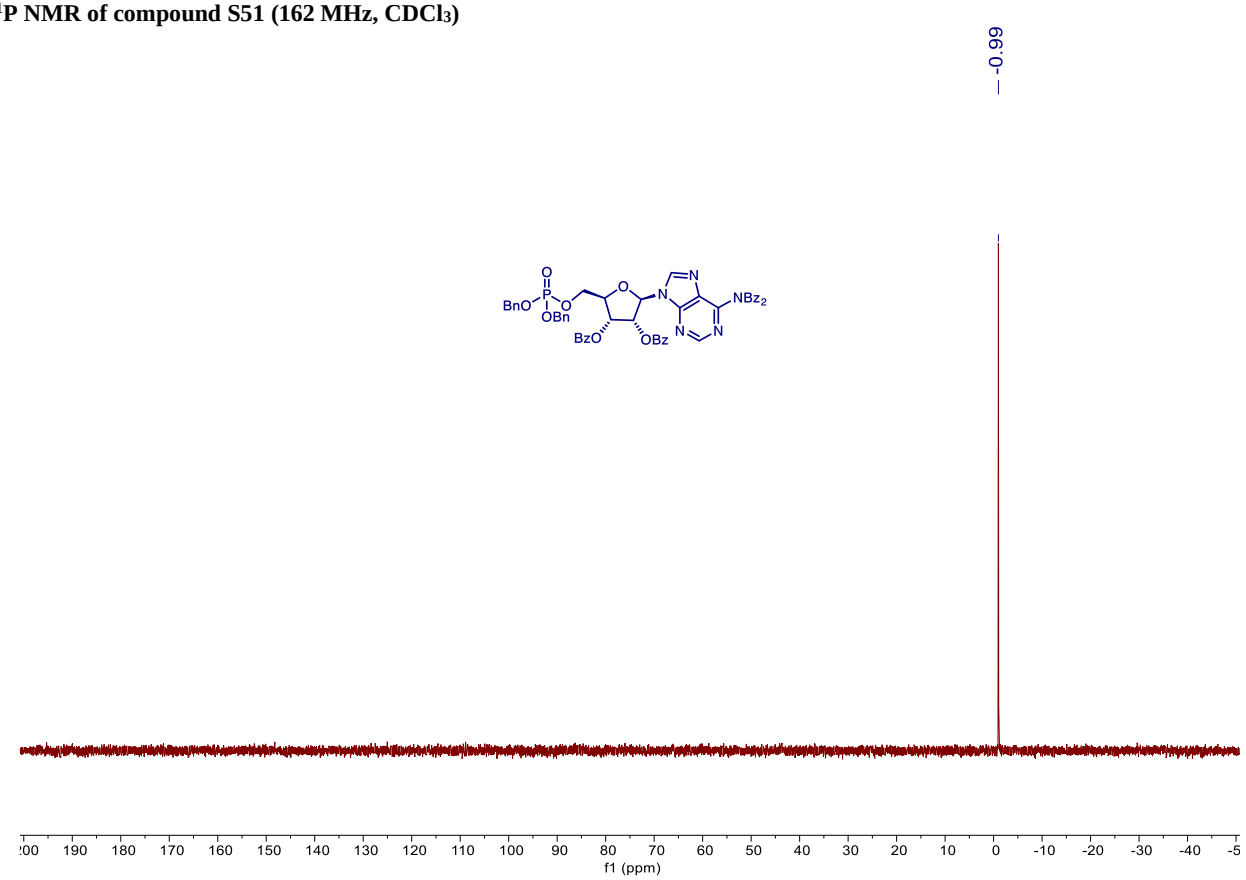
^{1}H NMR of compound S51 (600 MHz, $CDCl_3$)



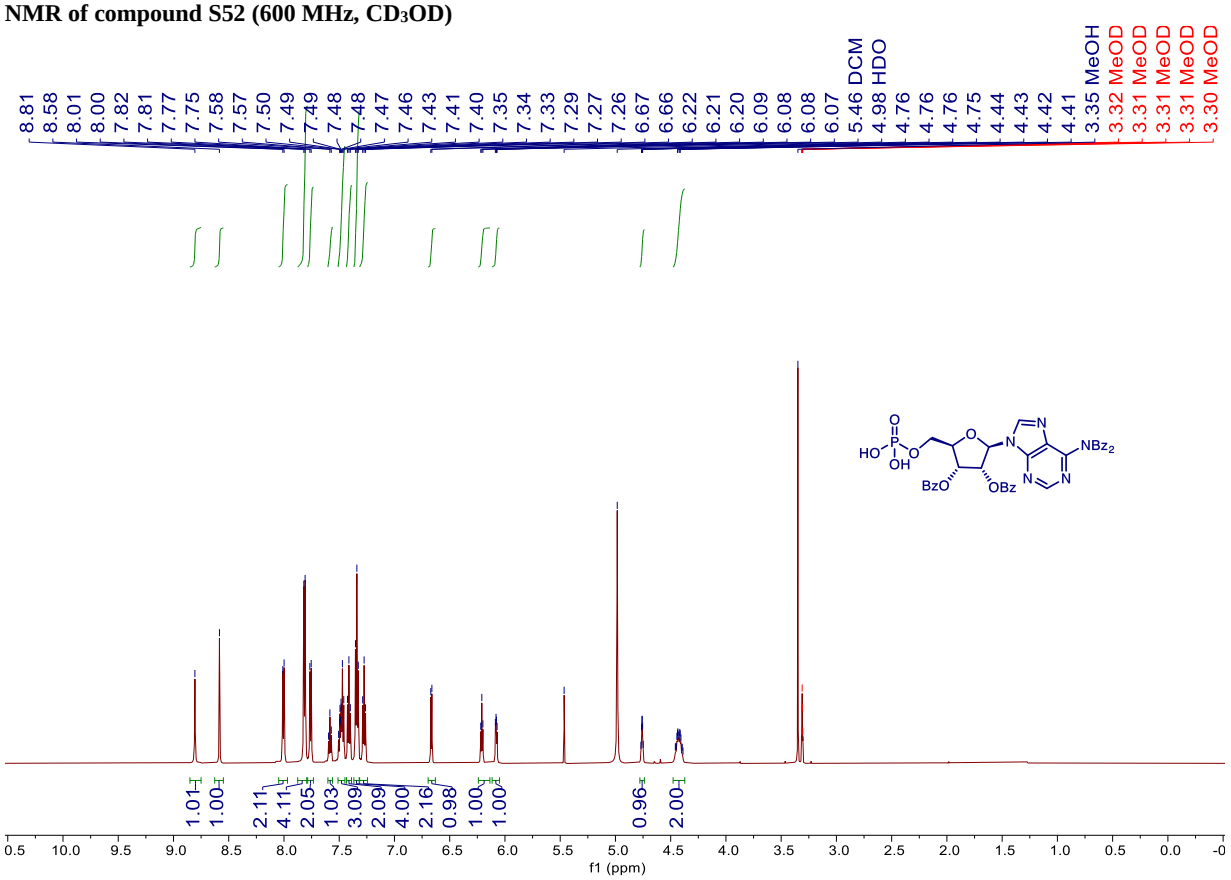
^{13}C NMR of compound S51 (150 MHz, $CDCl_3$)



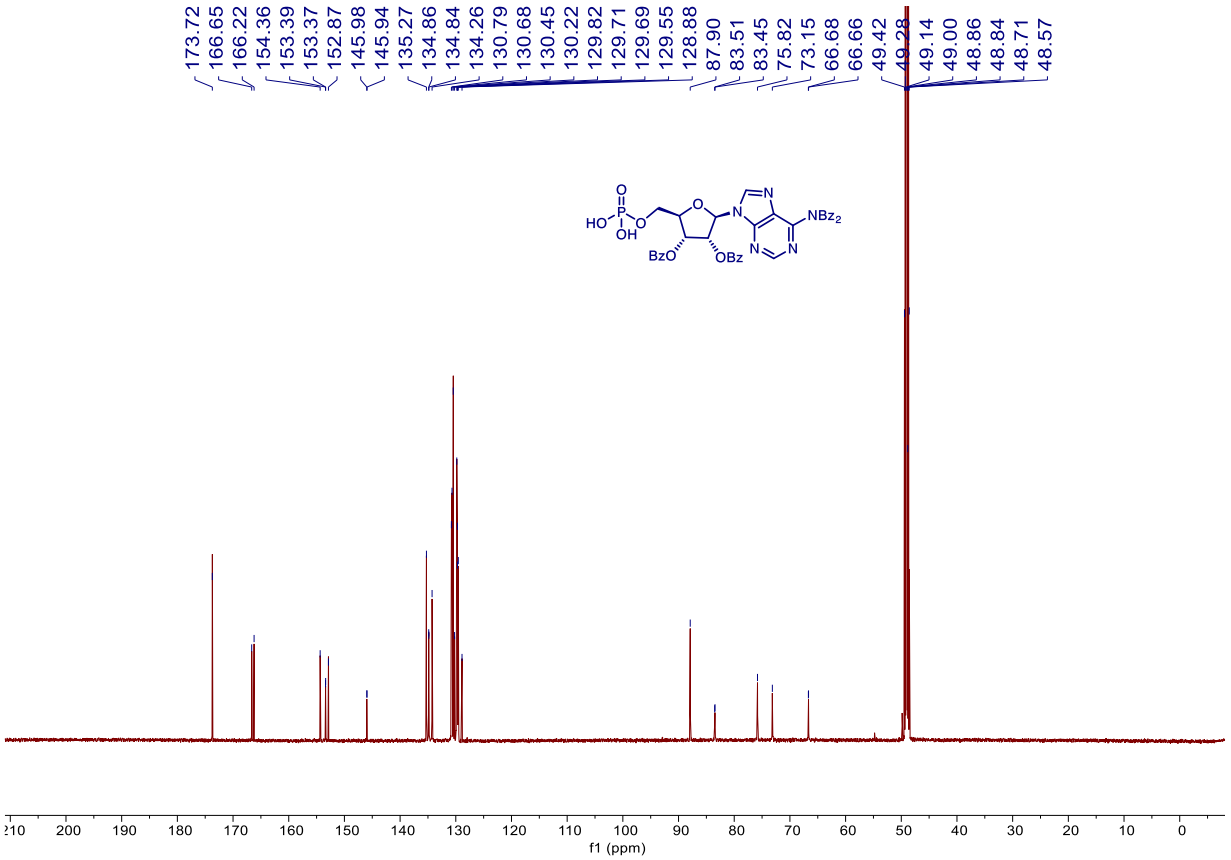
^{31}P NMR of compound S51 (162 MHz, $CDCl_3$)



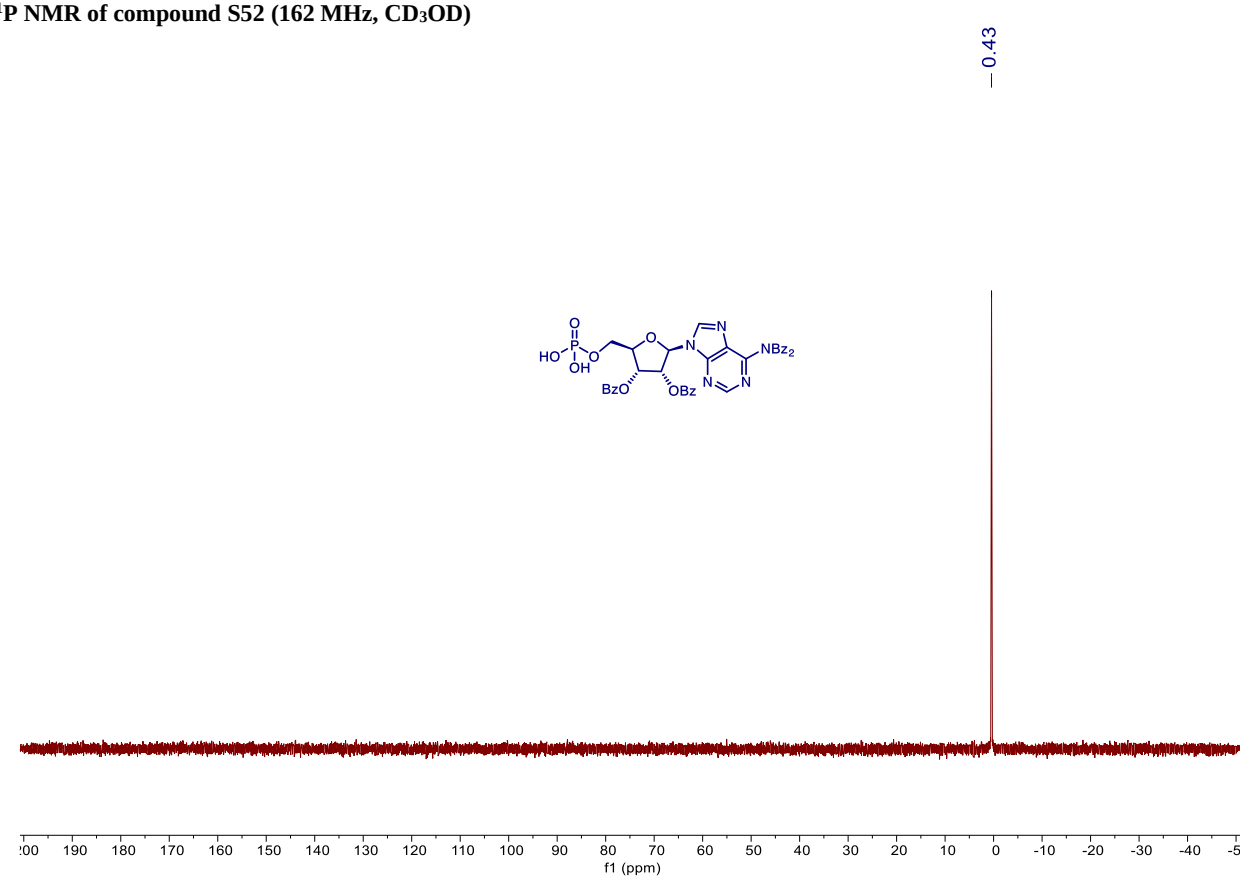
^{1}H NMR of compound S52 (600 MHz, CD_3OD)



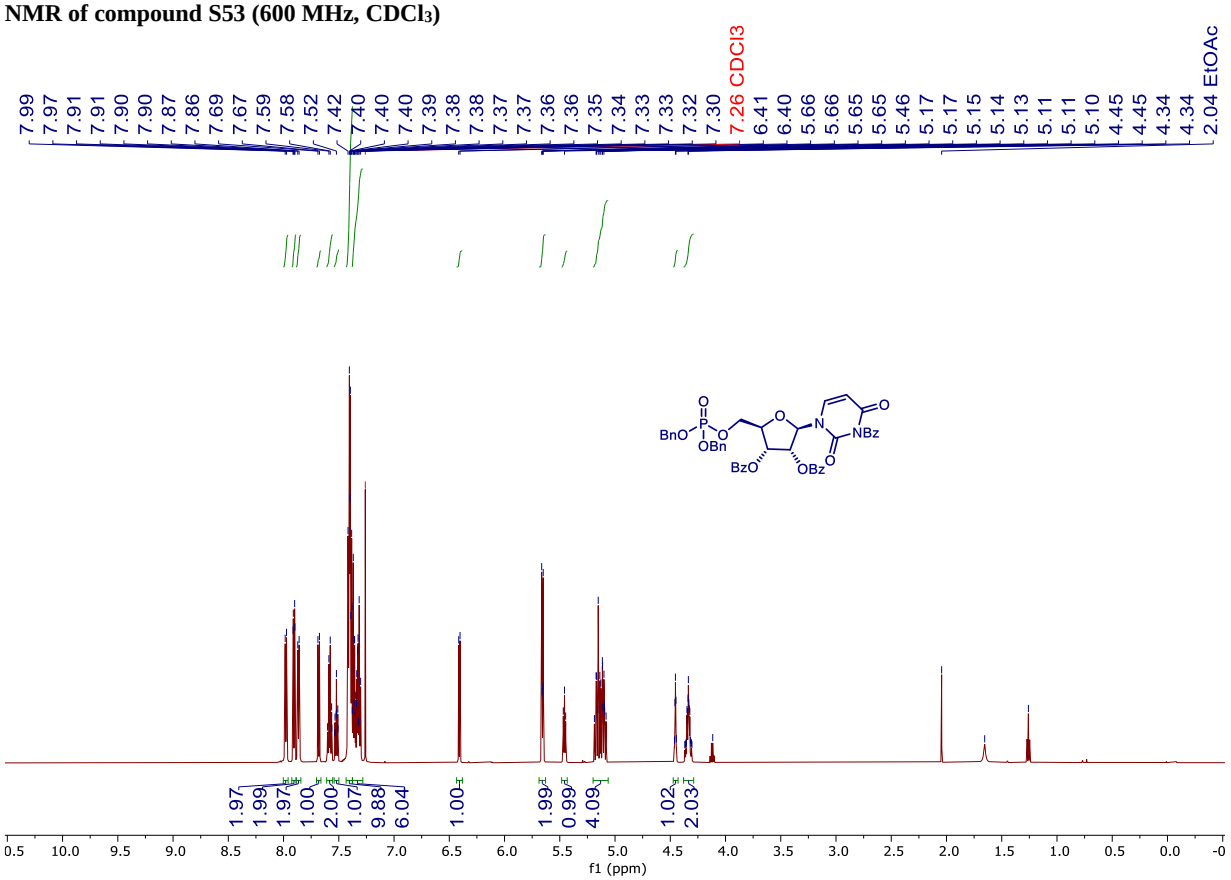
^{13}C NMR of compound S52 (150 MHz, CD_3OD)



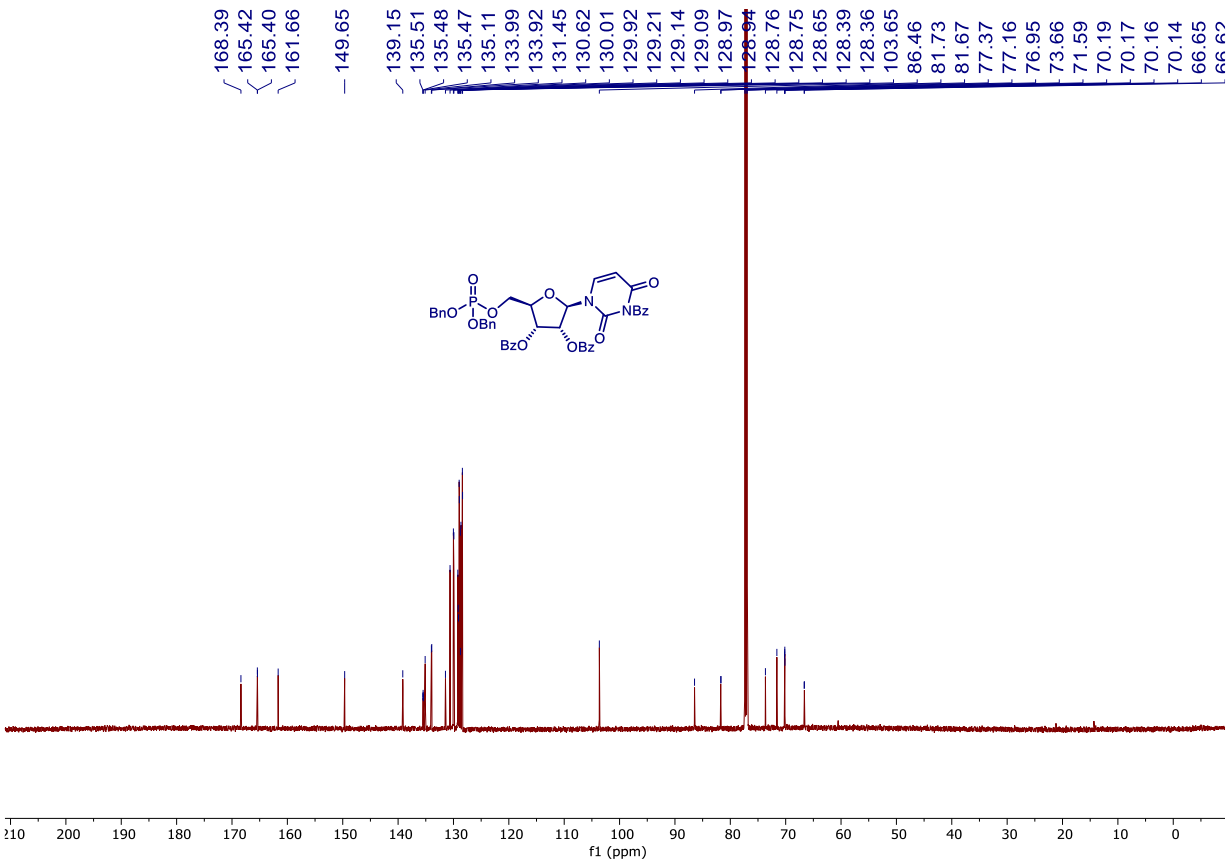
^{31}P NMR of compound S52 (162 MHz, CD_3OD)



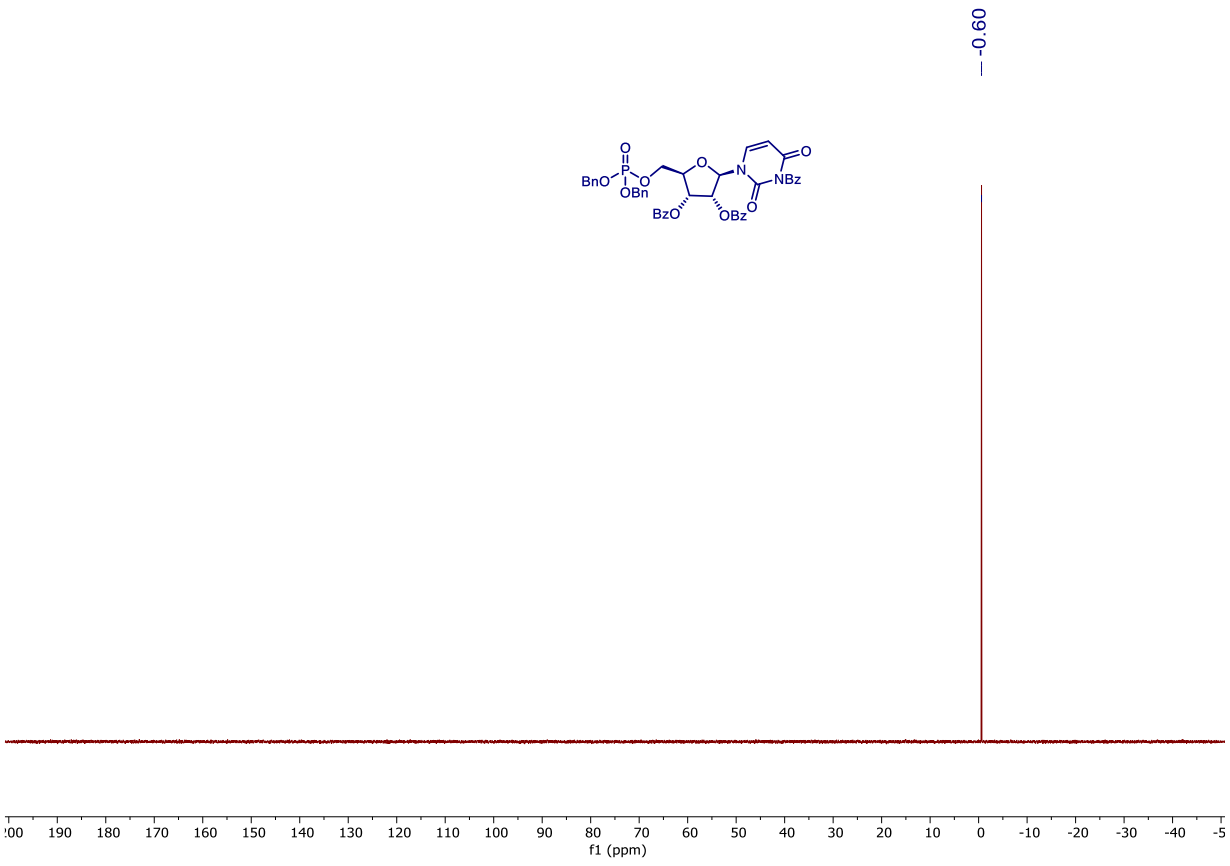
^{1}H NMR of compound S53 (600 MHz, $CDCl_3$)



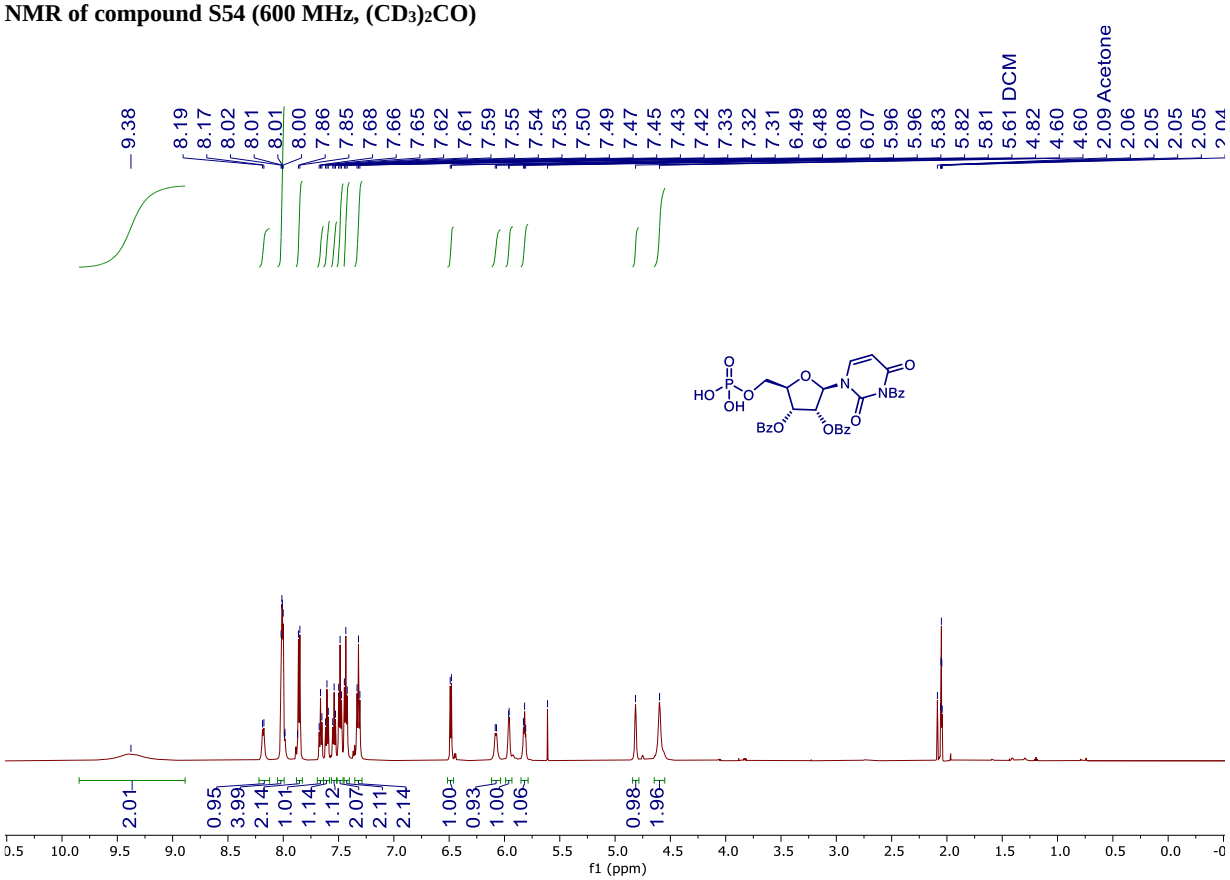
^{13}C NMR of compound S53 (150 MHz, $CDCl_3$)



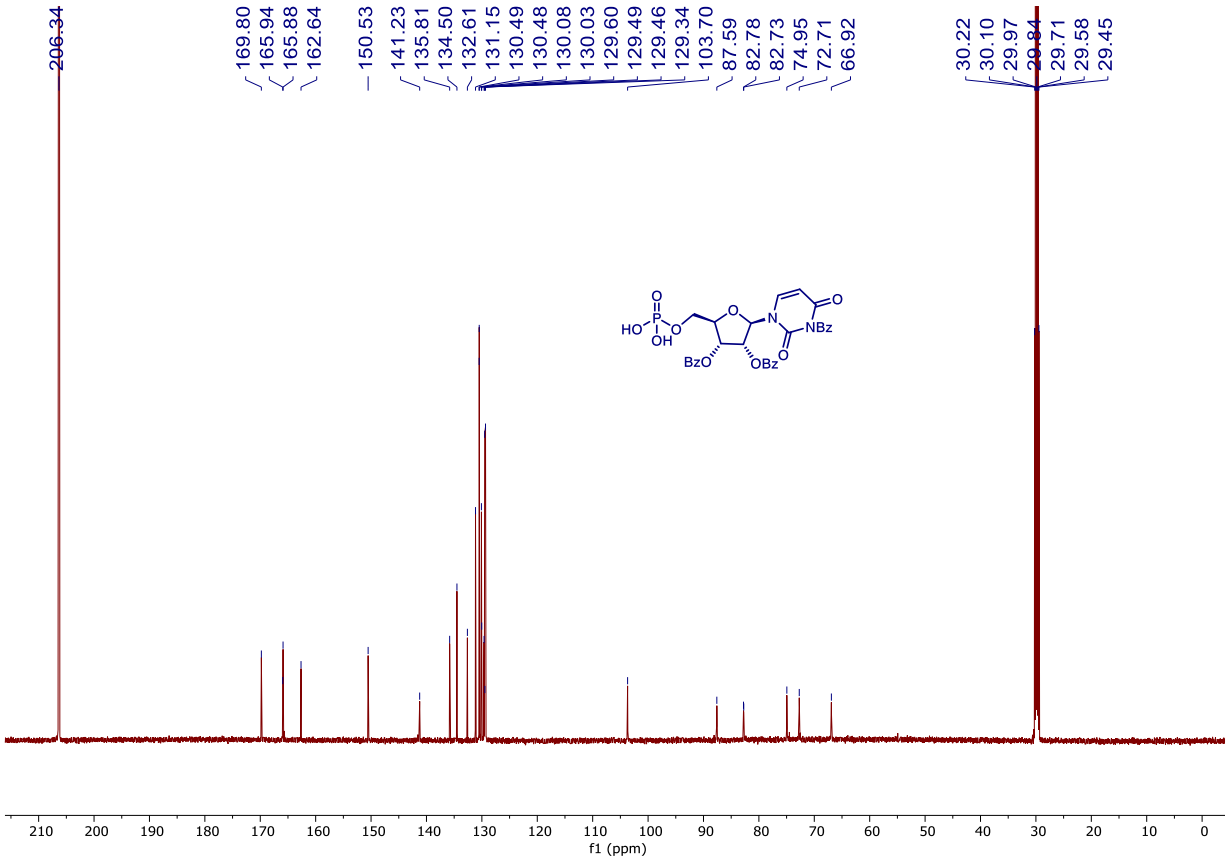
^{31}P NMR of compound S53 (162 MHz, $CDCl_3$)



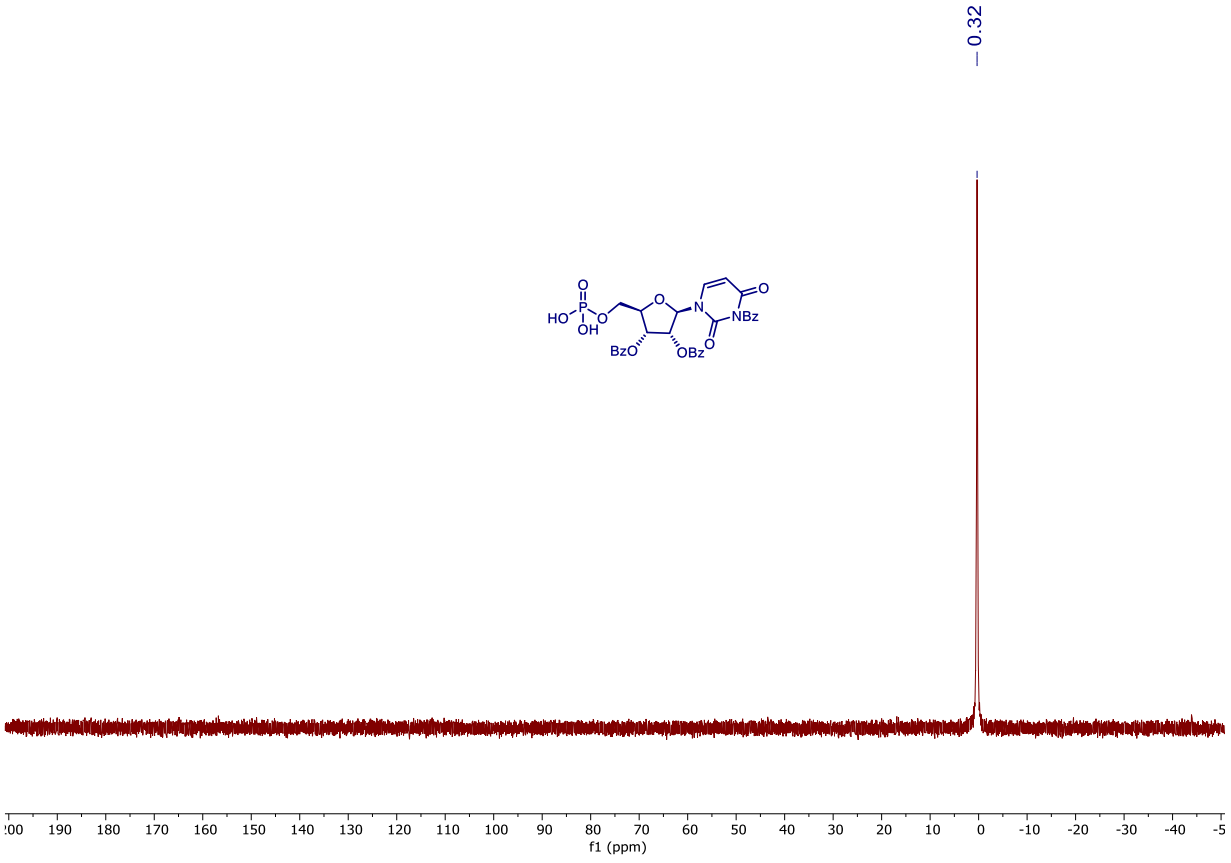
^{1}H NMR of compound S54 (600 MHz, $(CD_3)_2CO$)



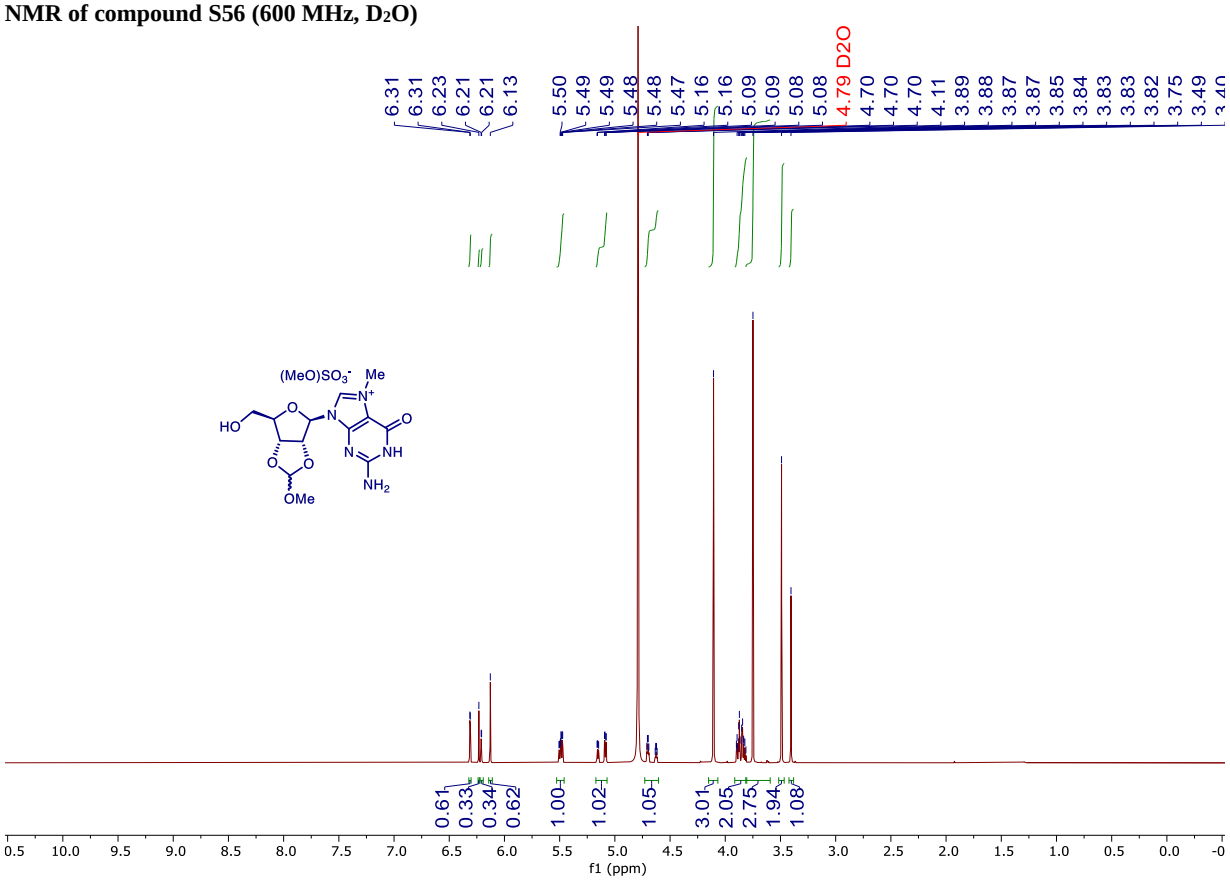
^{13}C NMR of compound S54 (150 MHz, $(CD_3)_2CO$)



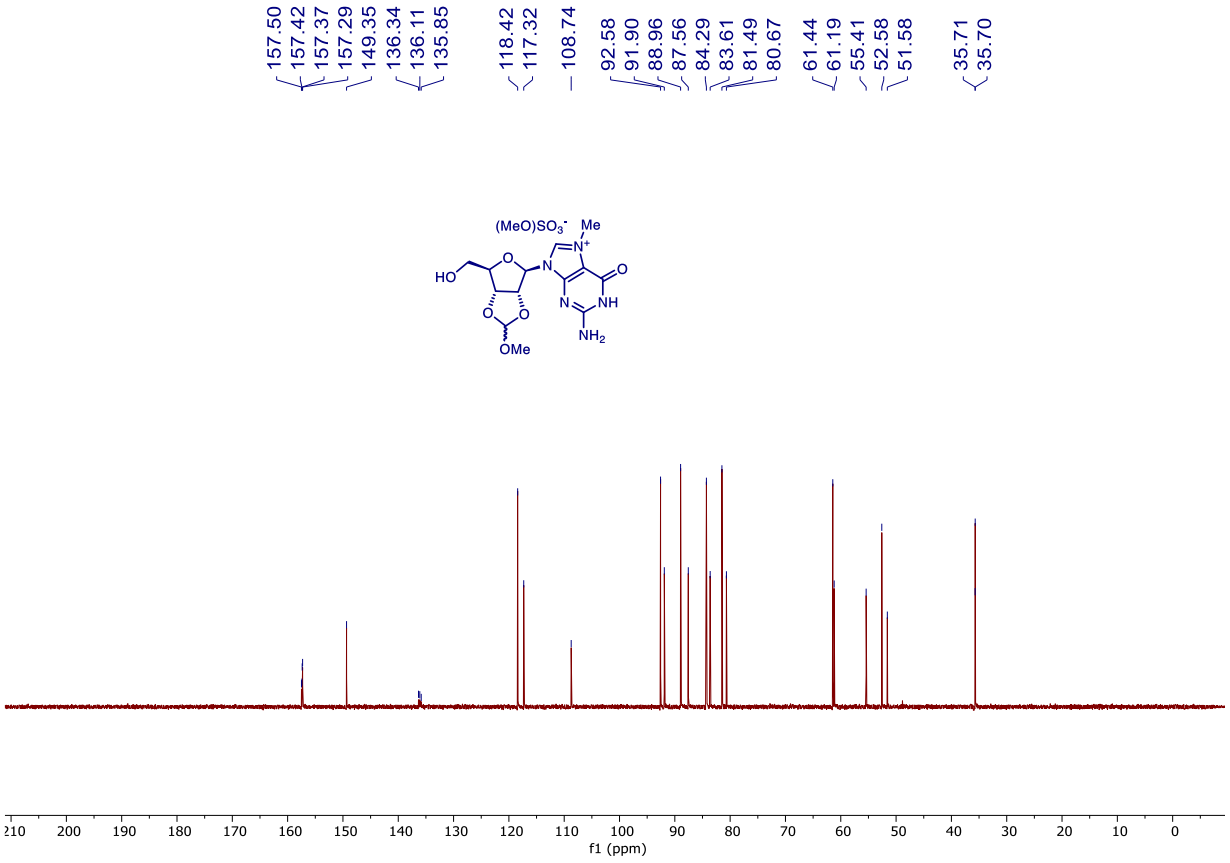
^{31}P NMR of compound S54 (162 MHz, $(CD_3)_2CO$)



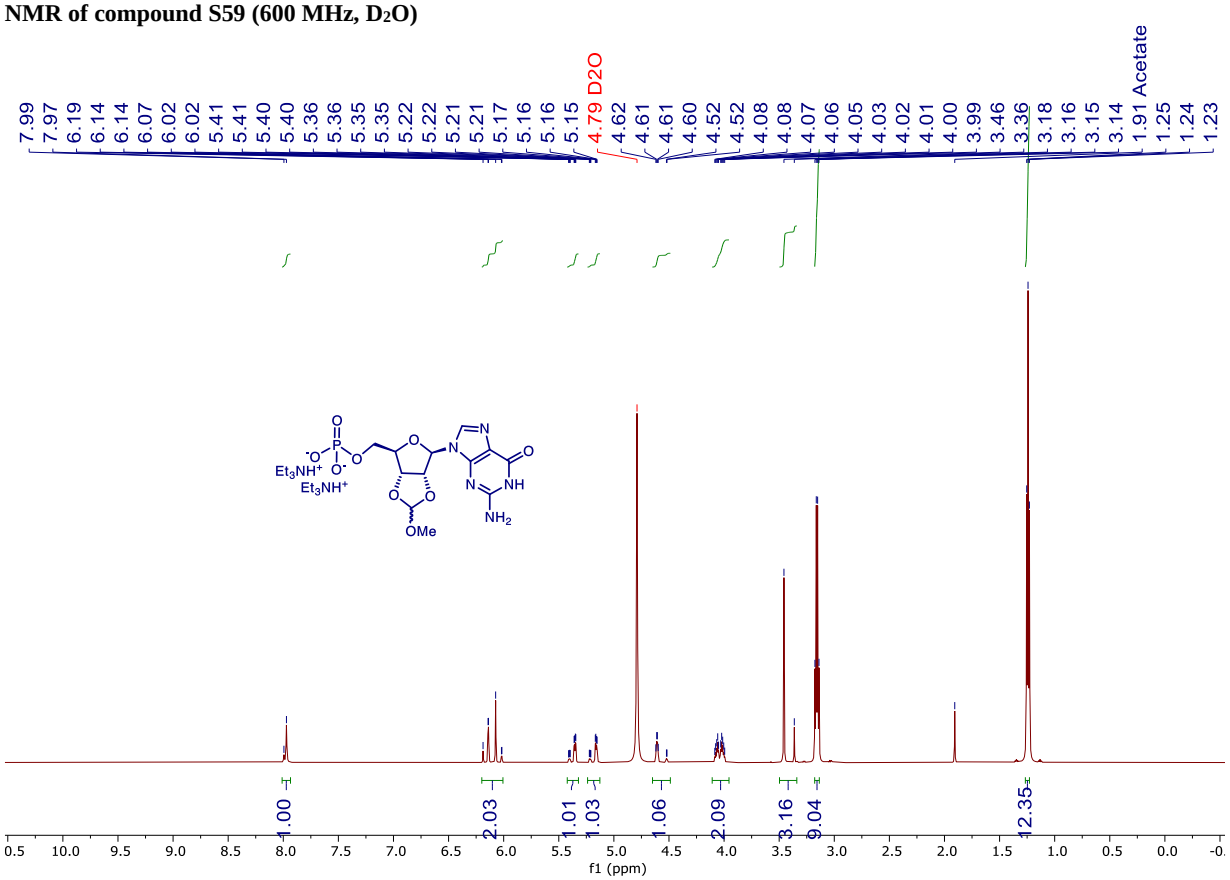
^{1}H NMR of compound S56 (600 MHz, D_2O)



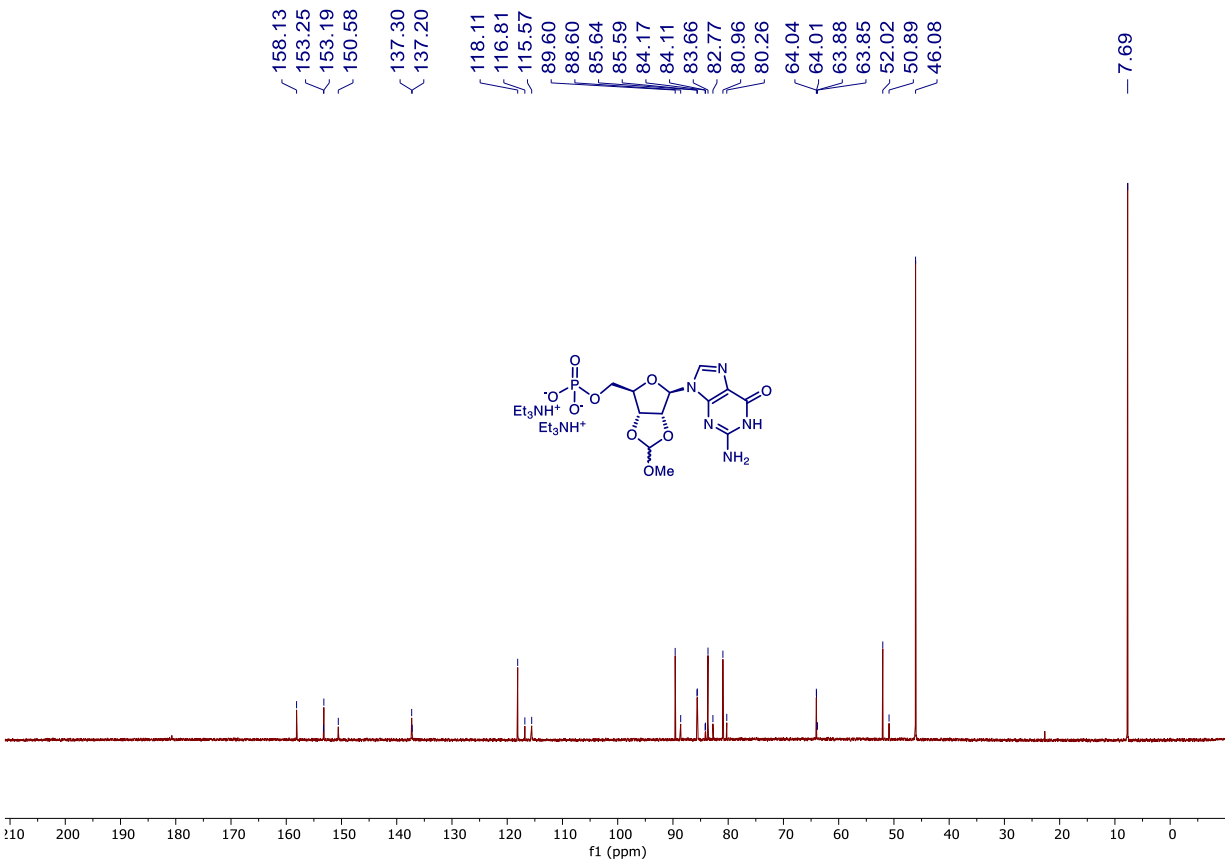
^{13}C NMR of compound S56 (150 MHz, D_2O)



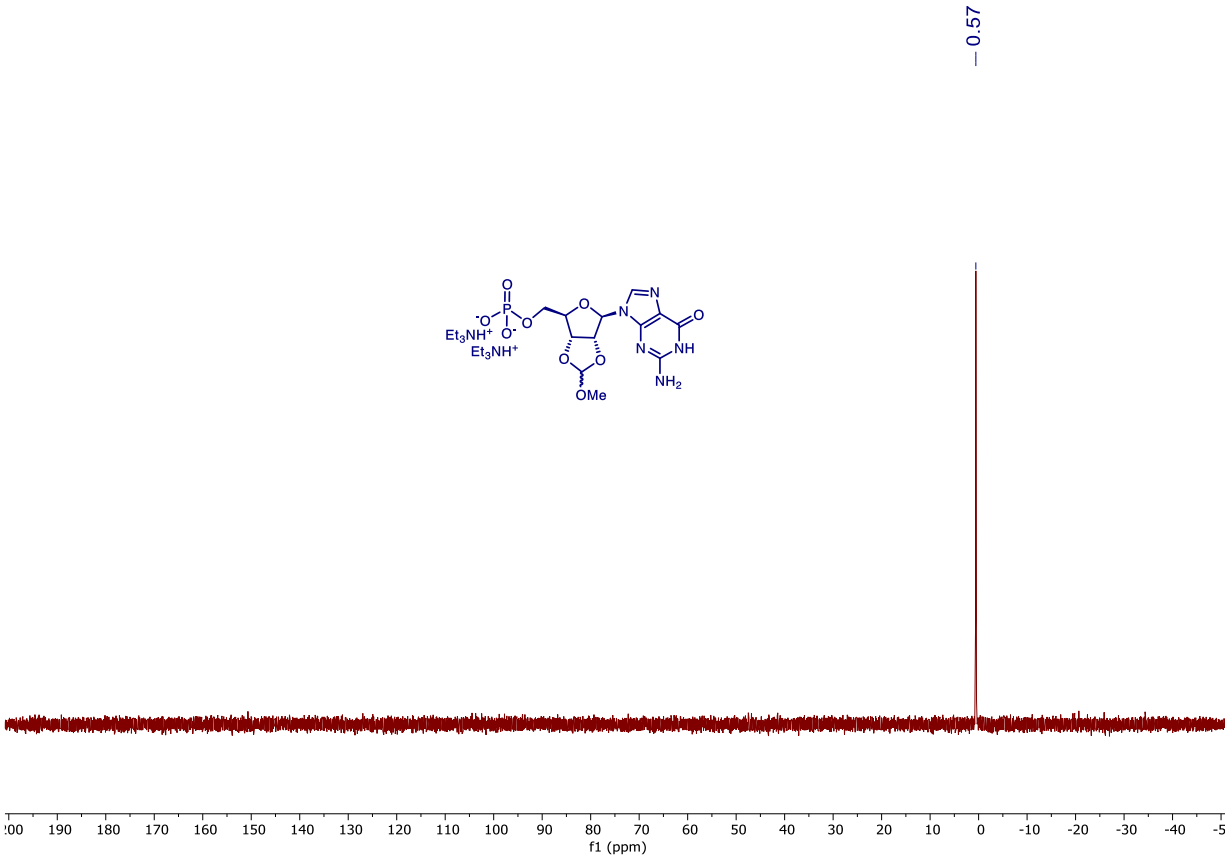
^{1}H NMR of compound S59 (600 MHz, D_2O)



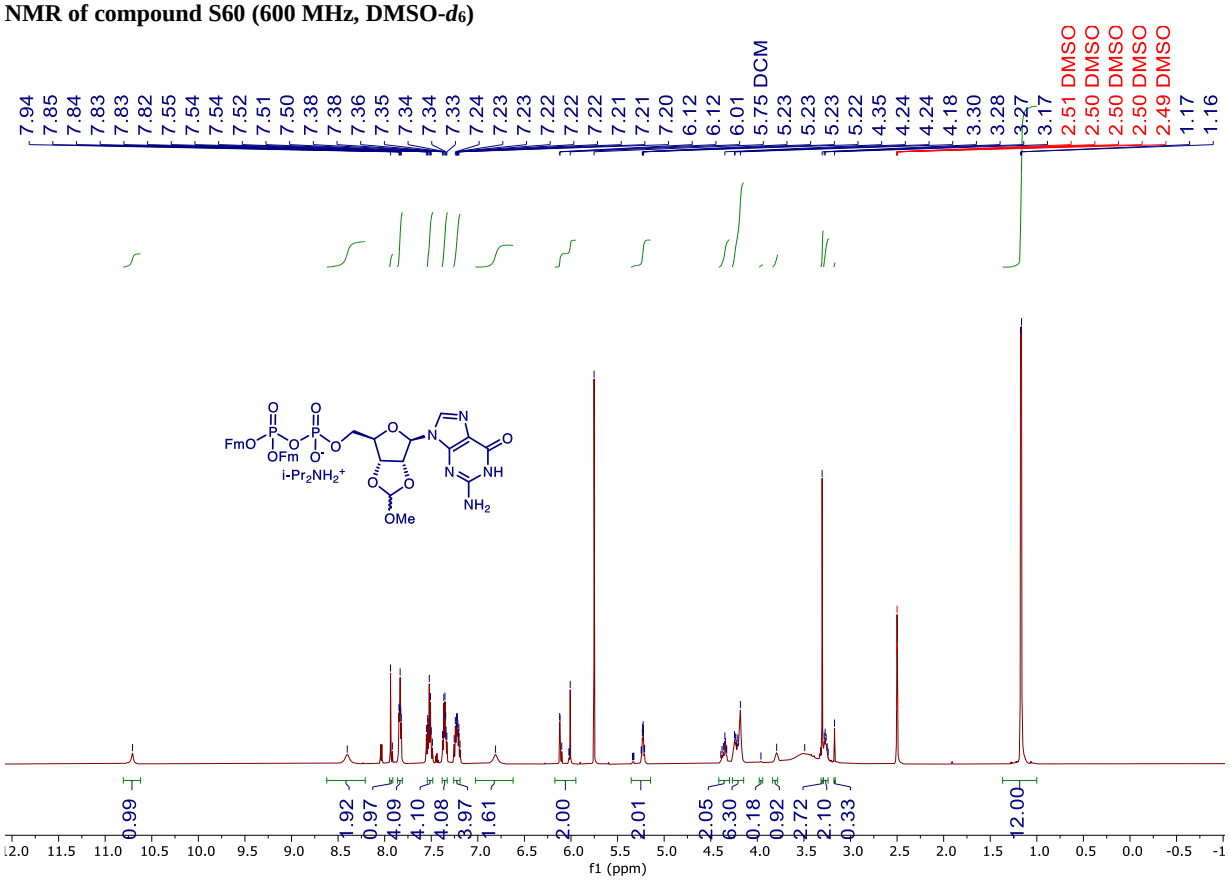
^{13}C NMR of compound S59 (150 MHz, D_2O)



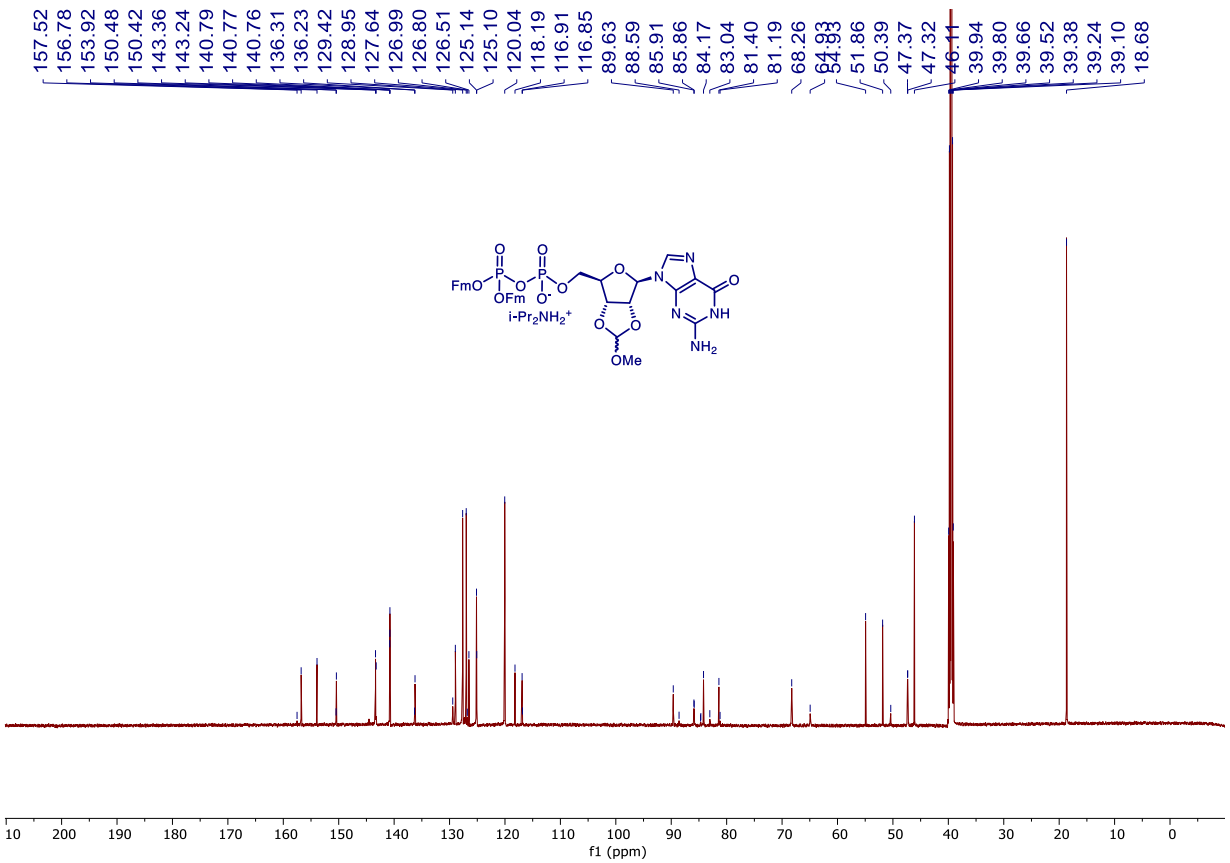
^{31}P NMR of compound S59 (162 MHz, D_2O)



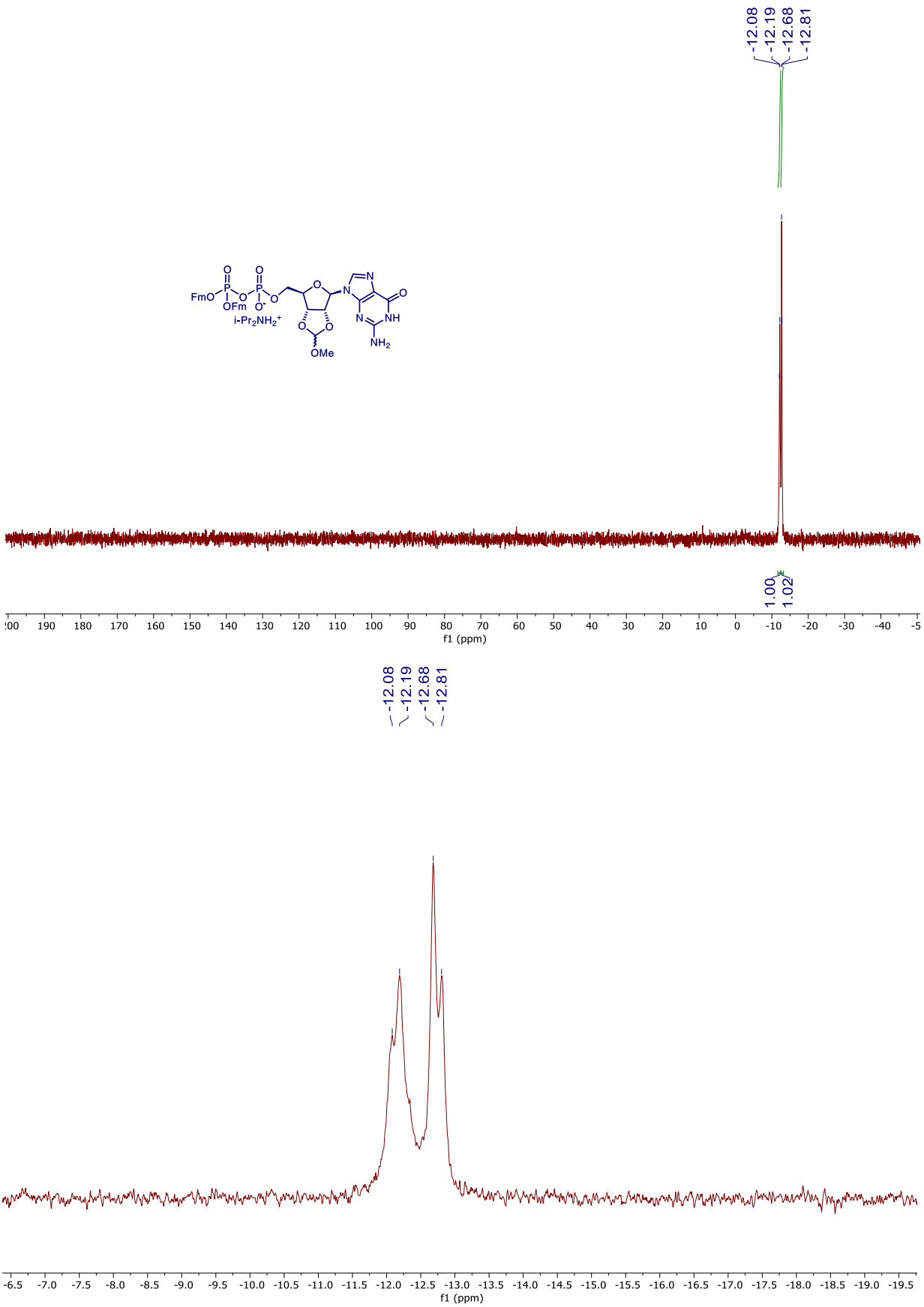
^{1}H NMR of compound S60 (600 MHz, DMSO-*d*$_6$)



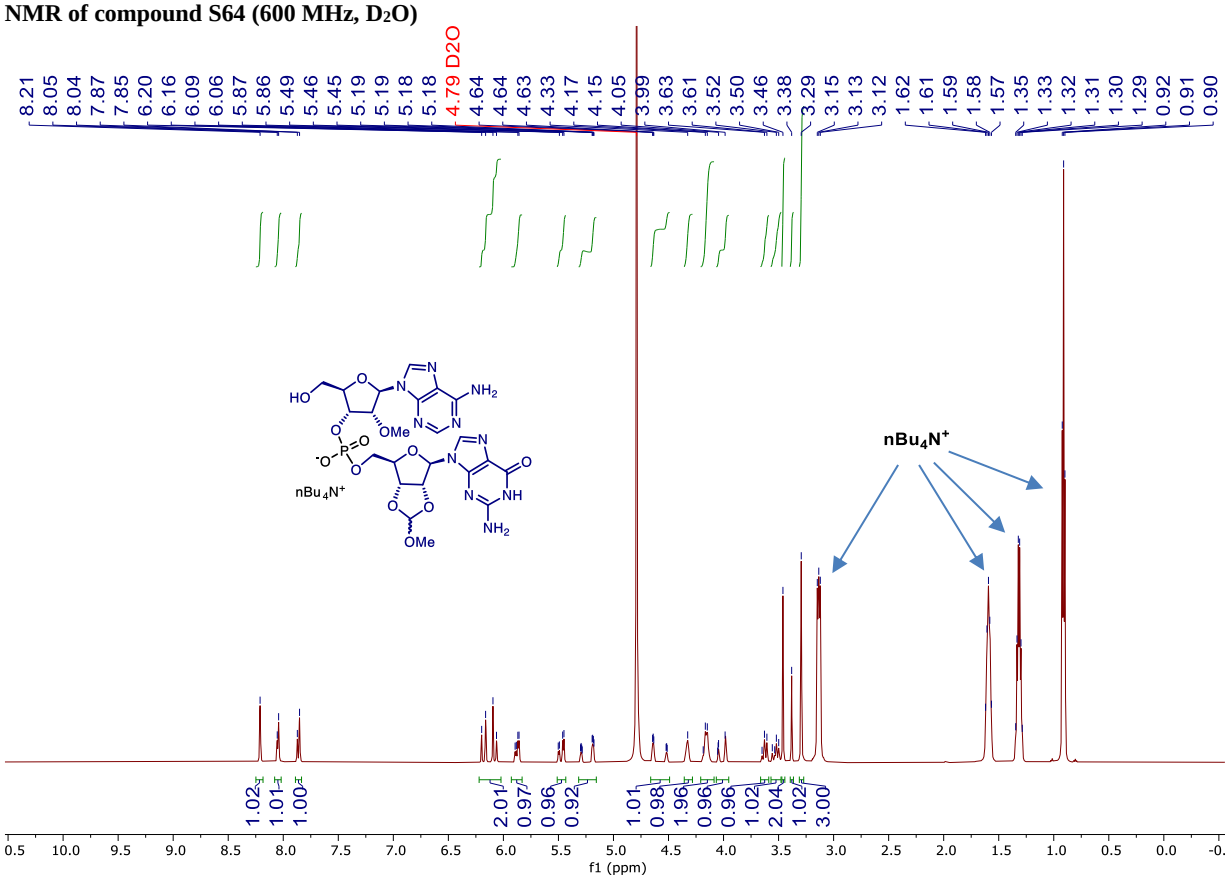
^{13}C NMR of compound S60 (150 MHz, DMSO-*d*$_6$)



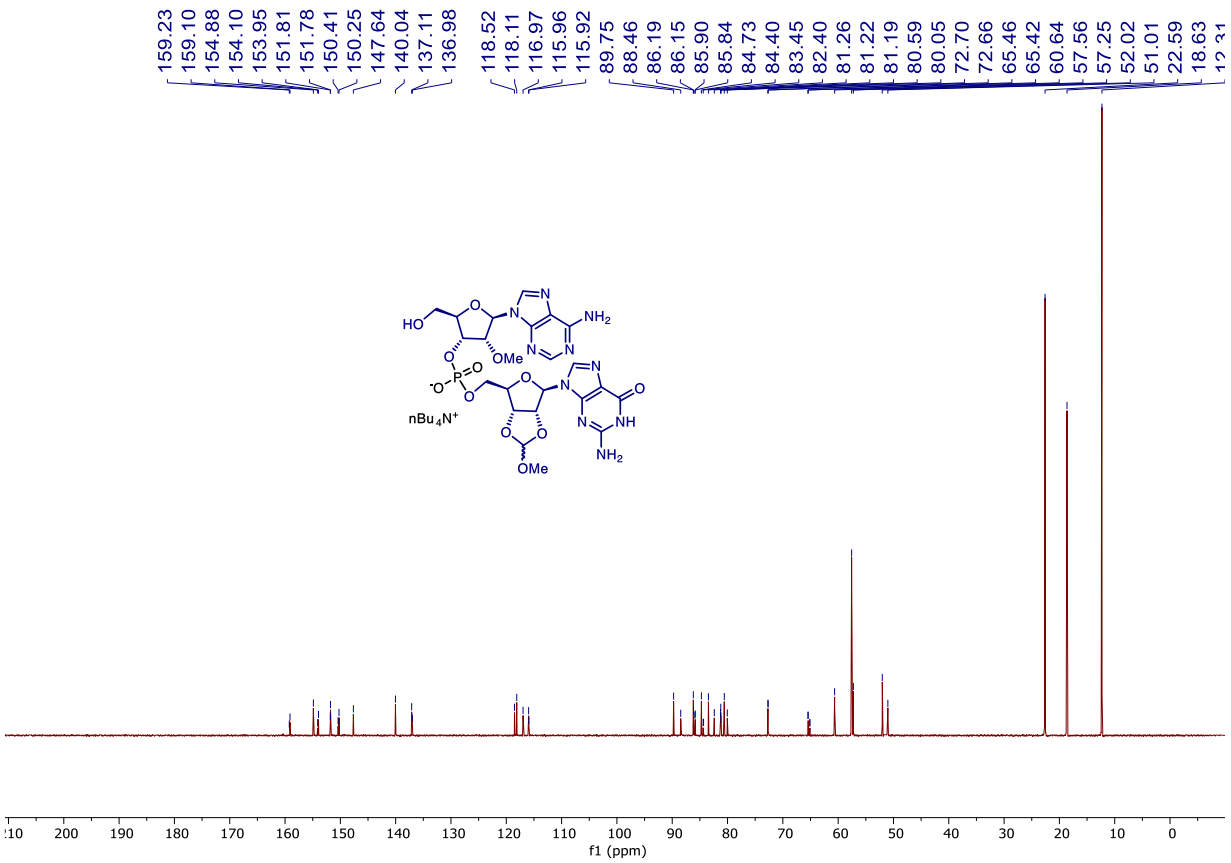
^{31}P NMR of compound S60 (162 MHz, DMSO-d_6)



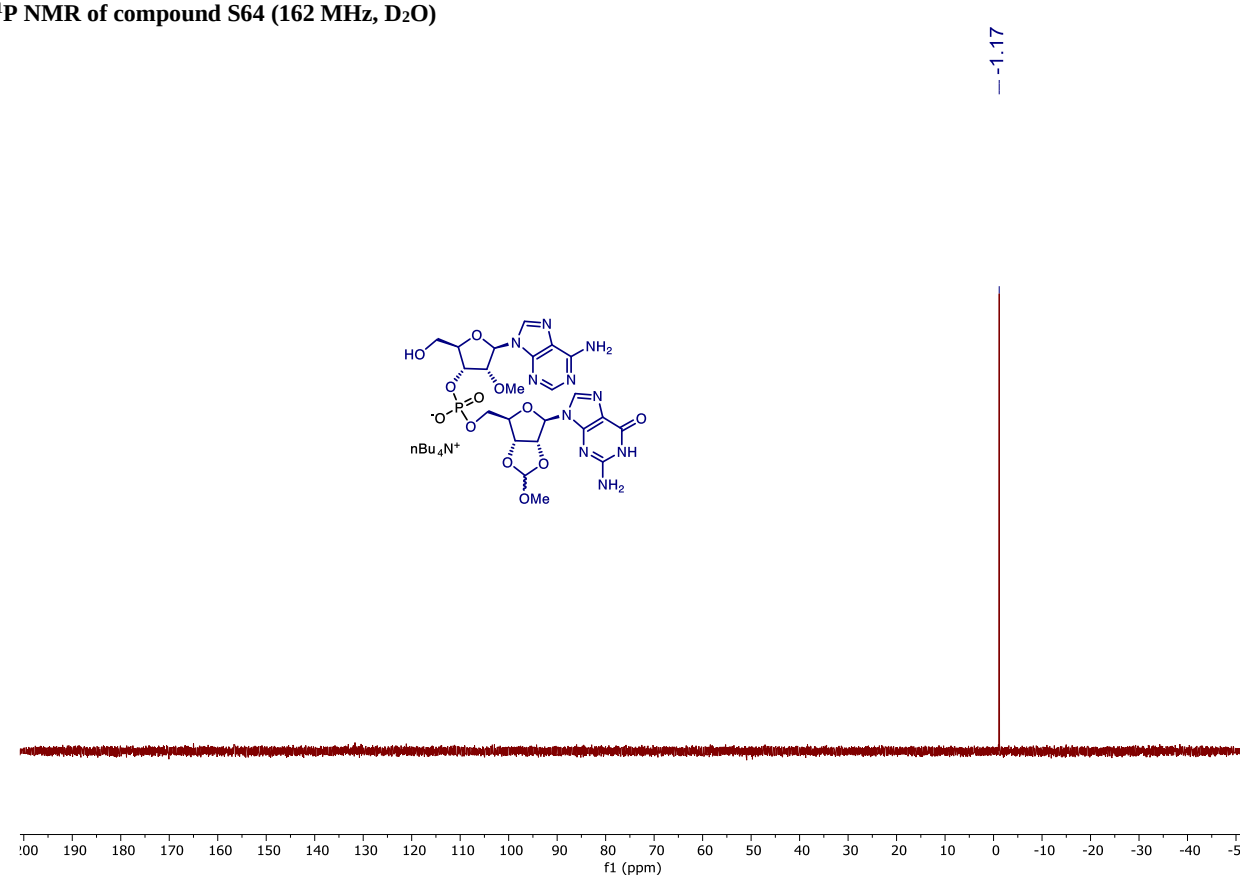
^{1}H NMR of compound S64 (600 MHz, D_2O)



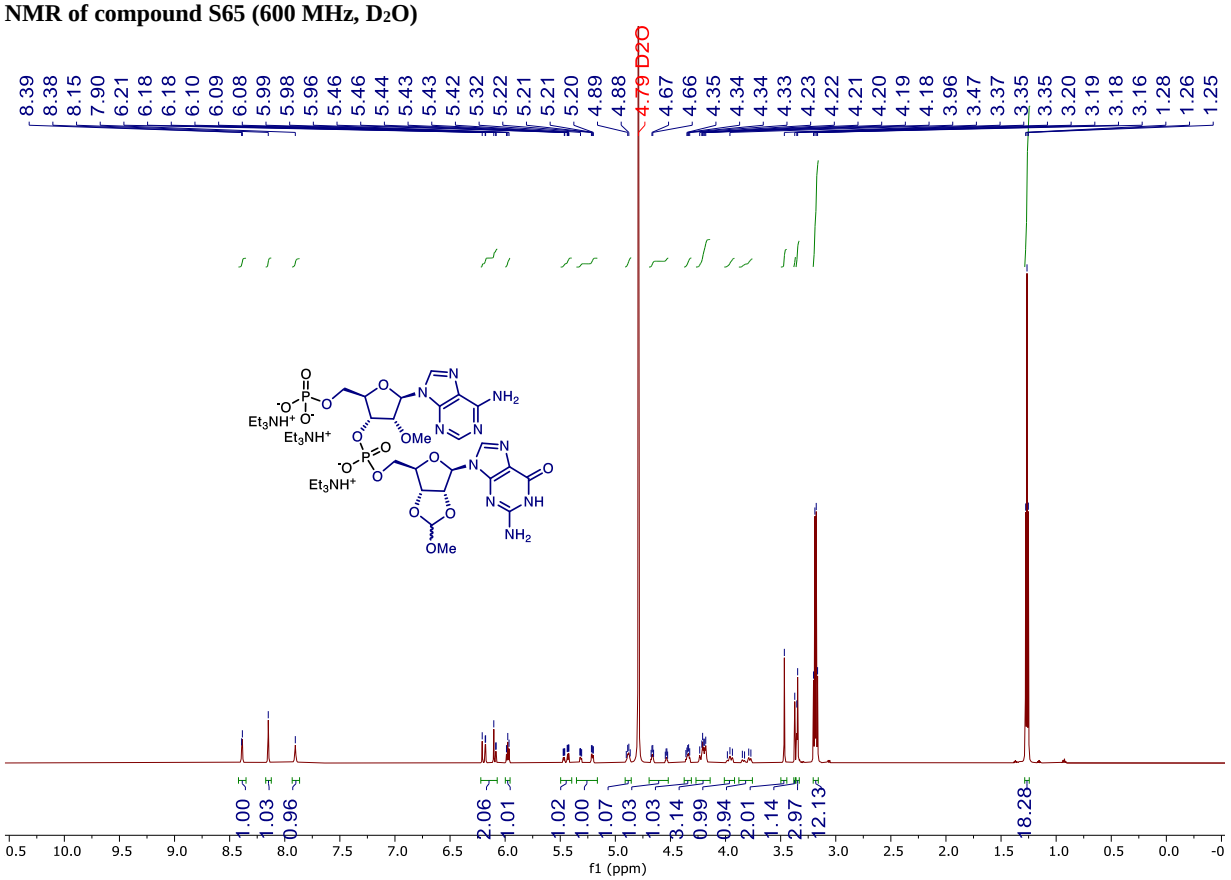
^{13}C NMR of compound S64 (150 MHz, D_2O)



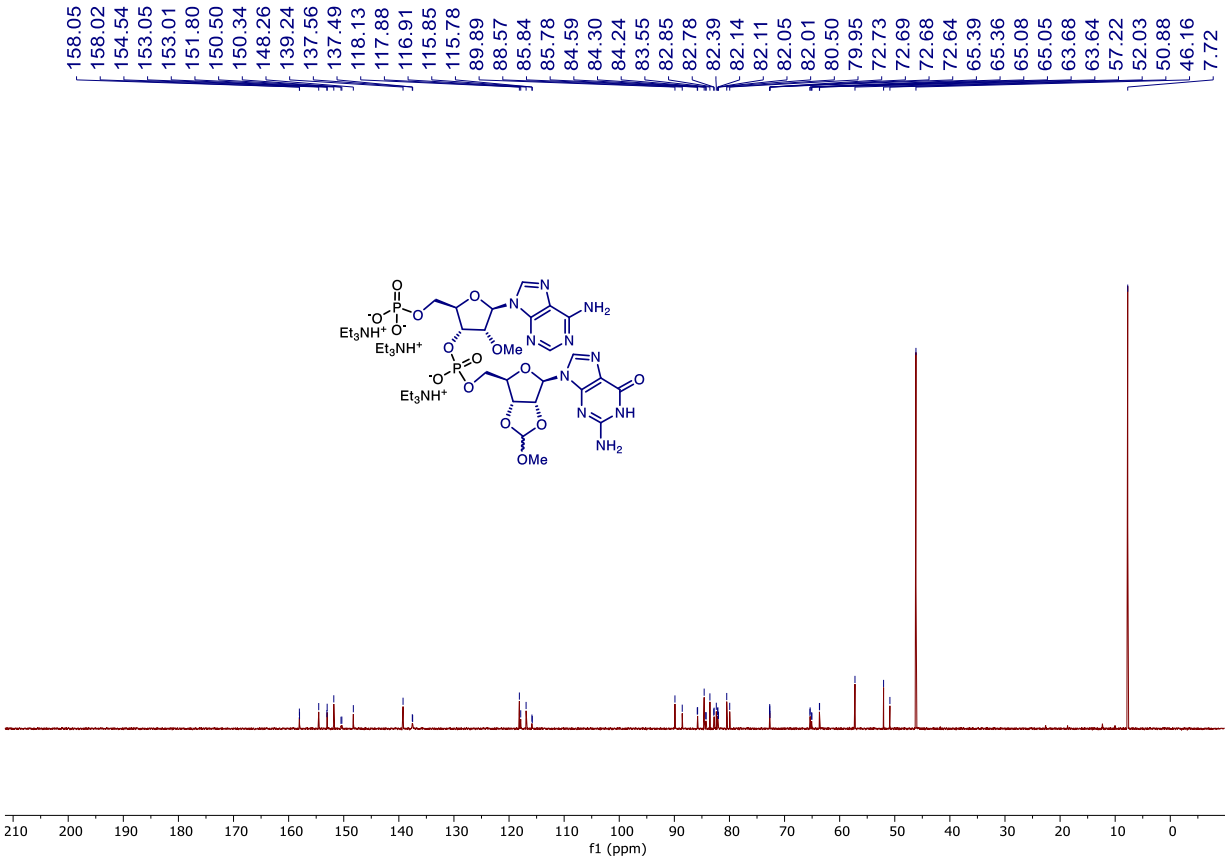
^{31}P NMR of compound S64 (162 MHz, D_2O)



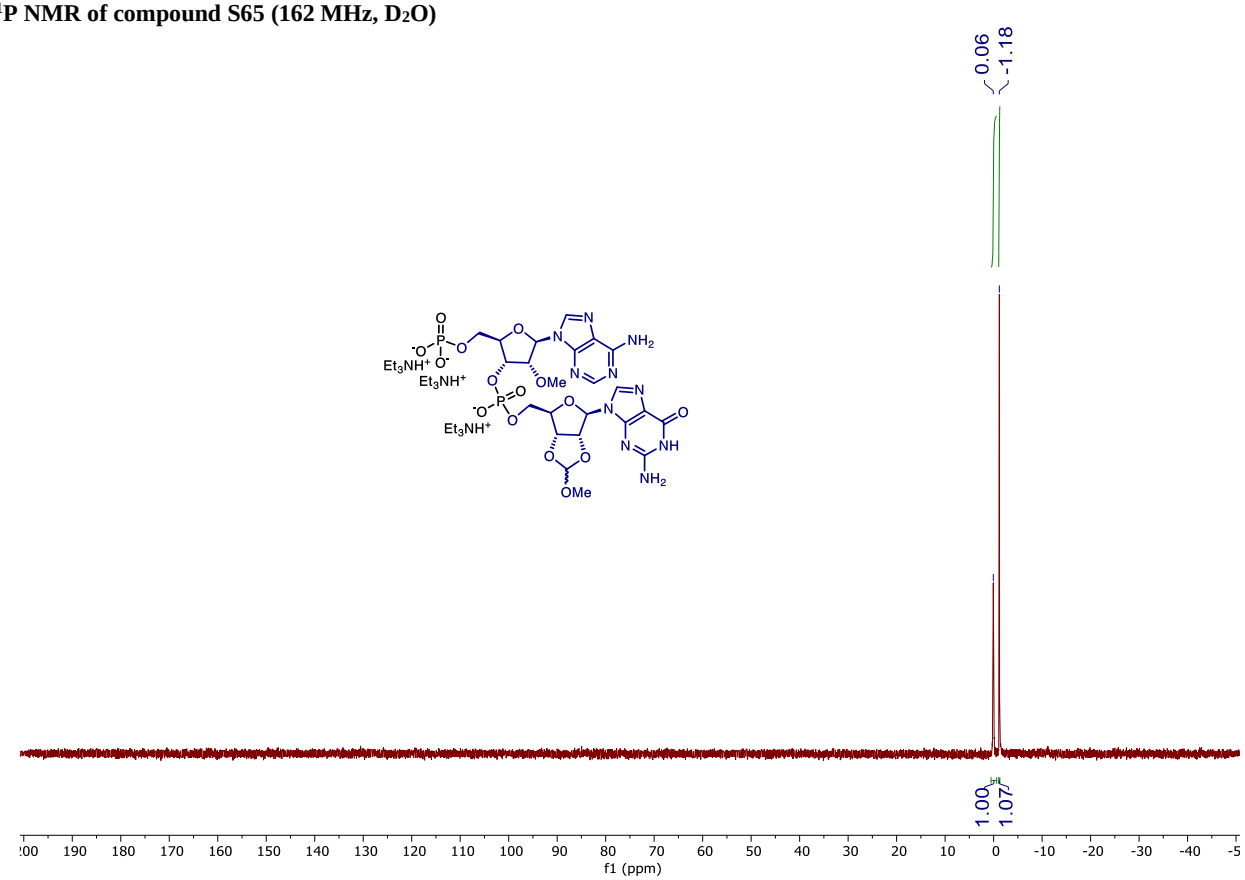
^{1}H NMR of compound S65 (600 MHz, D_2O)



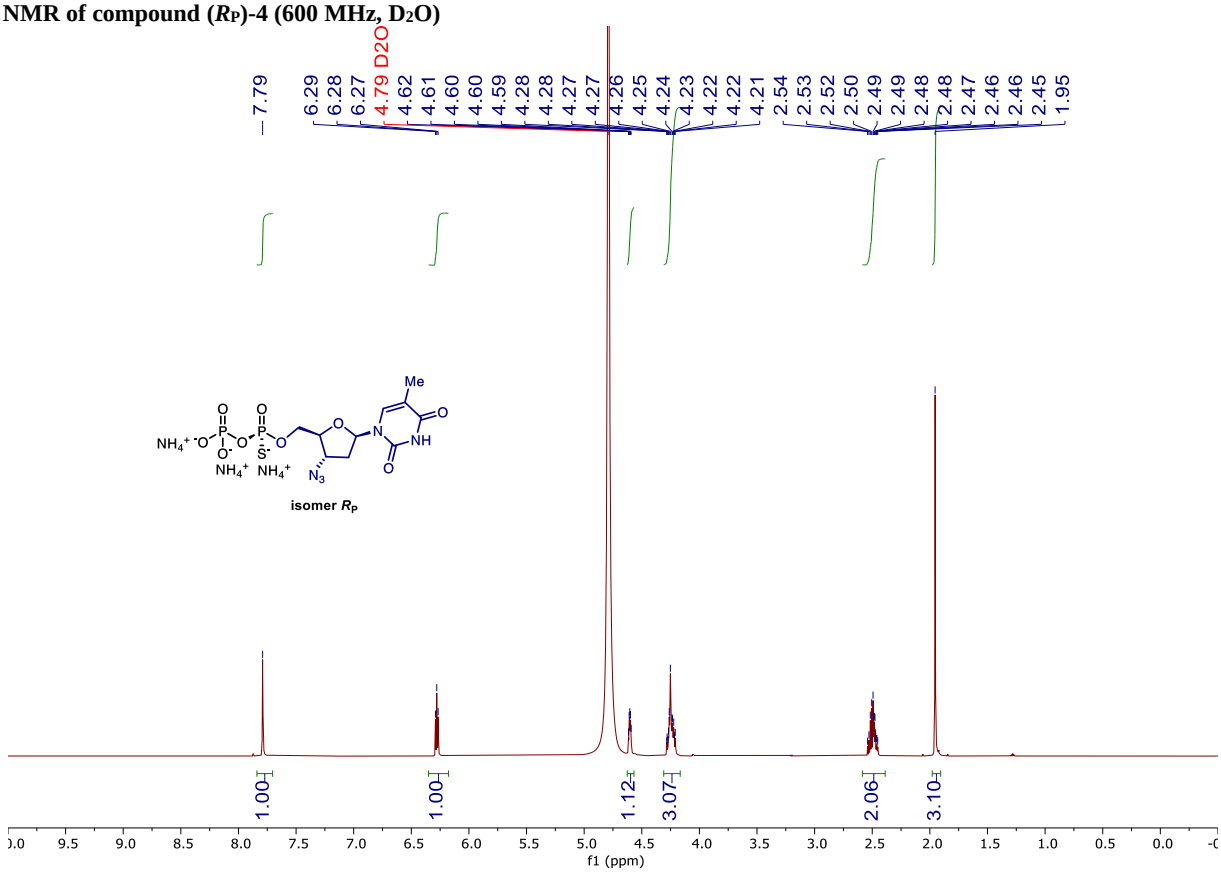
^{13}C NMR of compound S65 (150 MHz, D_2O)



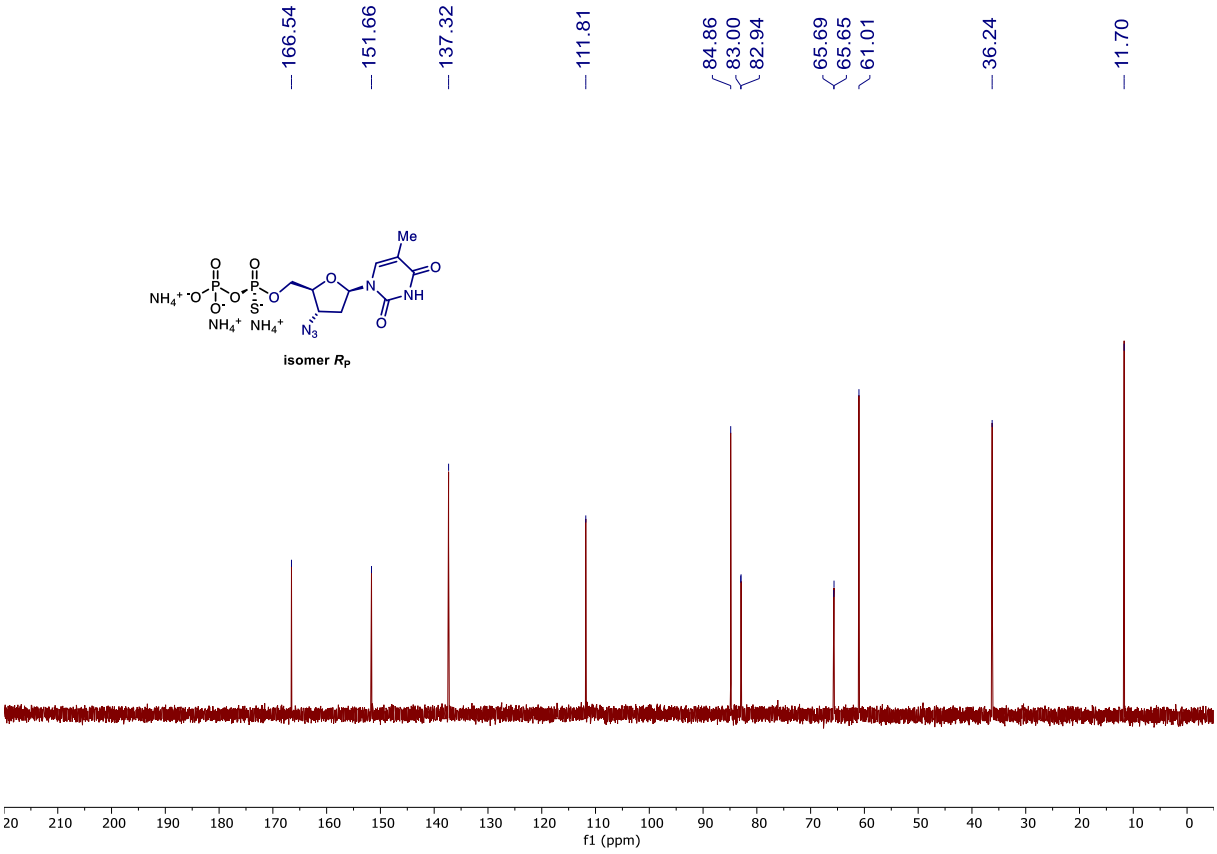
^{31}P NMR of compound S65 (162 MHz, D_2O)



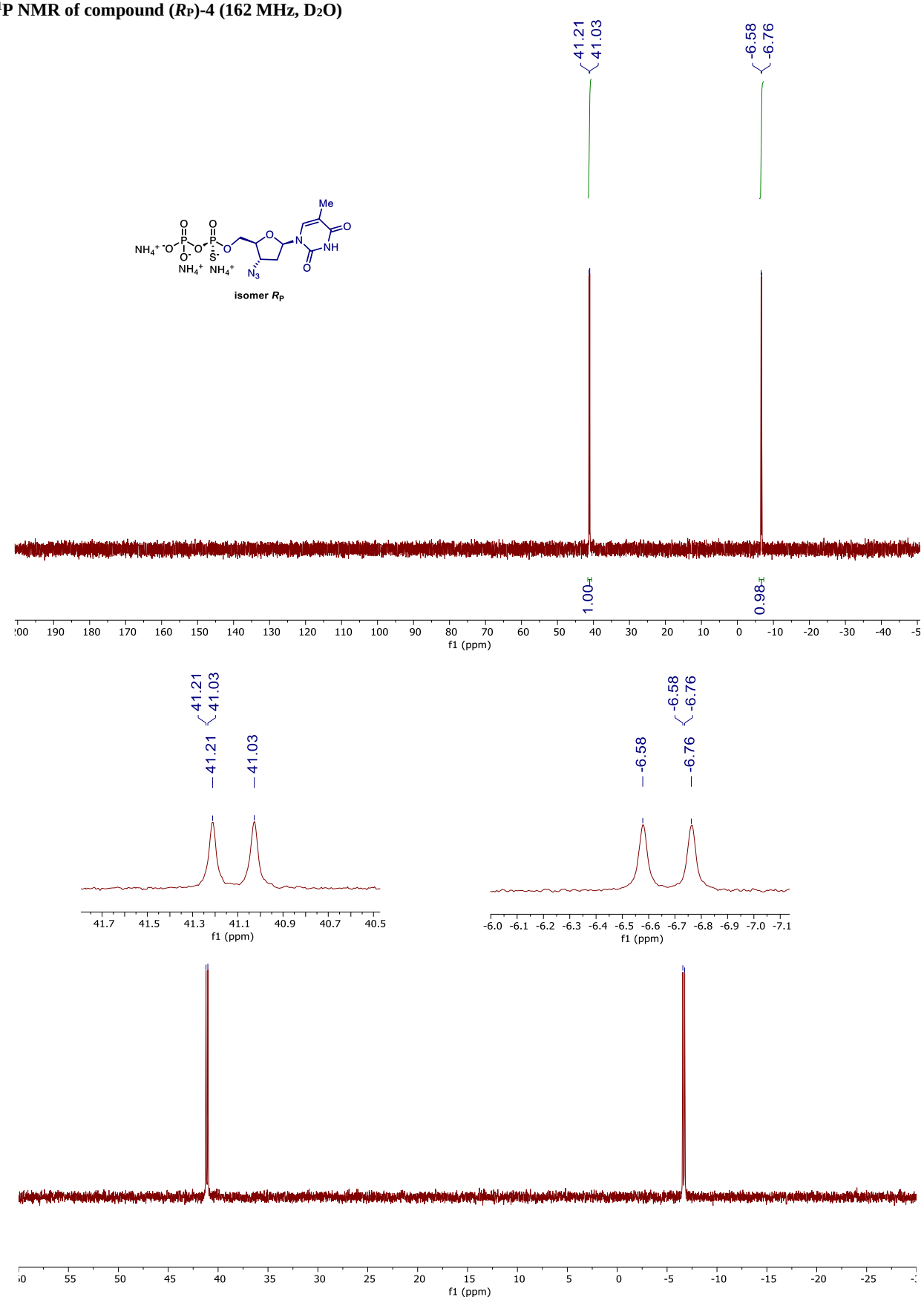
^{1}H NMR of compound (R_P)-4 (600 MHz, D_2O)



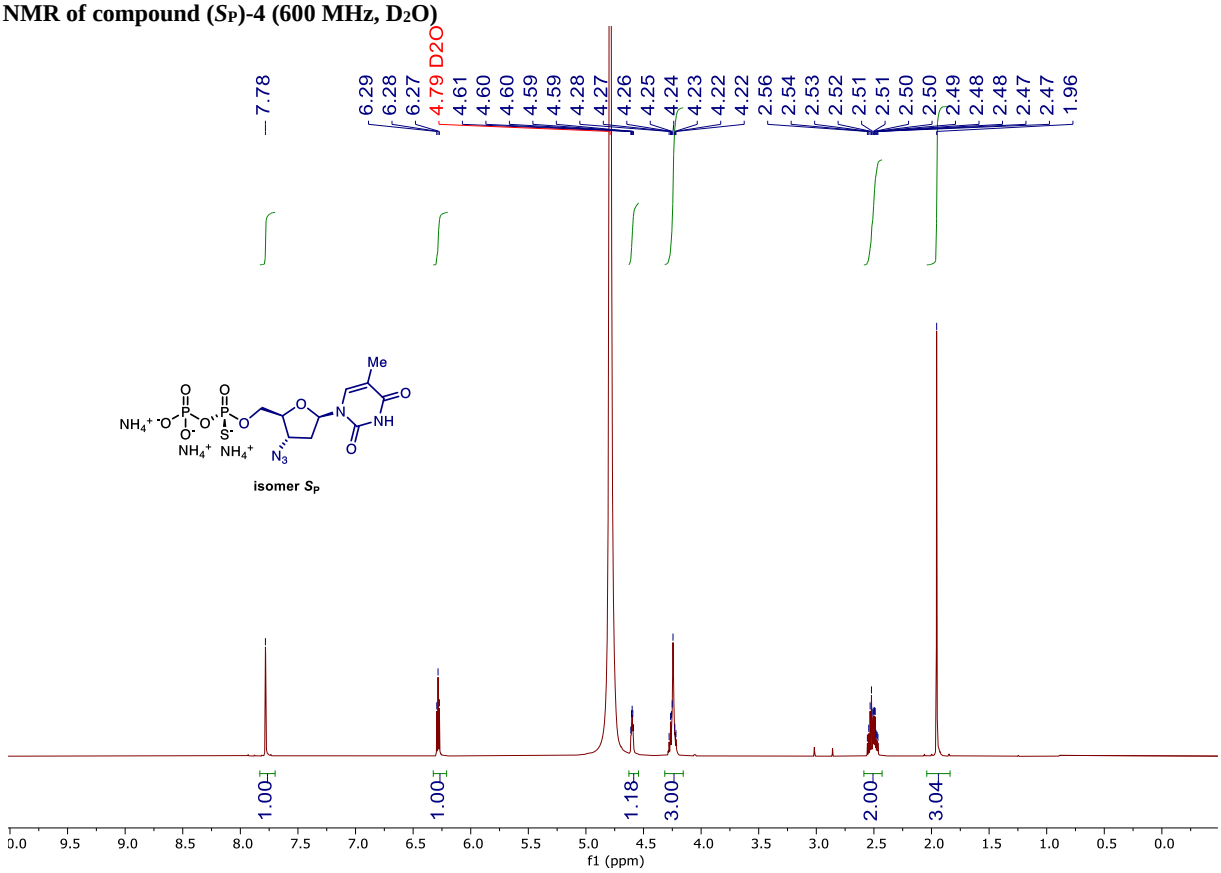
^{13}C NMR of compound (R_P)-4 (150 MHz, D_2O)



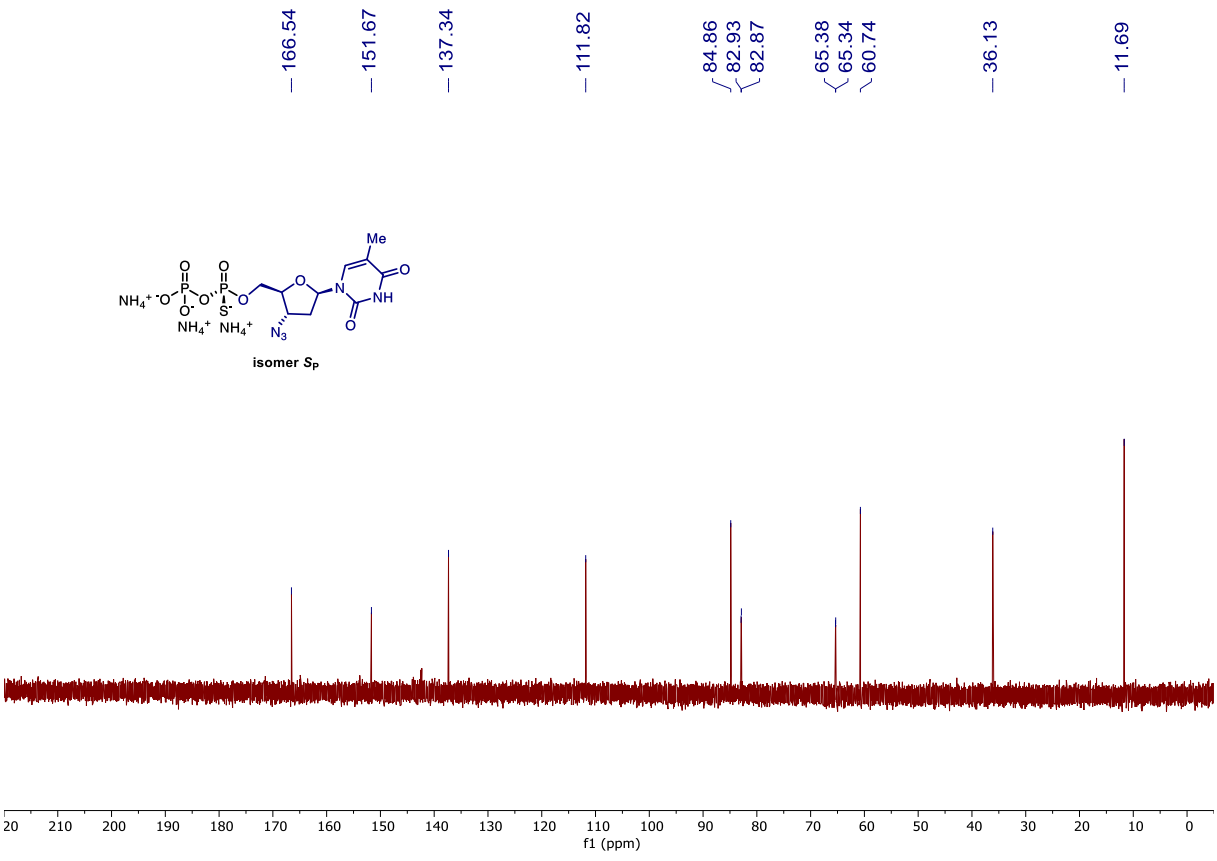
^{31}P NMR of compound (R_P)-4 (162 MHz, D_2O)



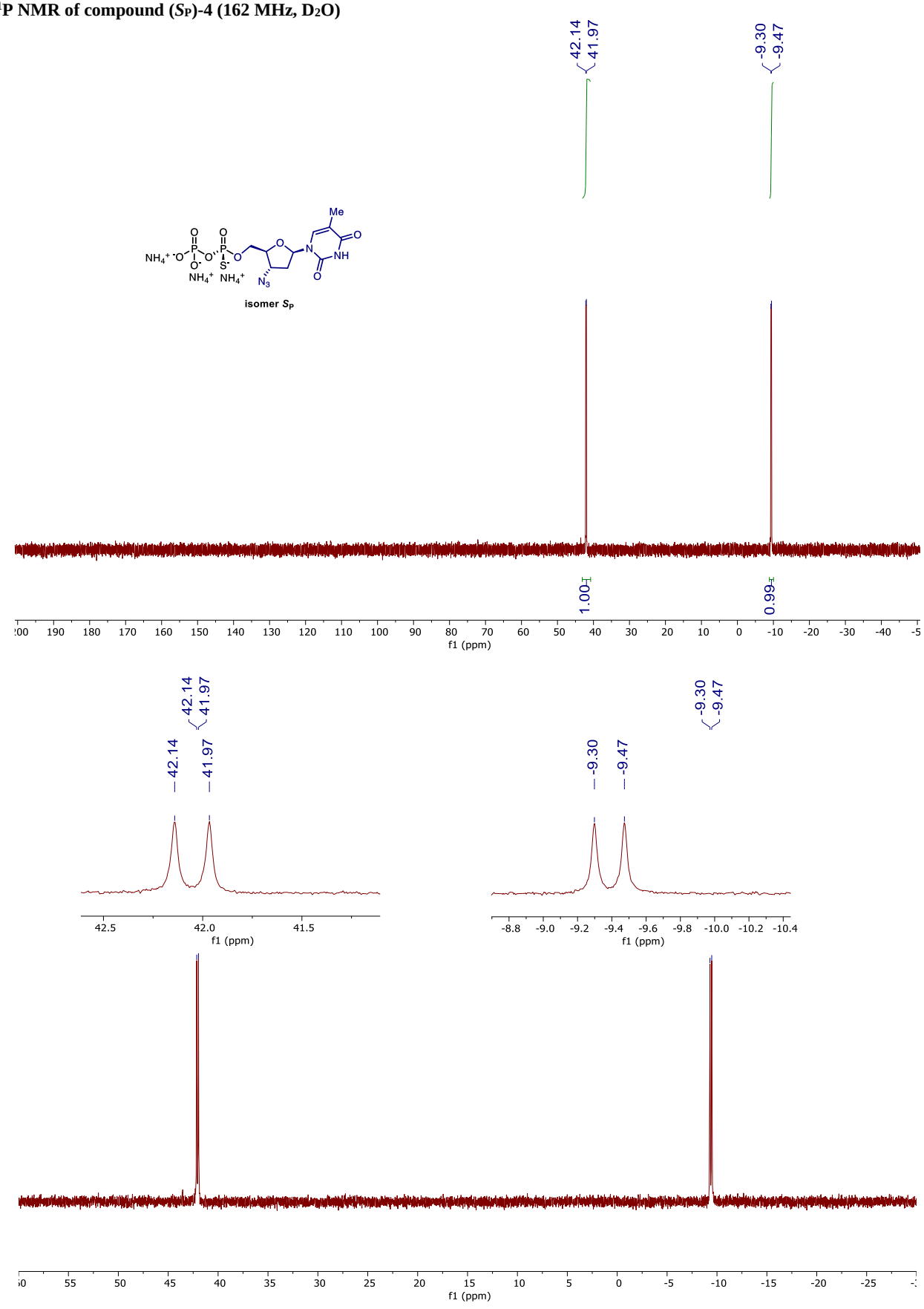
^{1}H NMR of compound (S_P)-4 (600 MHz, D_2O)



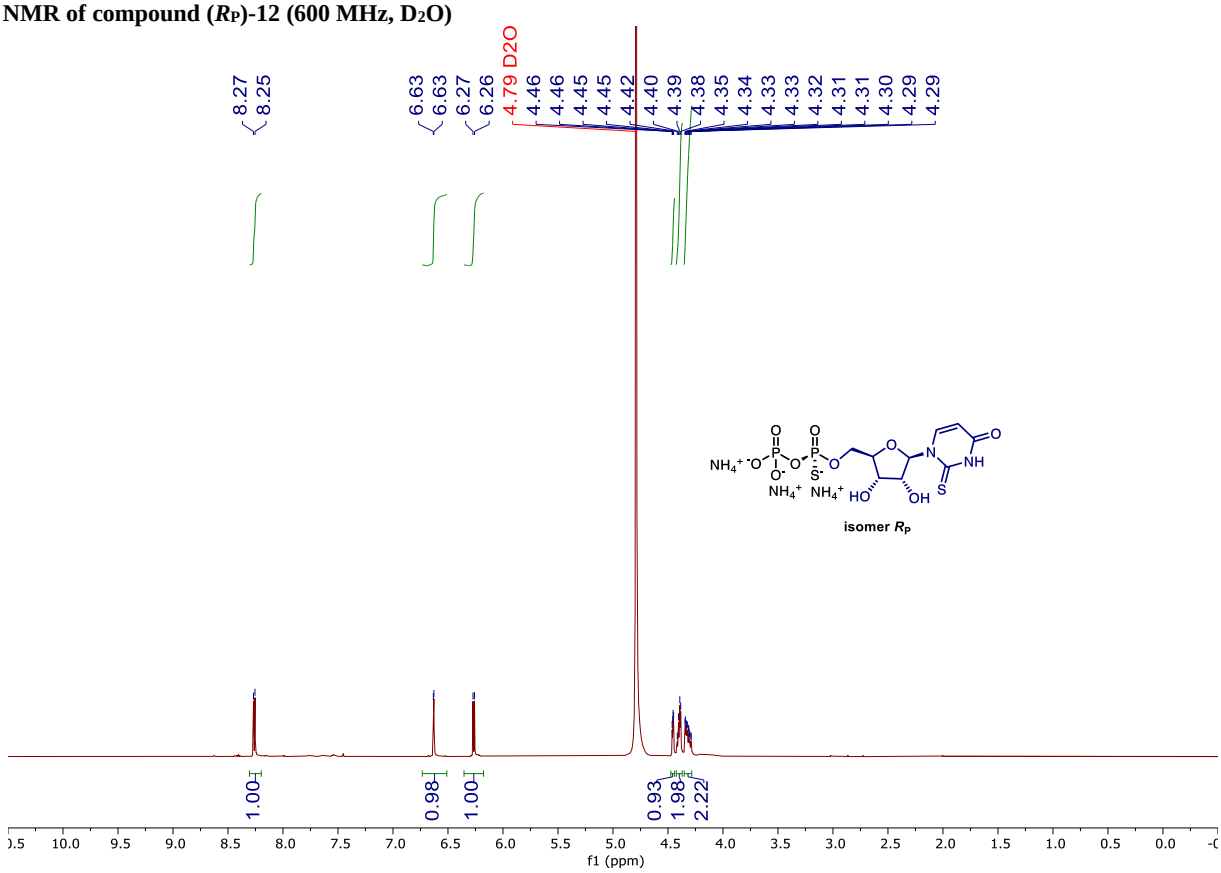
^{13}C NMR of compound (S_P)-4 (150 MHz, D_2O)



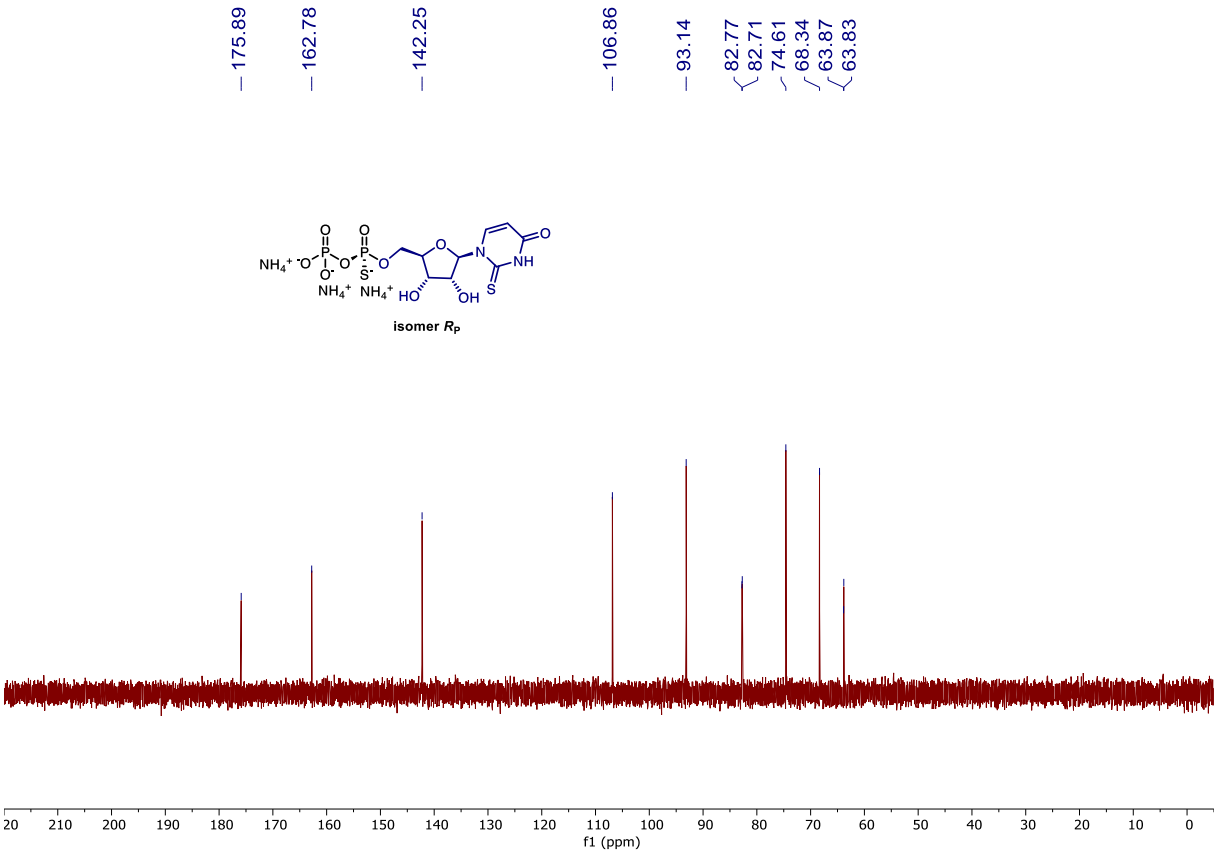
^{31}P NMR of compound (S_P)-4 (162 MHz, D_2O)



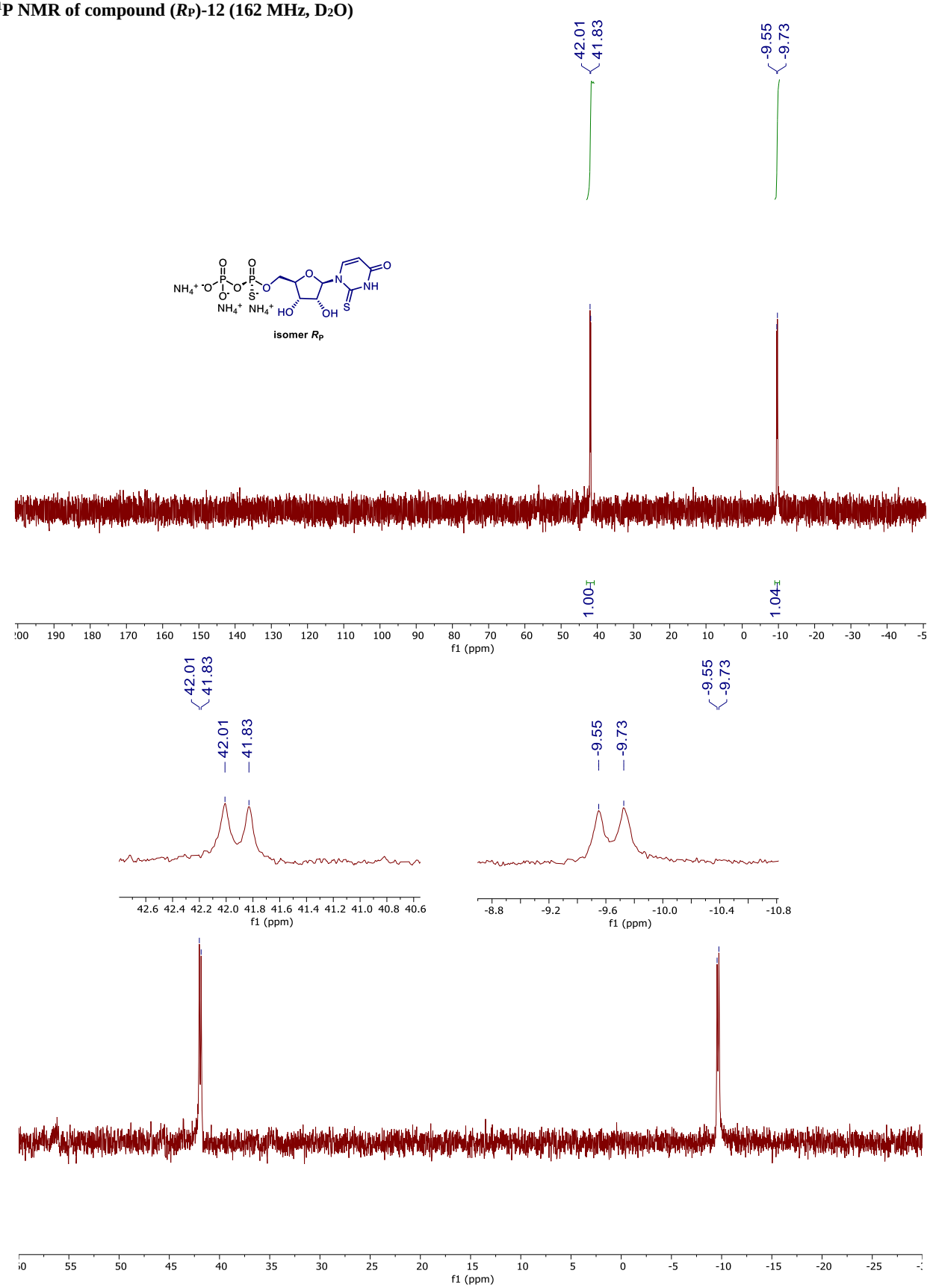
^{1}H NMR of compound (R_P)-12 (600 MHz, D_2O)



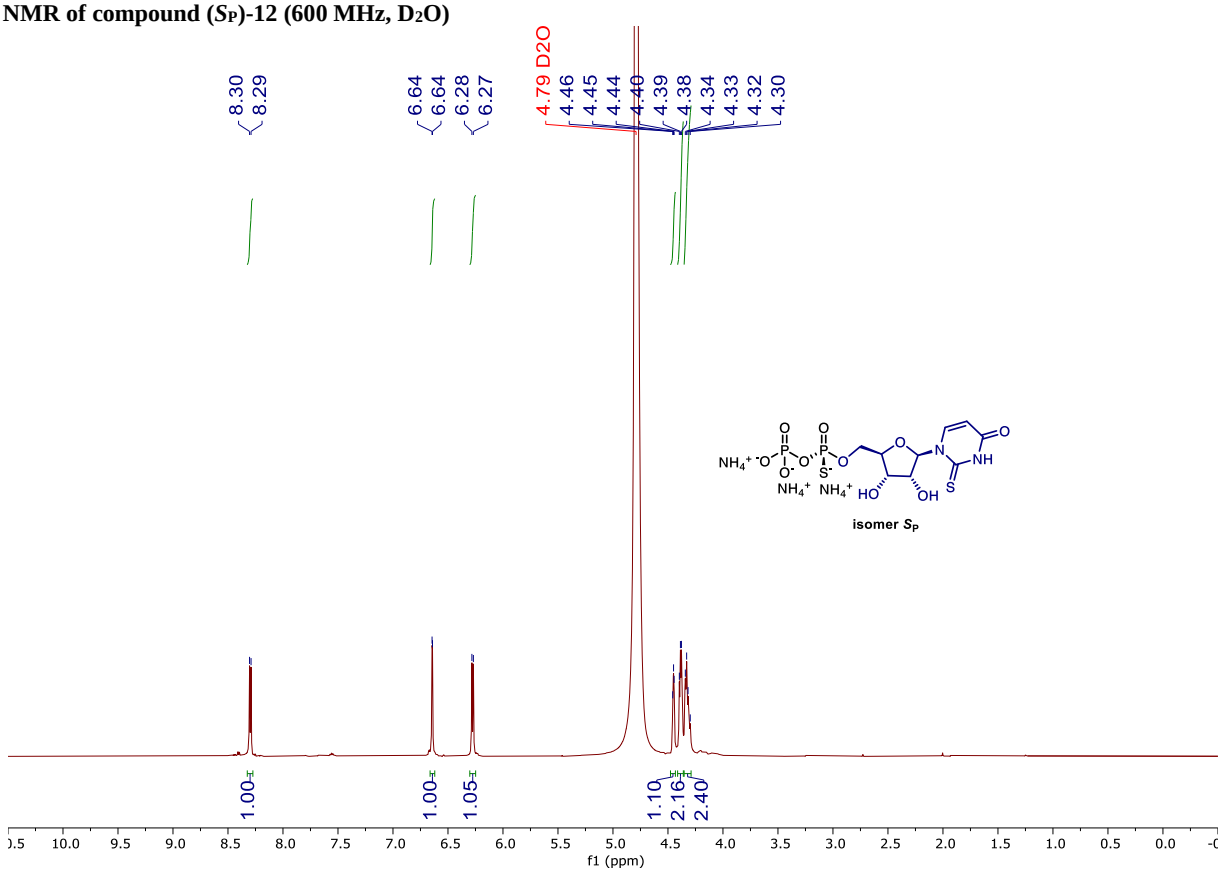
^{13}C NMR of compound (R_P)-12 (150 MHz, D_2O)



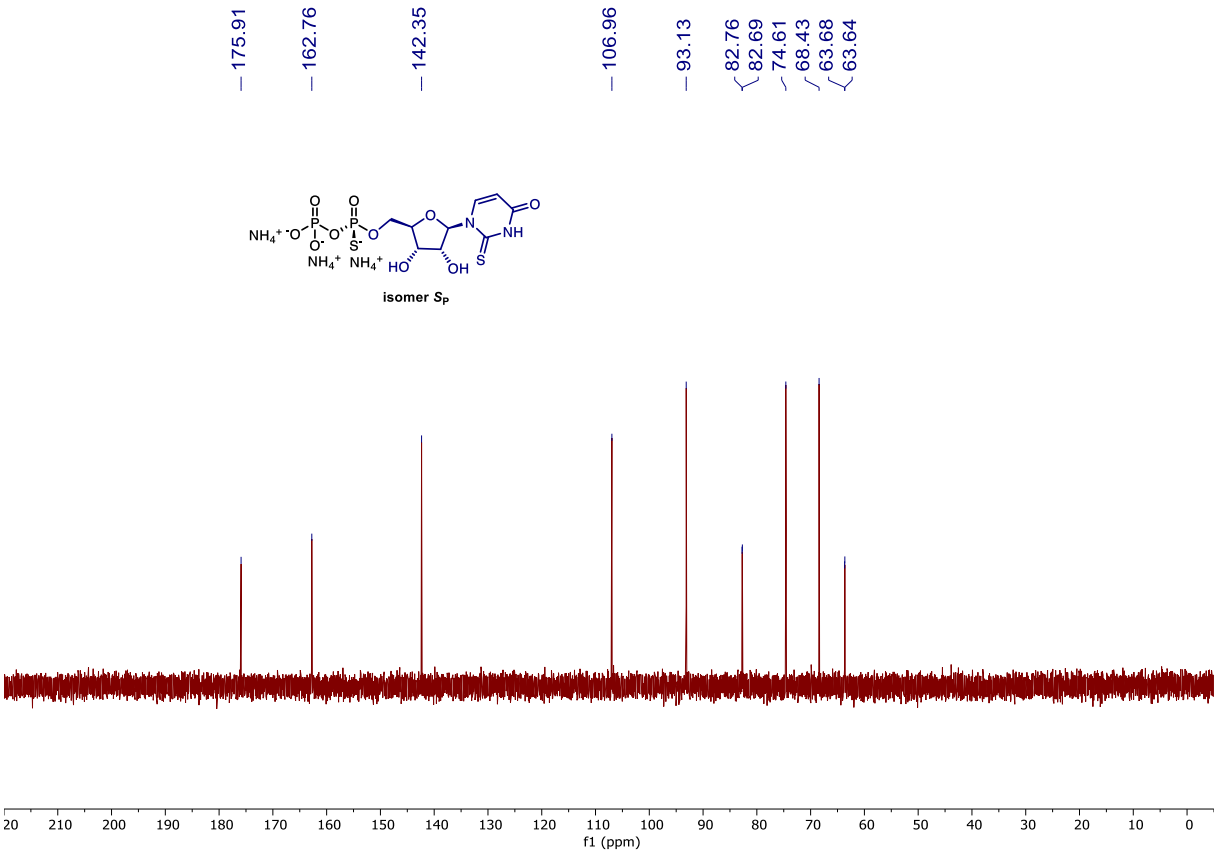
^{31}P NMR of compound (R_P)-12 (162 MHz, D_2O)



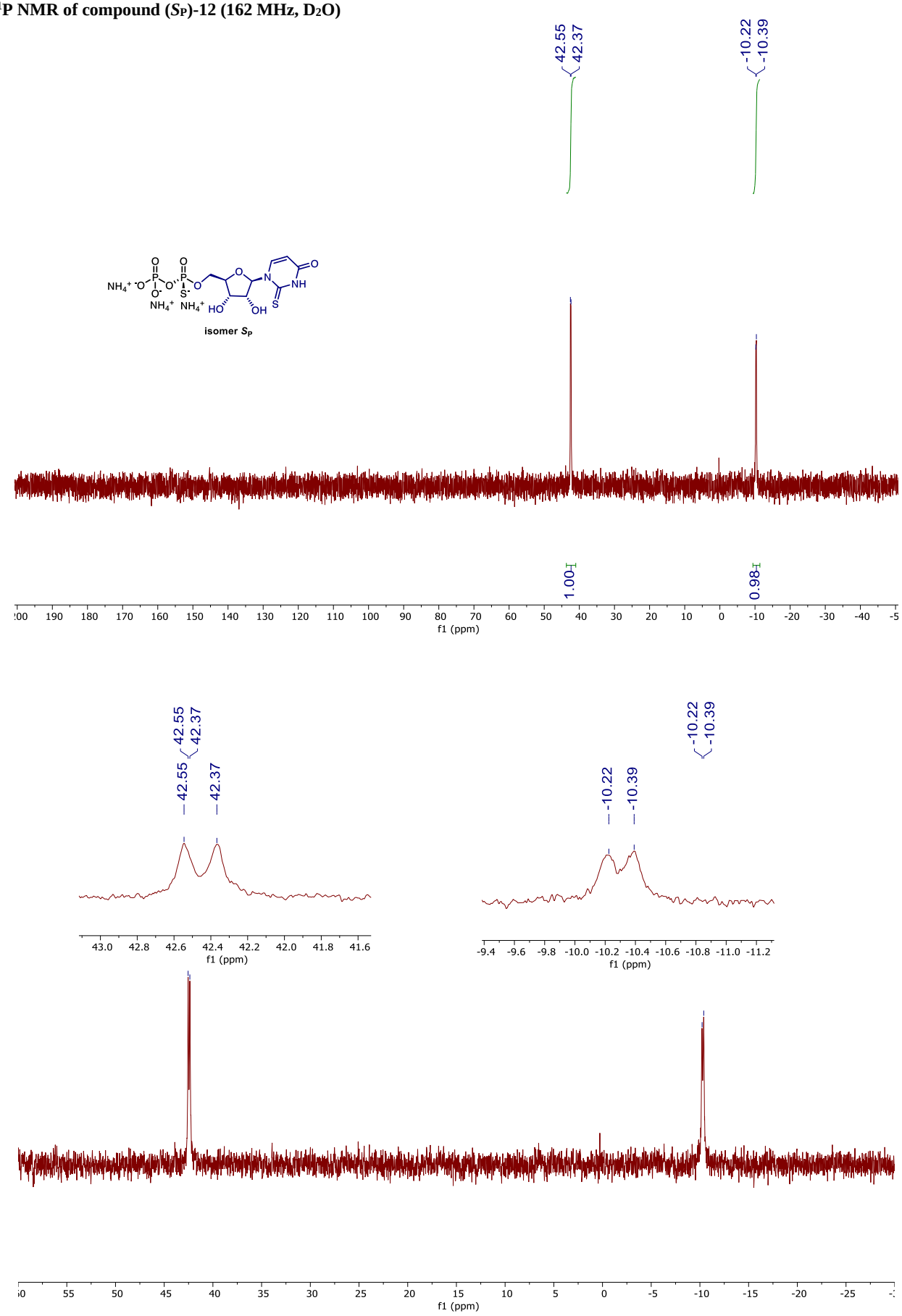
^{1}H NMR of compound (S_P)-12 (600 MHz, D_2O)



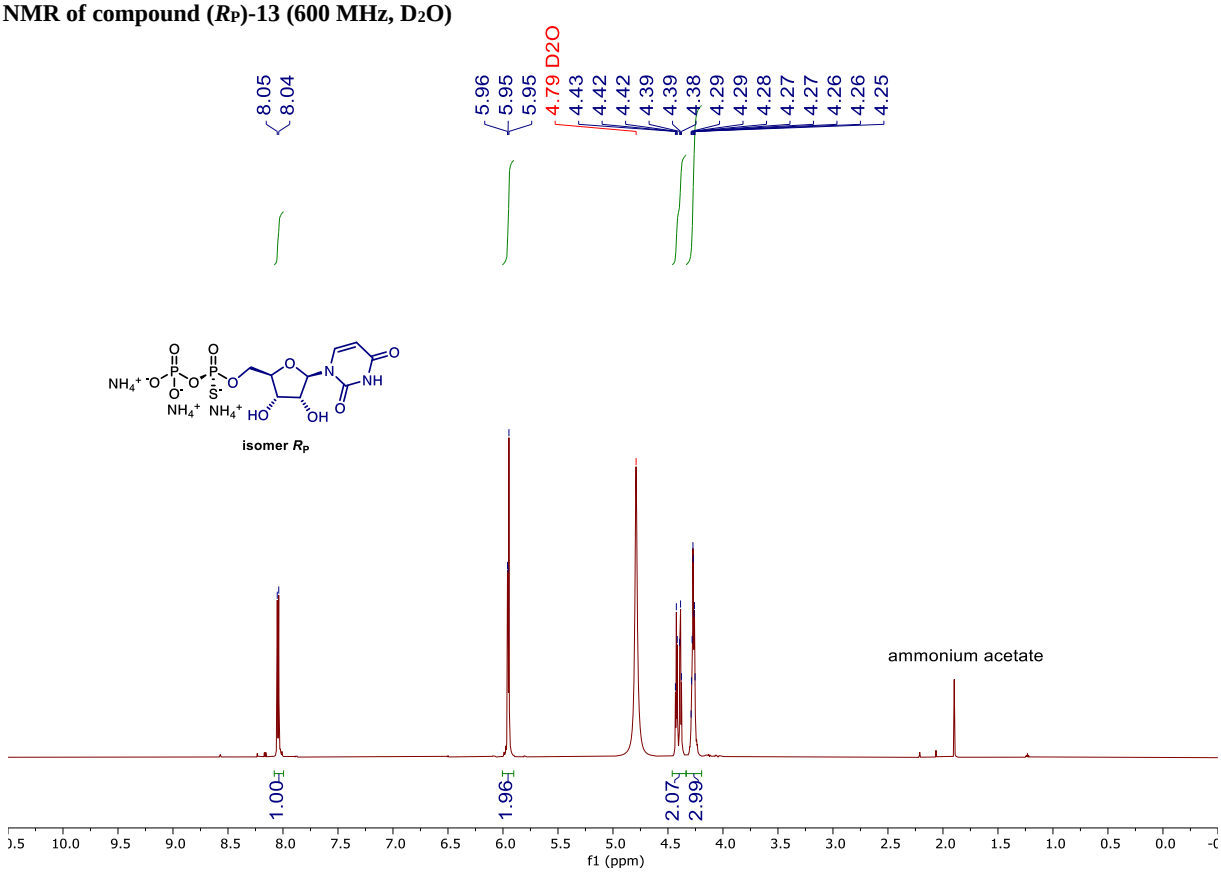
^{13}C NMR of compound (S_P)-12 (150 MHz, D_2O)



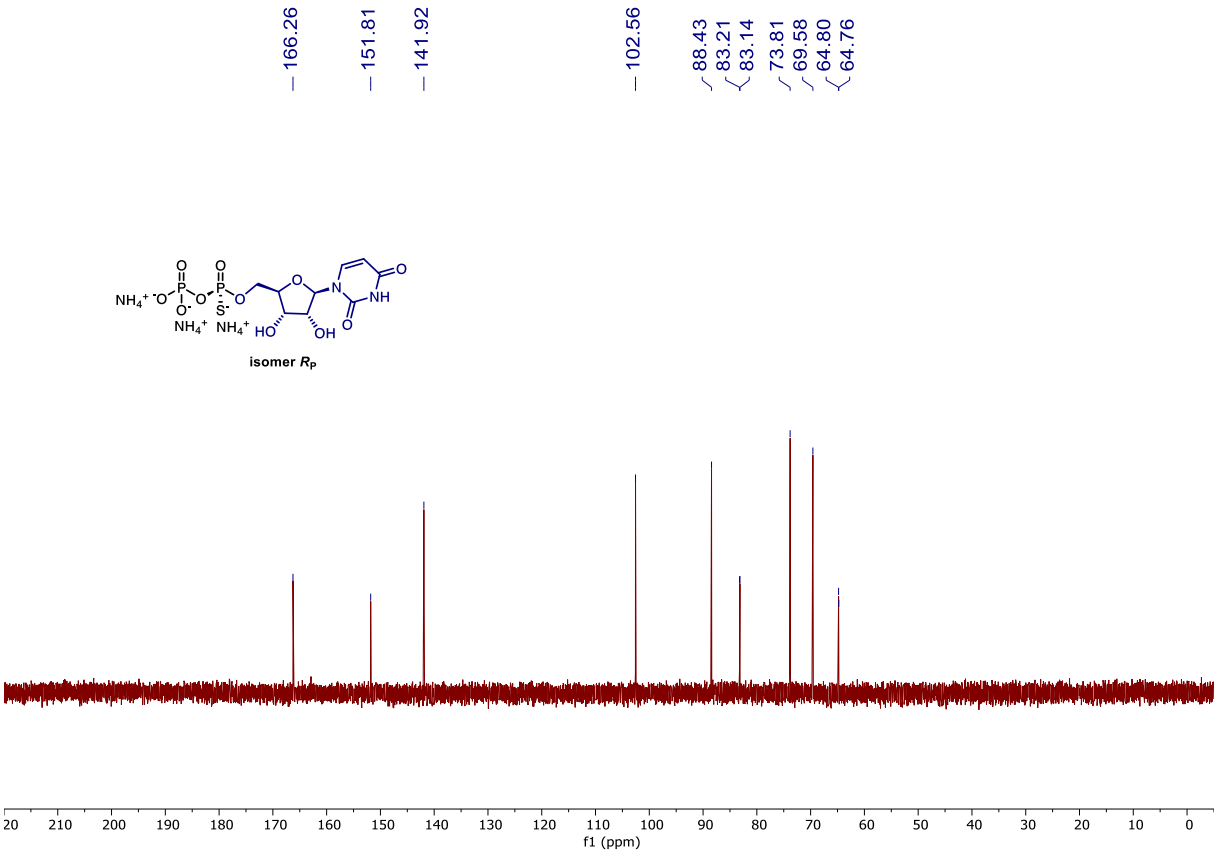
^{31}P NMR of compound (S_P)-12 (162 MHz, D_2O)



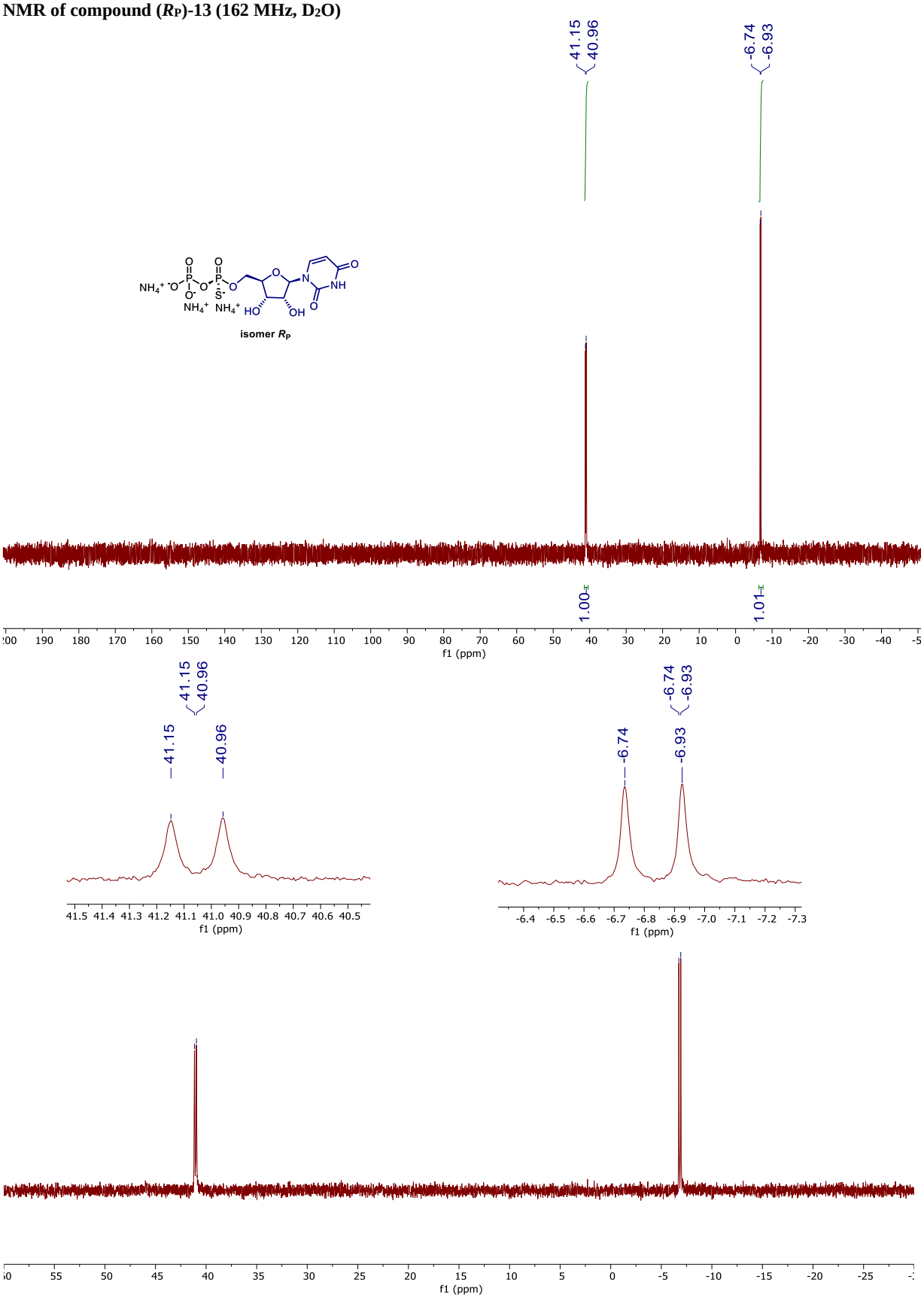
^{1}H NMR of compound (R_P)-13 (600 MHz, D_2O)



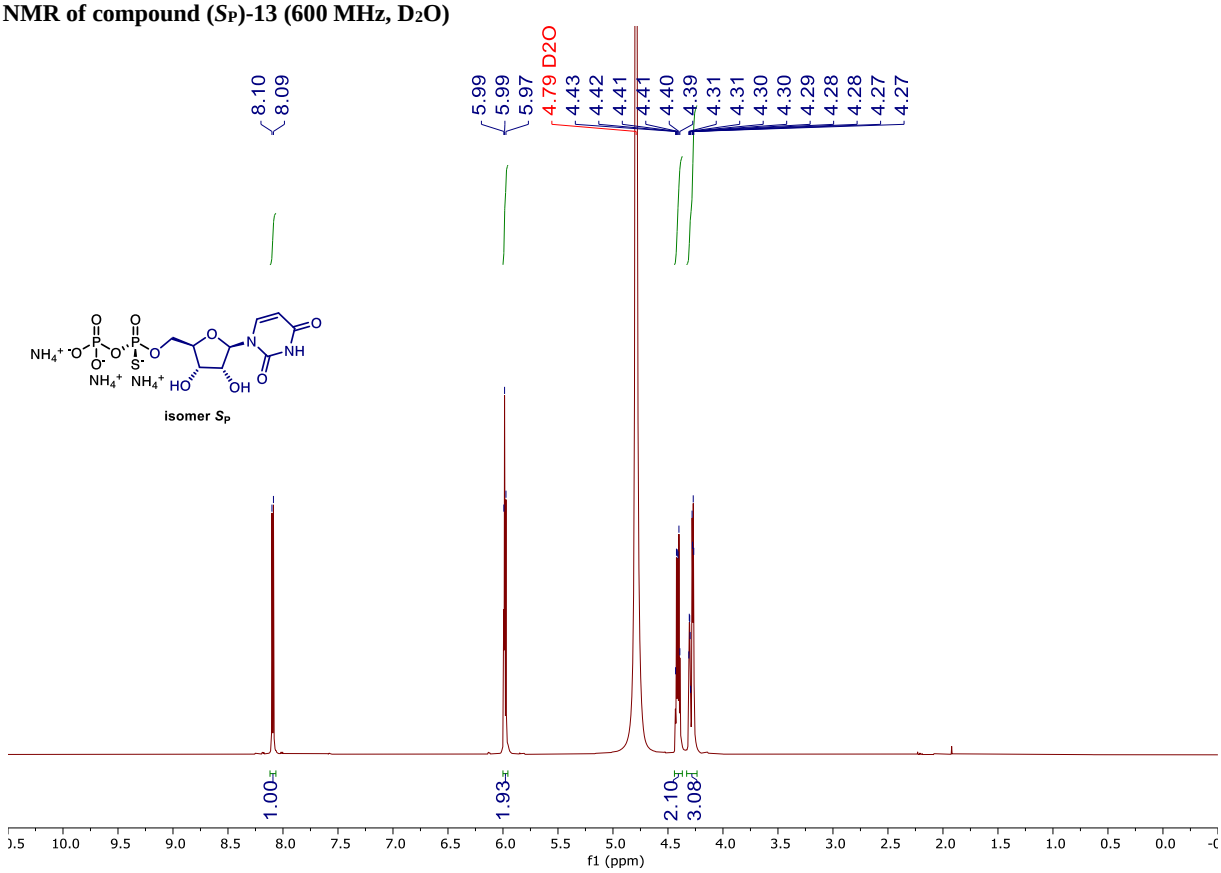
^{13}C NMR of compound (R_P)-13 (150 MHz, D_2O)



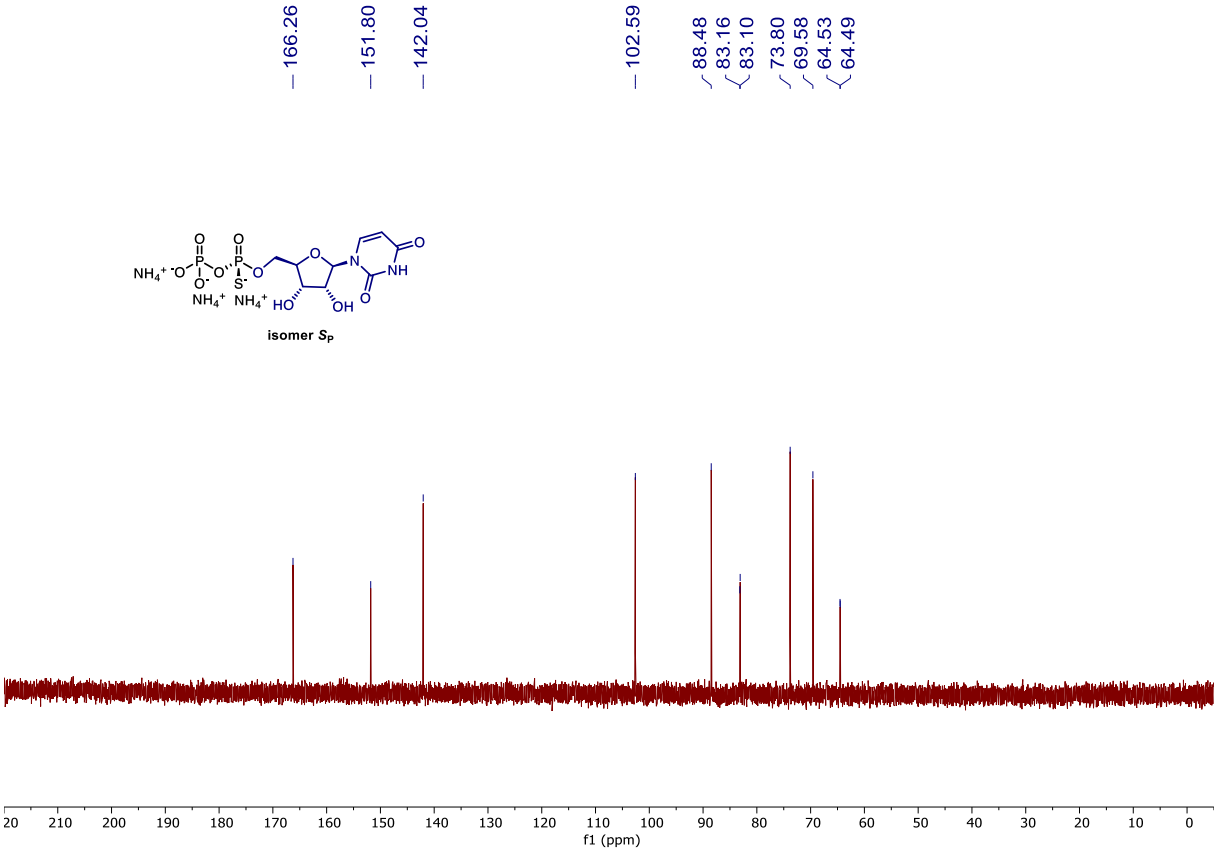
^{31}P NMR of compound (R_P)-13 (162 MHz, D_2O)



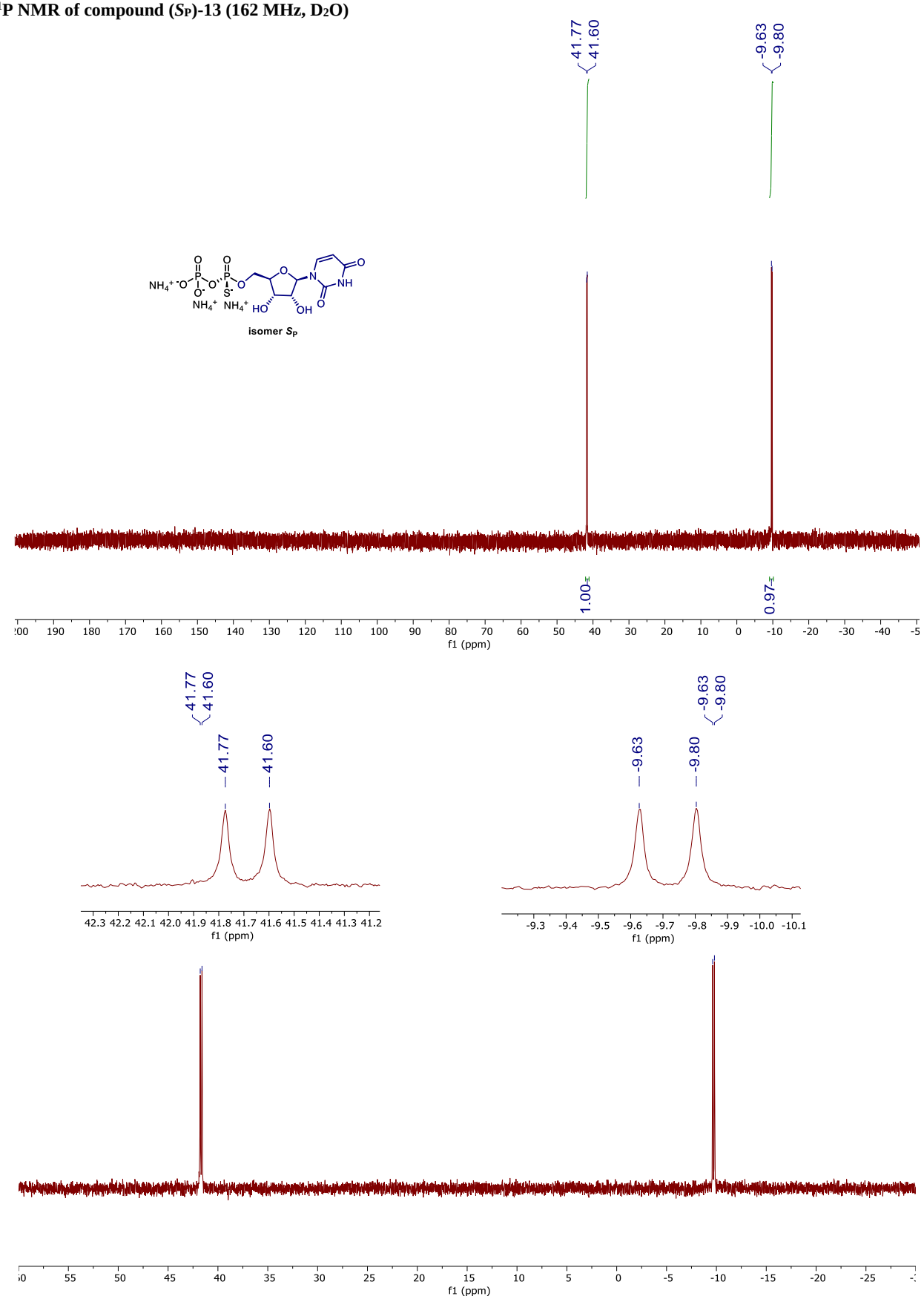
^{1}H NMR of compound (S_P)-13 (600 MHz, D_2O)



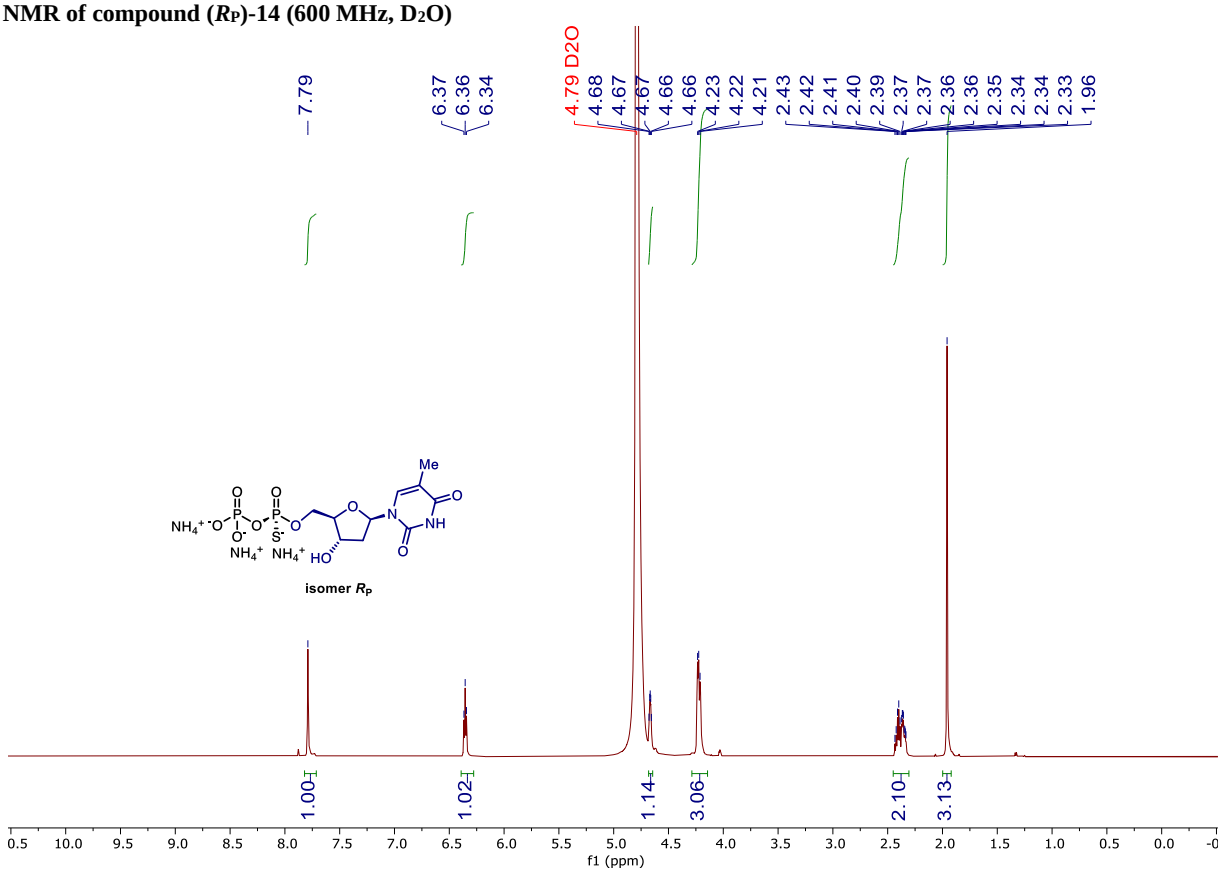
^{13}C NMR of compound (S_P)-13 (150 MHz, D_2O)



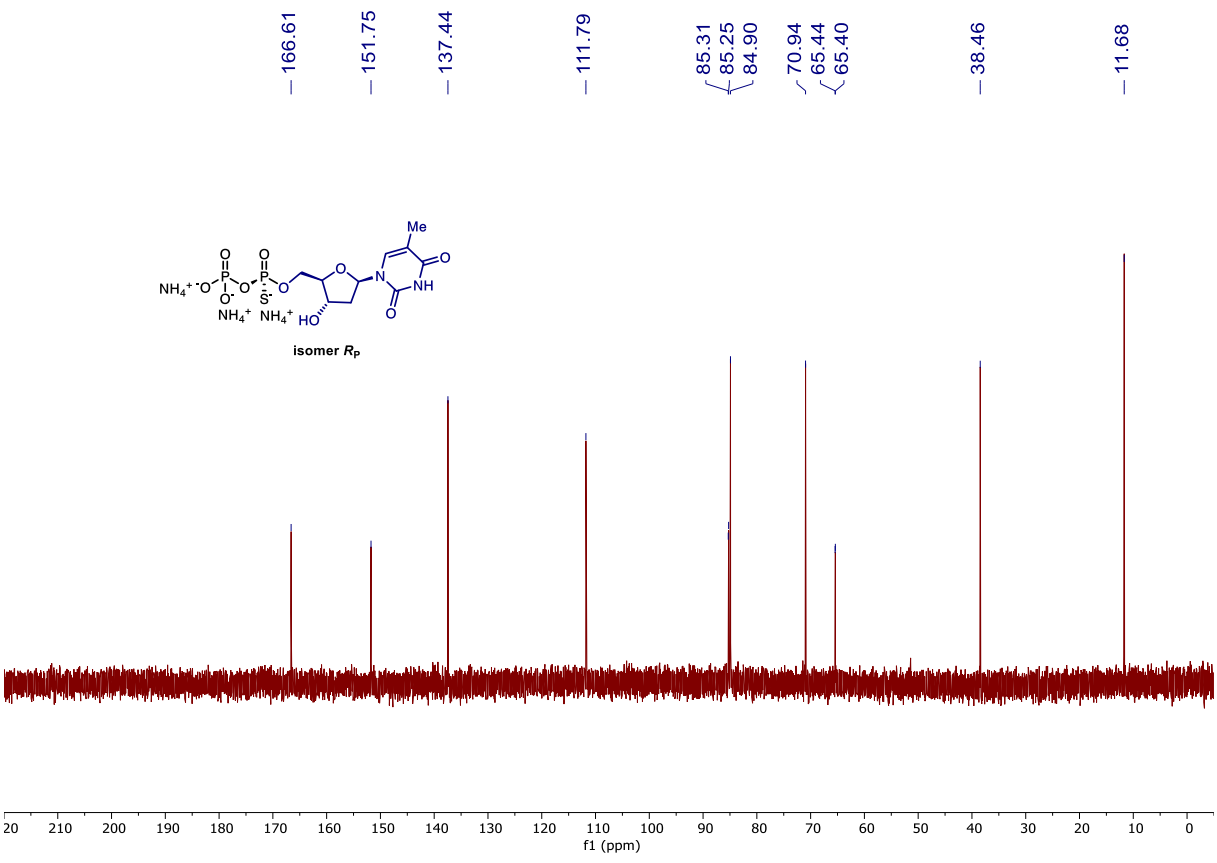
^{31}P NMR of compound (S_P)-13 (162 MHz, D_2O)



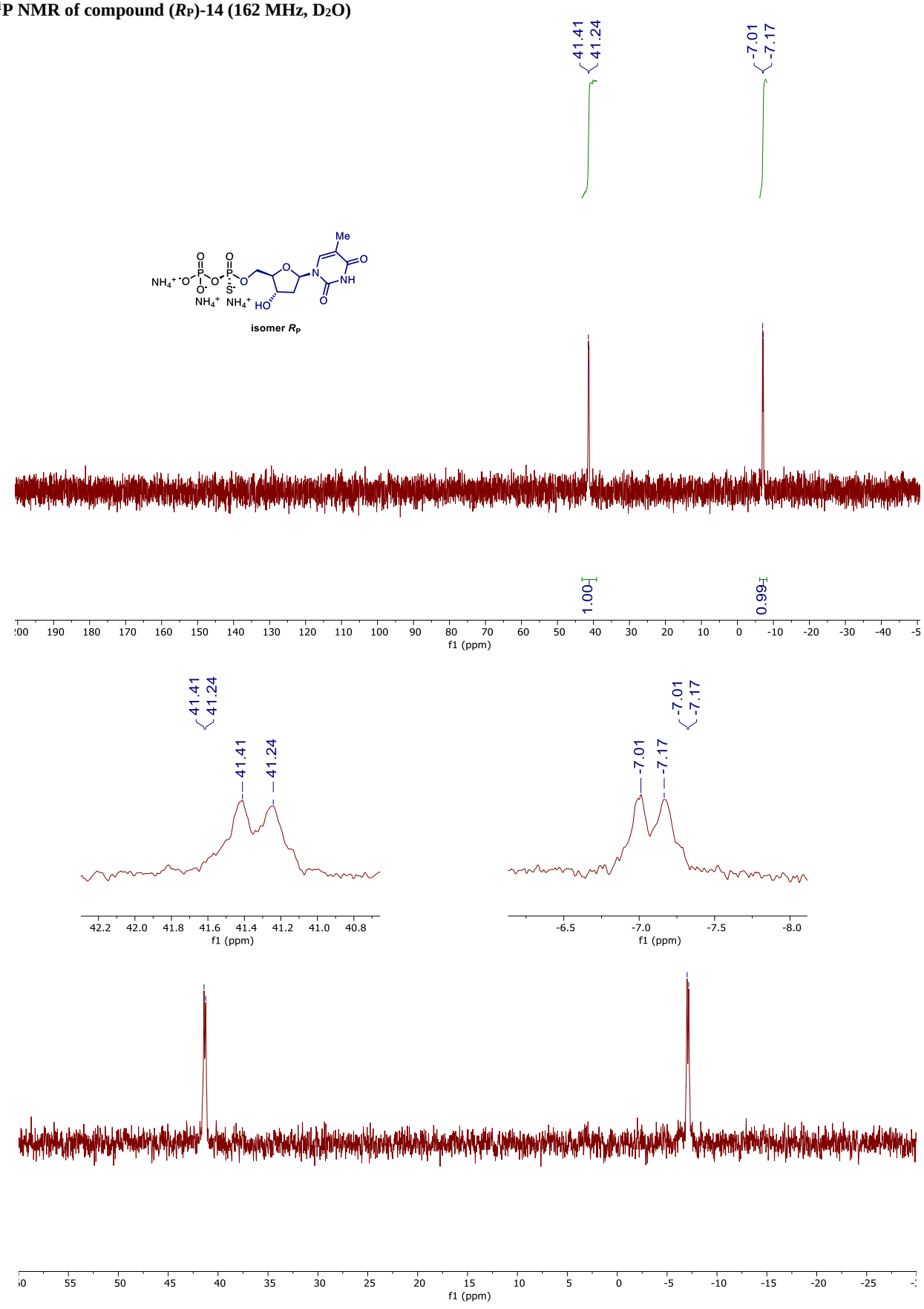
^{1}H NMR of compound (R_P)-14 (600 MHz, D_2O)



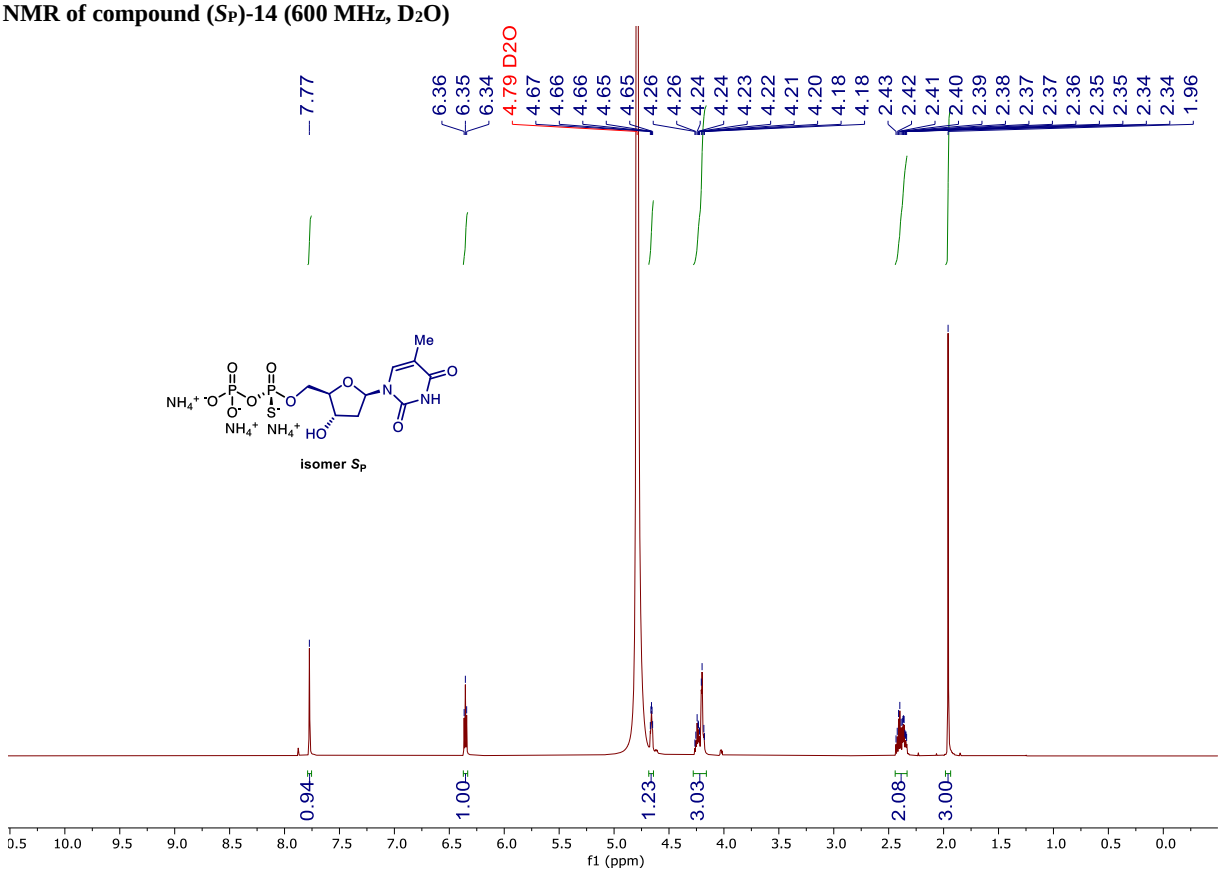
^{13}C NMR of compound (R_P)-14 (150 MHz, D_2O)



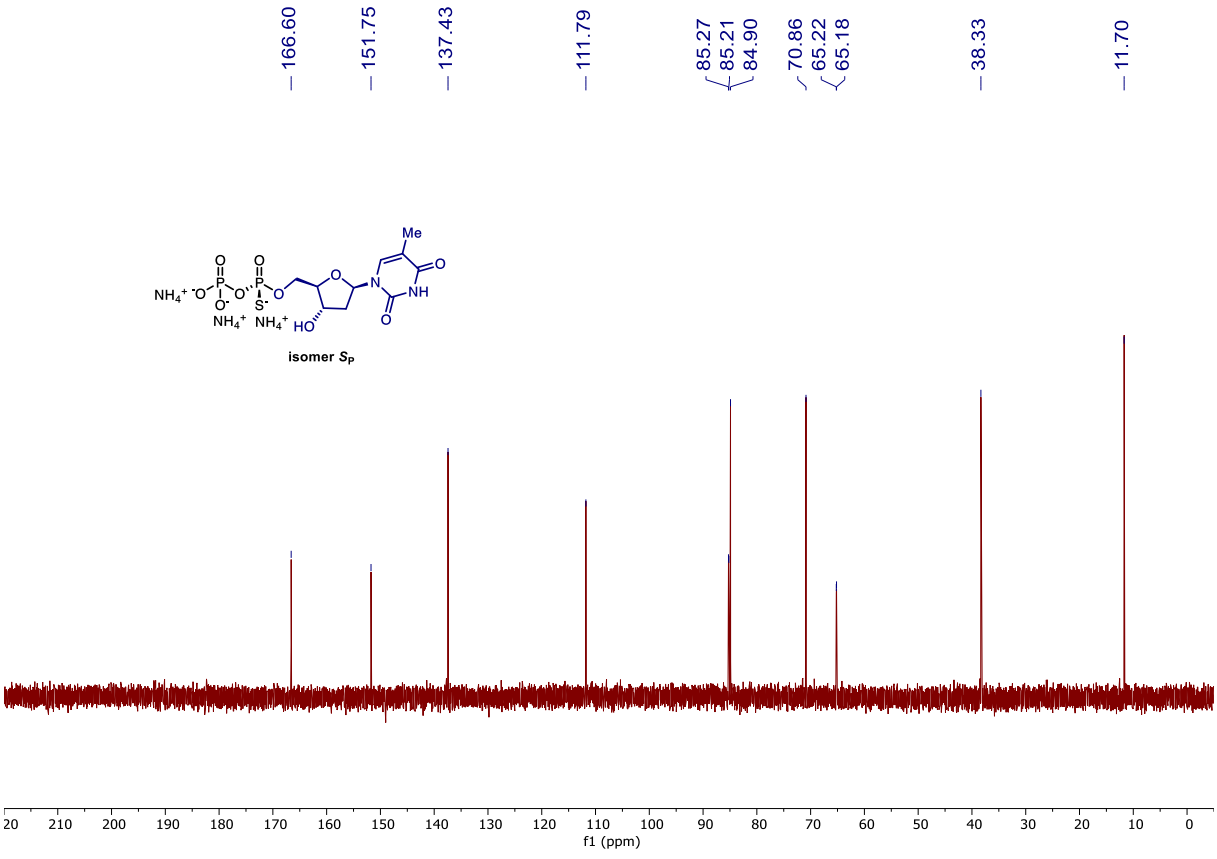
^{31}P NMR of compound (R_P)-14 (162 MHz, D_2O)



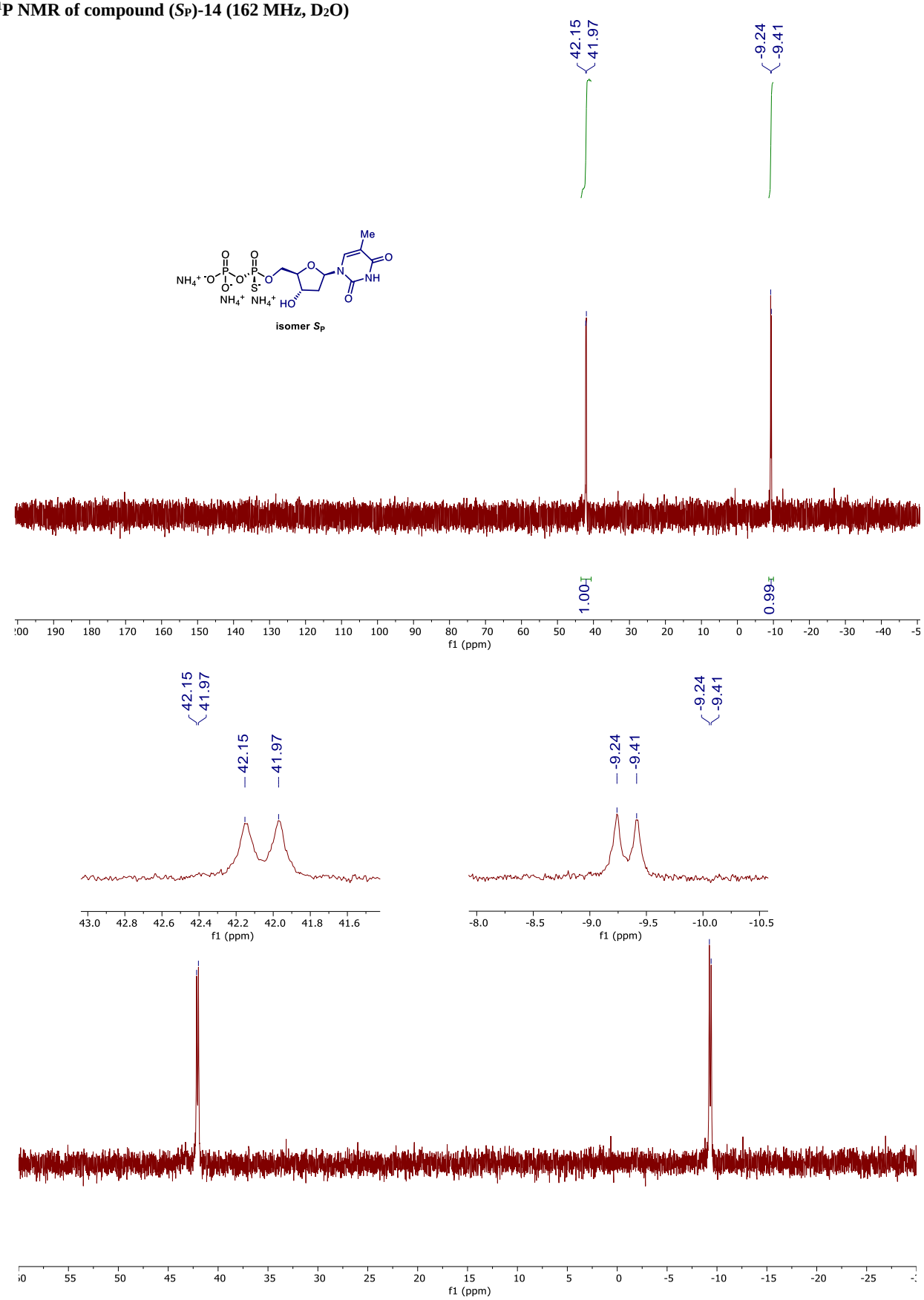
^{1}H NMR of compound (S_P)-14 (600 MHz, D_2O)



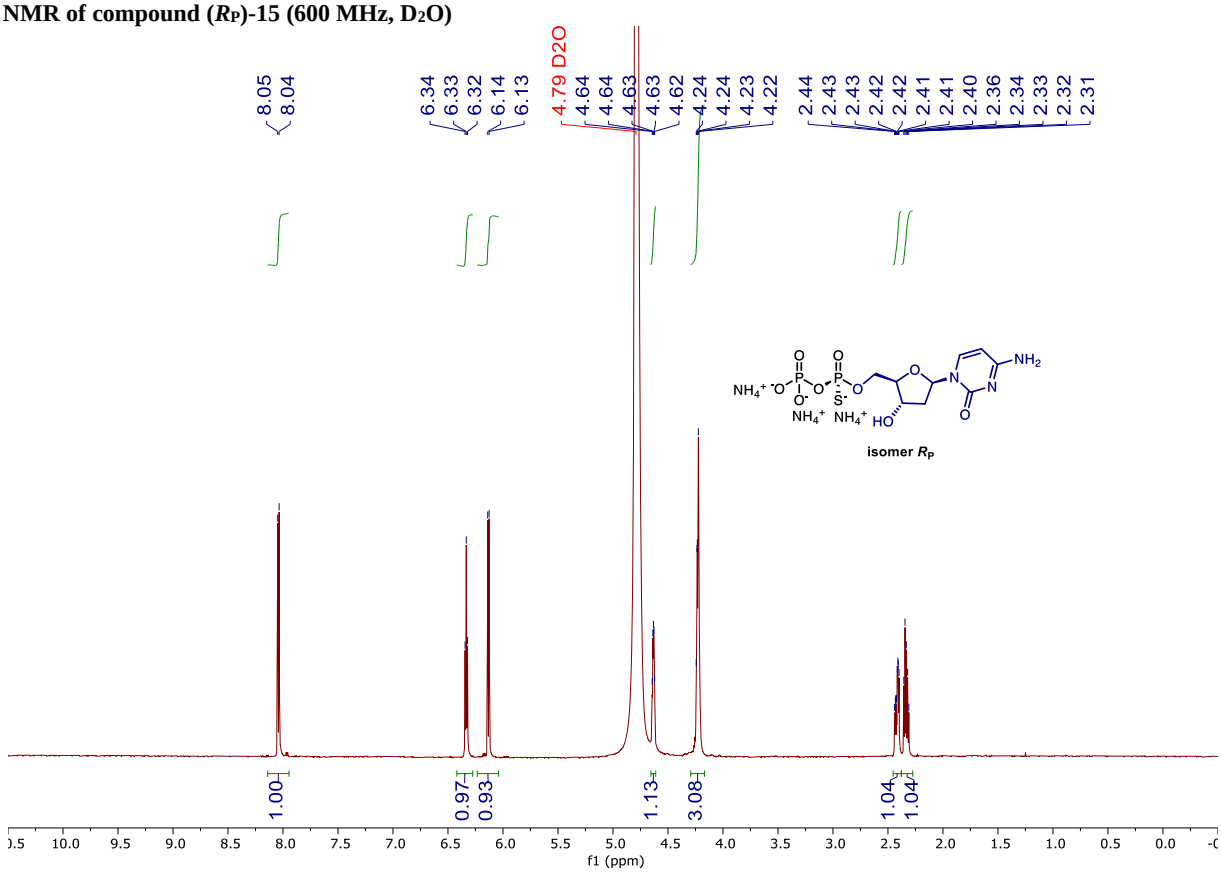
^{13}C NMR of compound (S_P)-14 (150 MHz, D_2O)



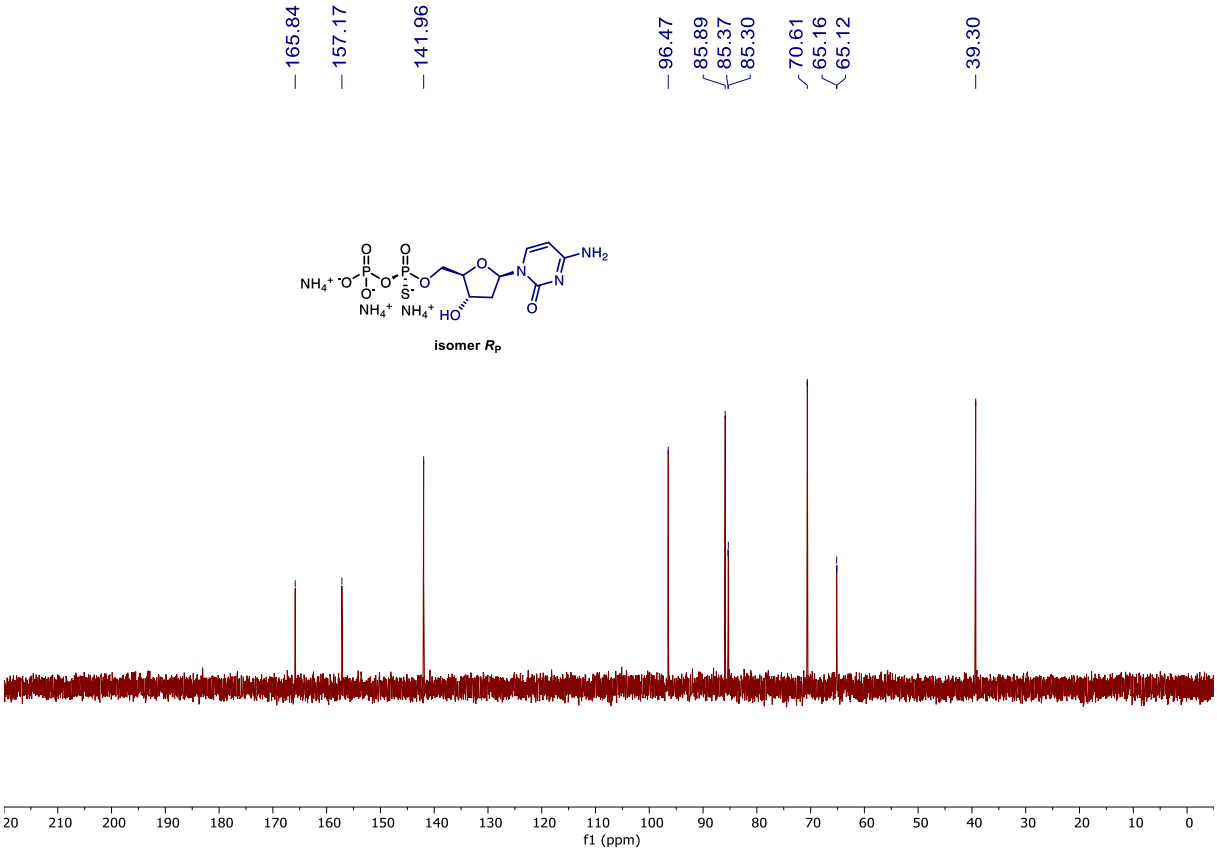
^{31}P NMR of compound (S_P)-14 (162 MHz, D_2O)



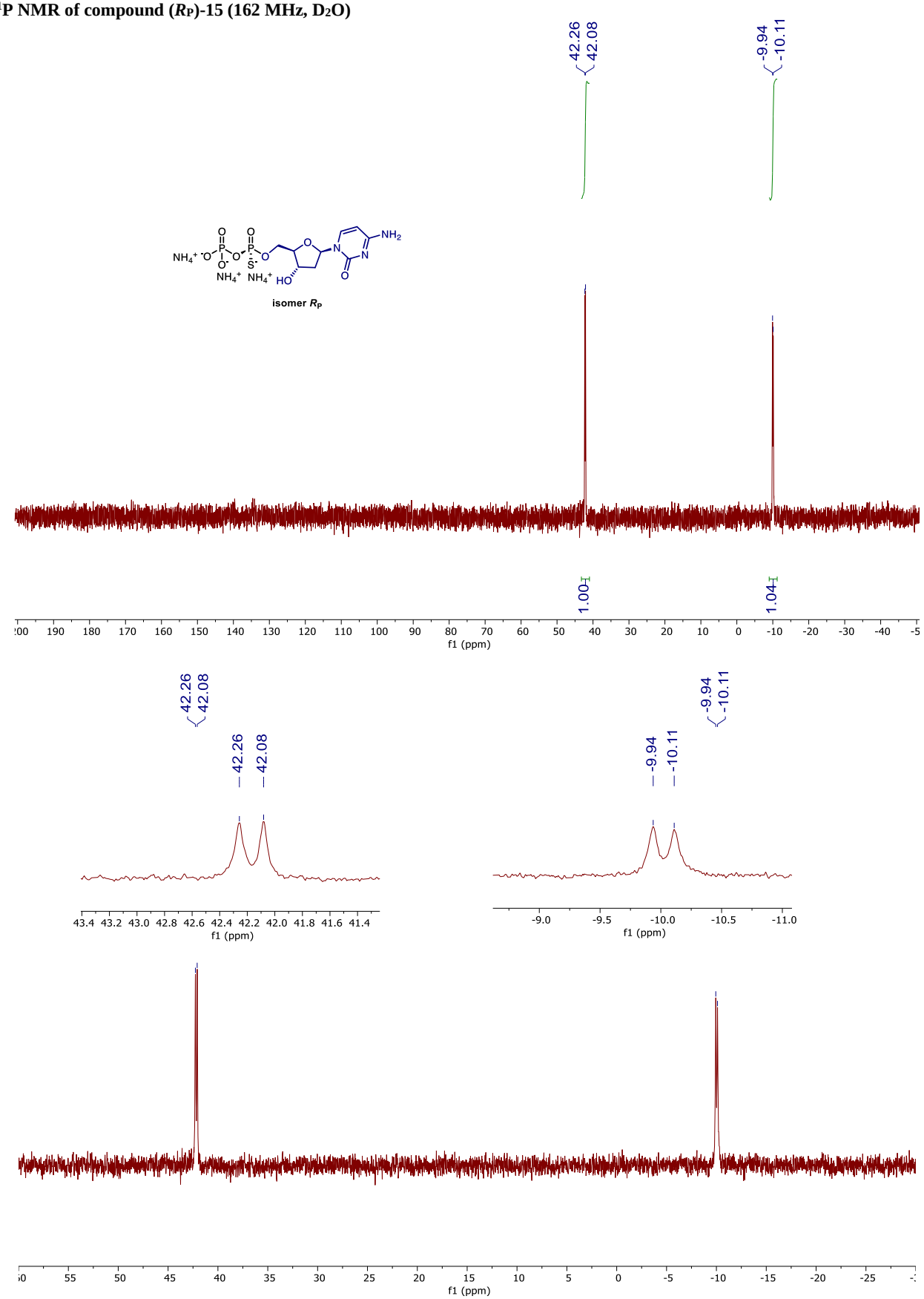
^{1}H NMR of compound (R_P)-15 (600 MHz, D_2O)



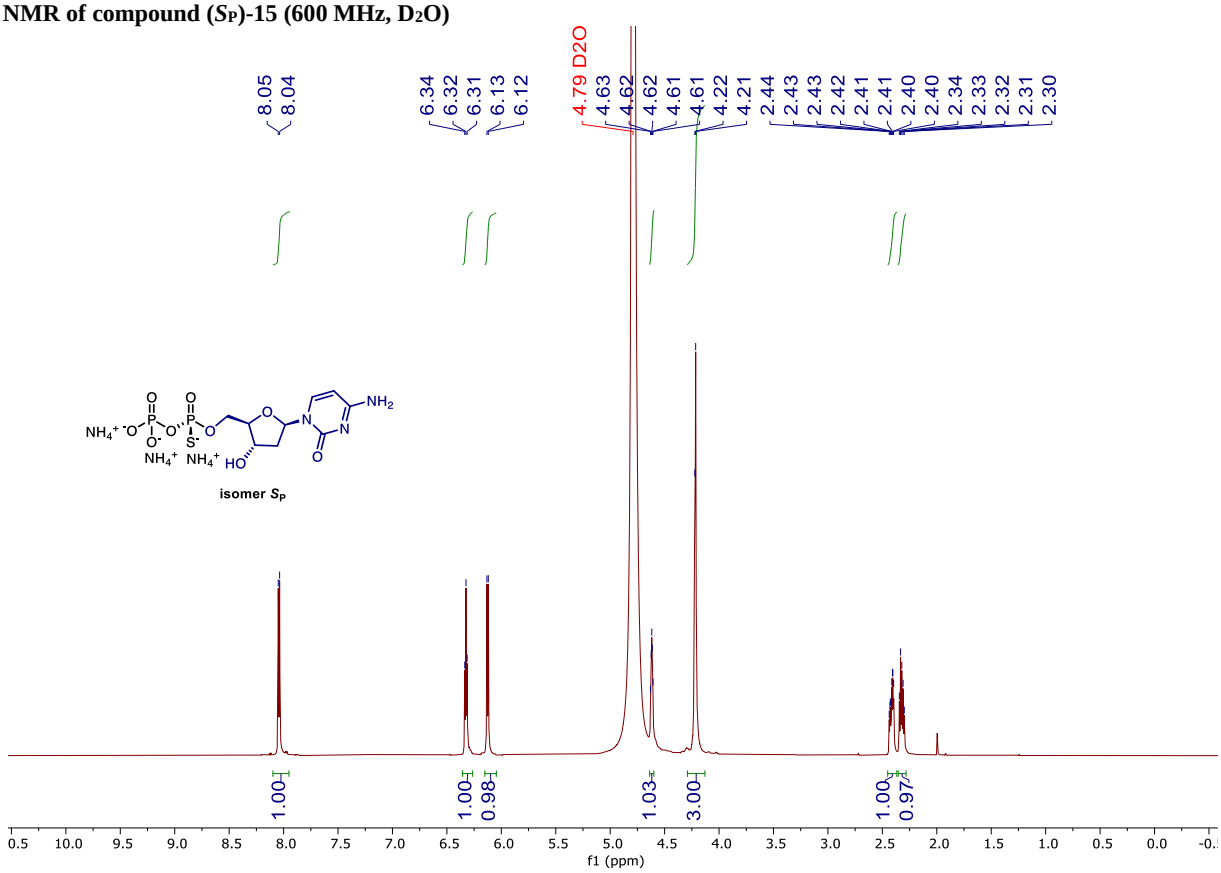
^{13}C NMR of compound (R_P)-15 (150 MHz, D_2O)



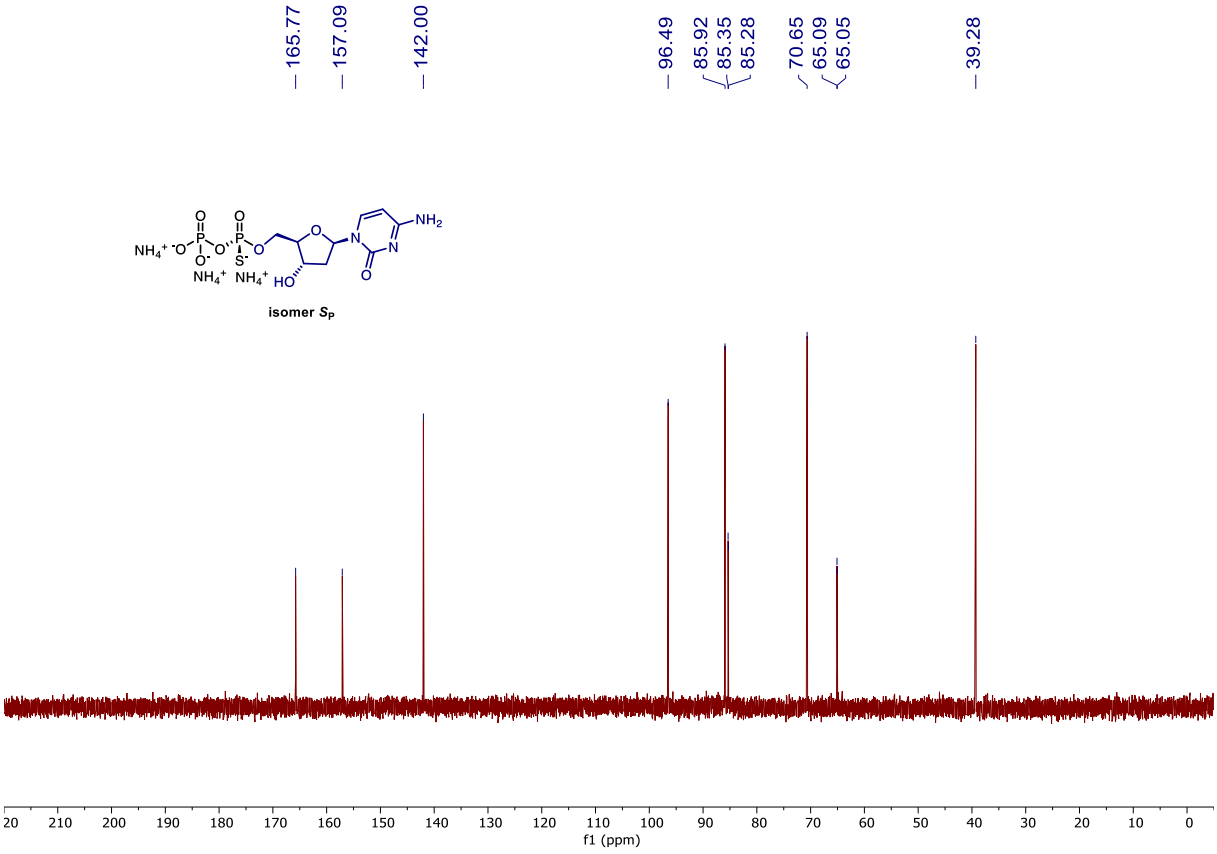
^{31}P NMR of compound (R_P)-15 (162 MHz, D_2O)



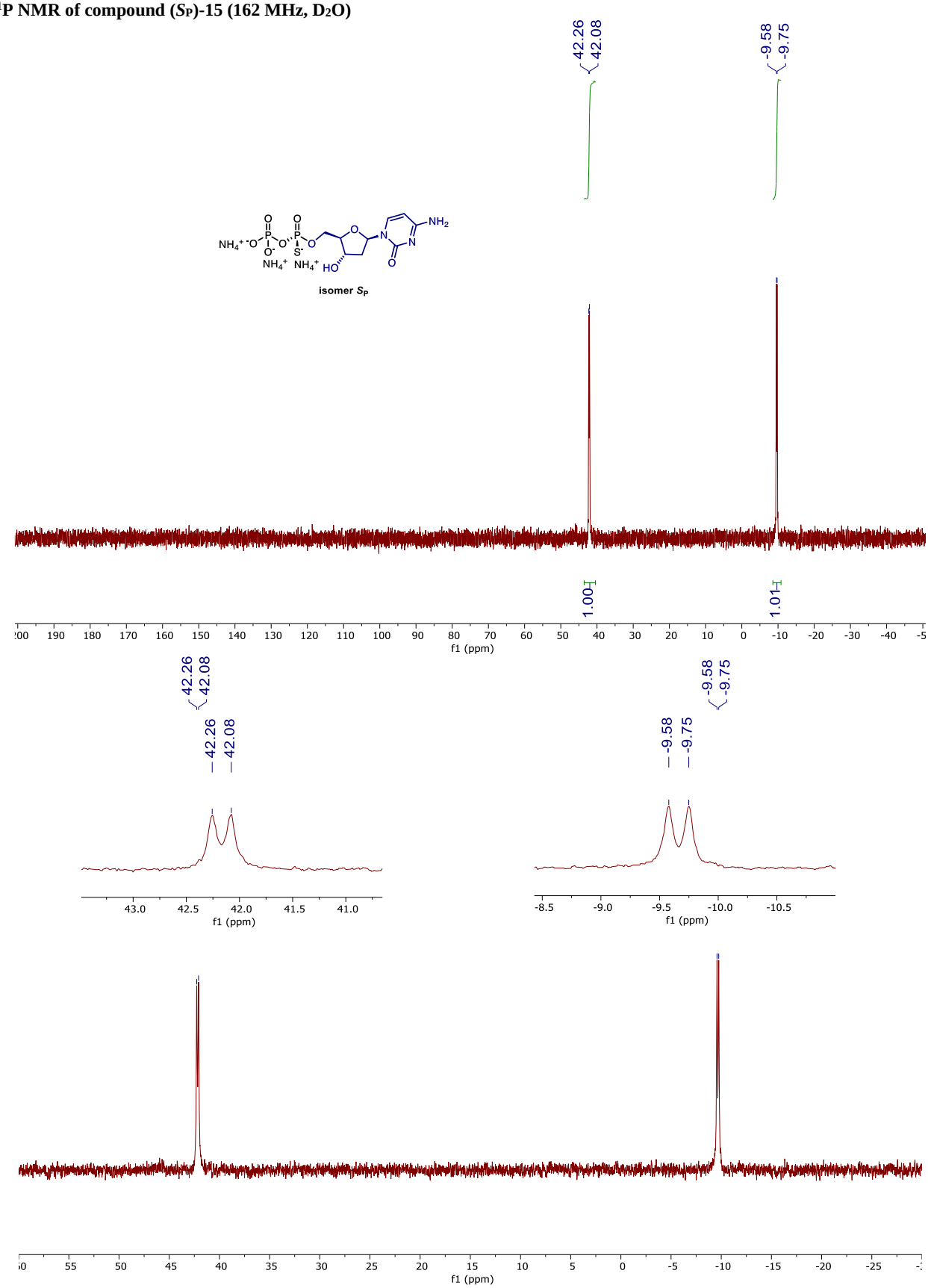
^{1}H NMR of compound (S_P)-15 (600 MHz, D_2O)



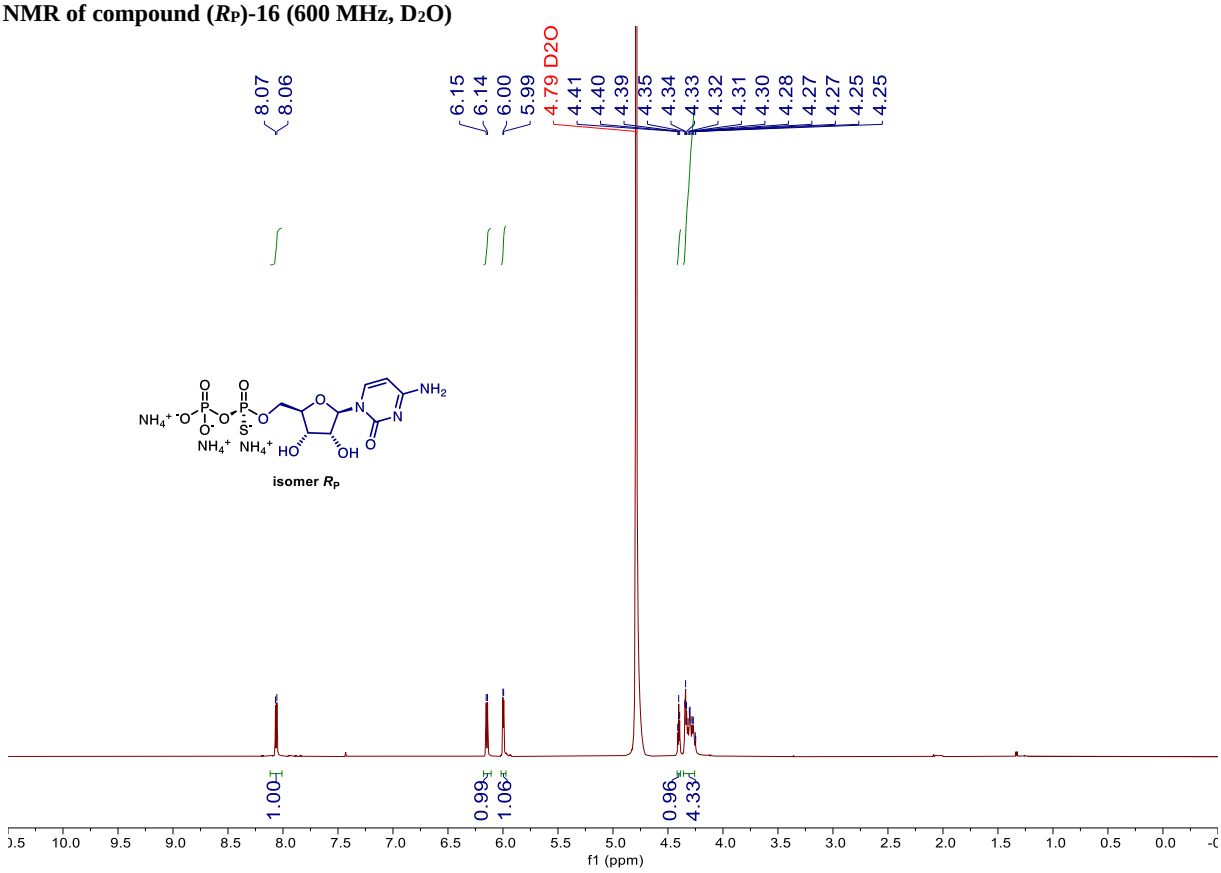
^{13}C NMR of compound (S_P)-15 (150 MHz, D_2O)



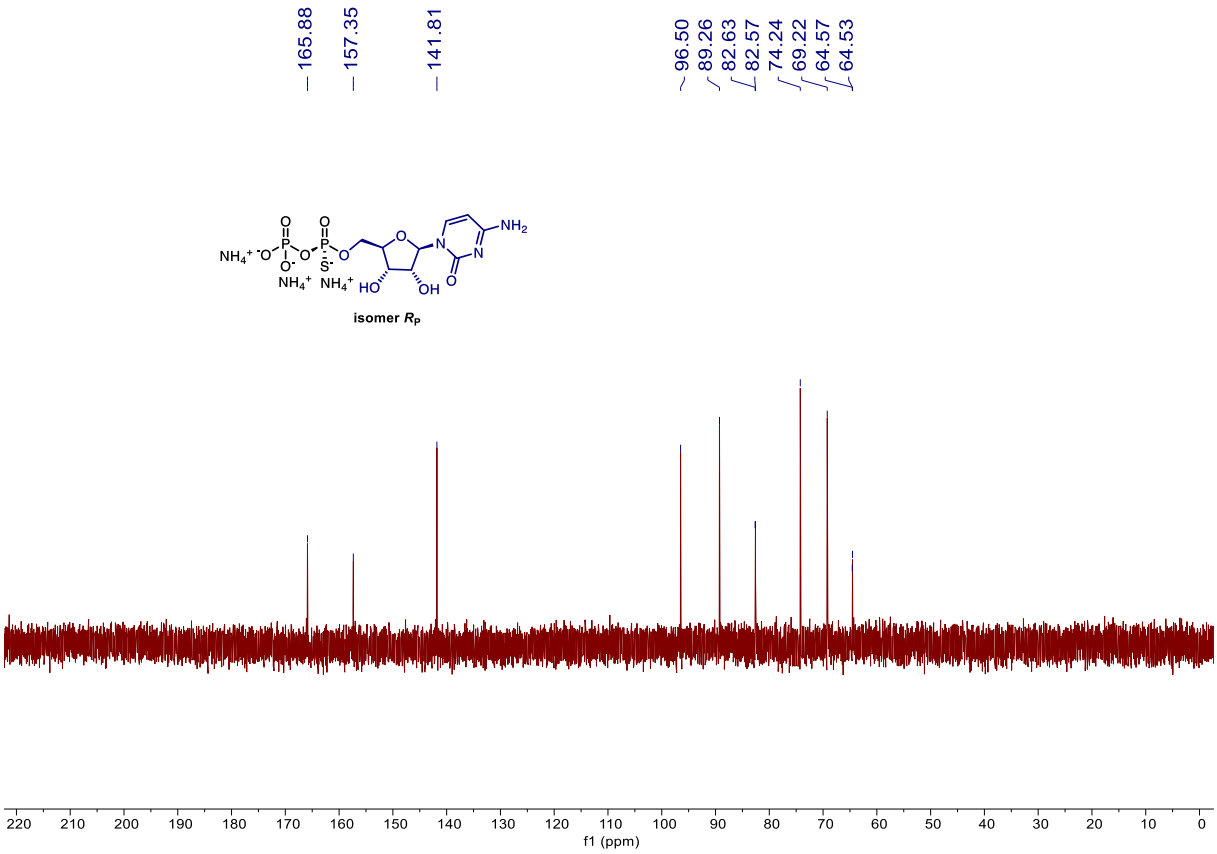
^{31}P NMR of compound (S_P)-15 (162 MHz, D_2O)



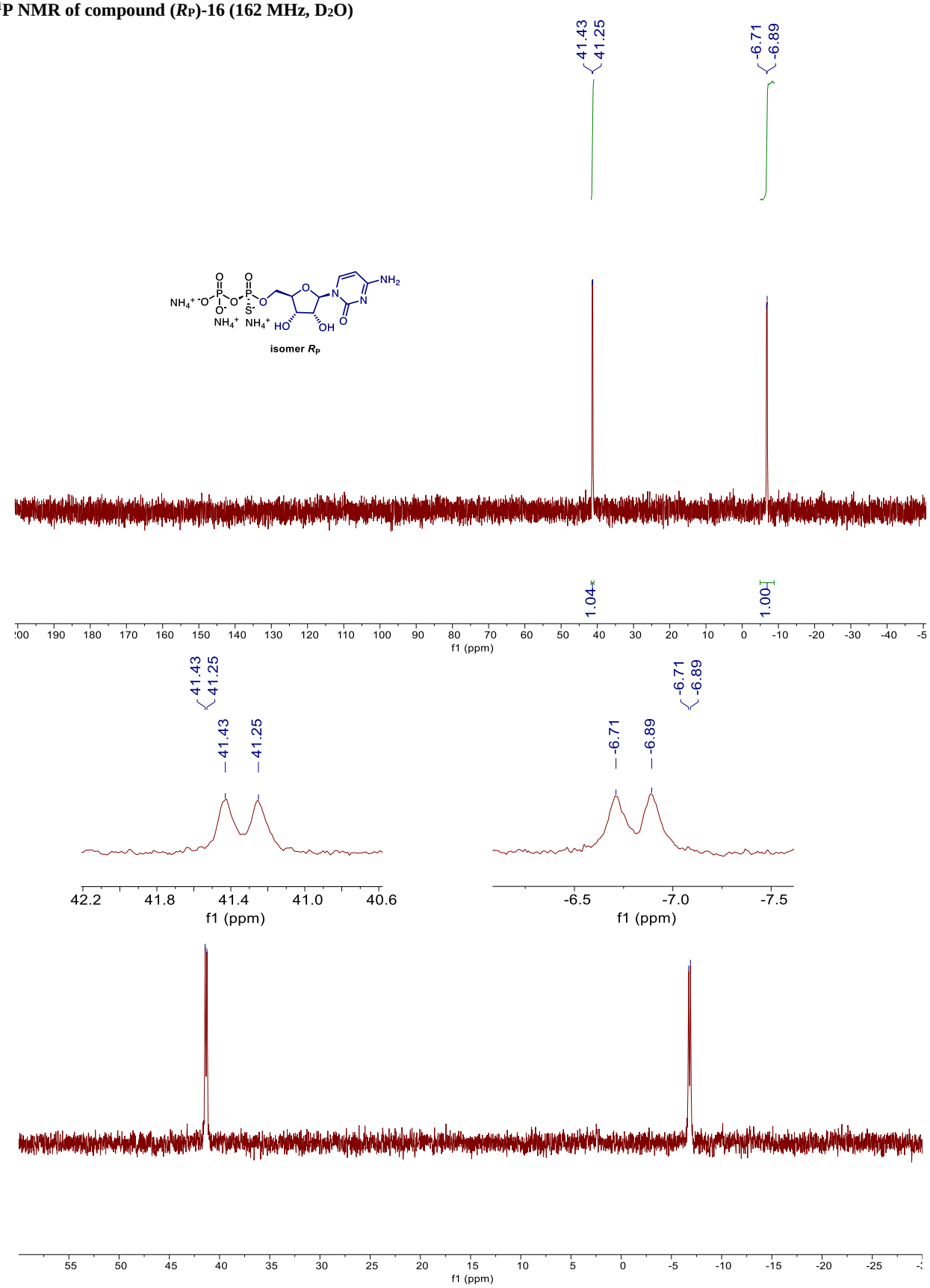
^{1}H NMR of compound (R_P)-16 (600 MHz, D_2O)



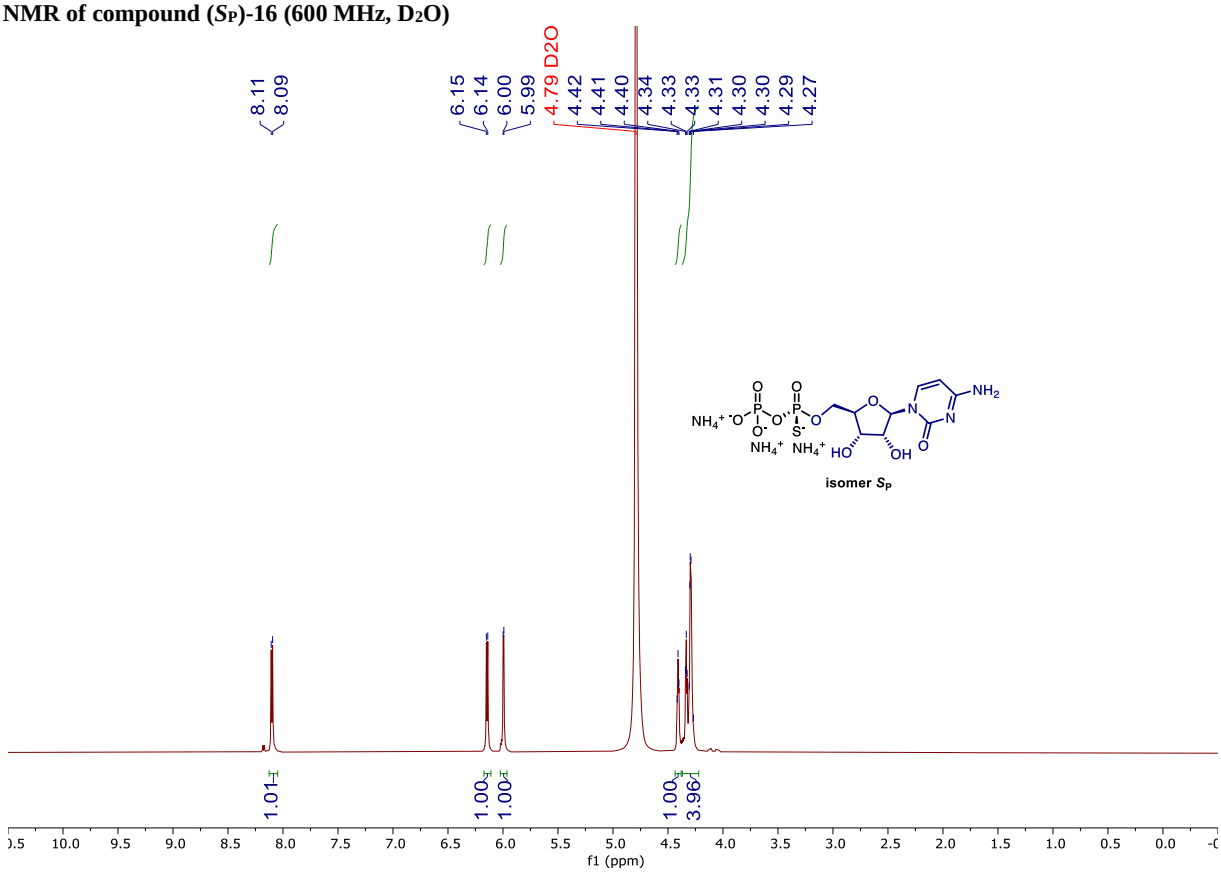
^{13}C NMR of compound (R_P)-16 (150 MHz, D_2O)



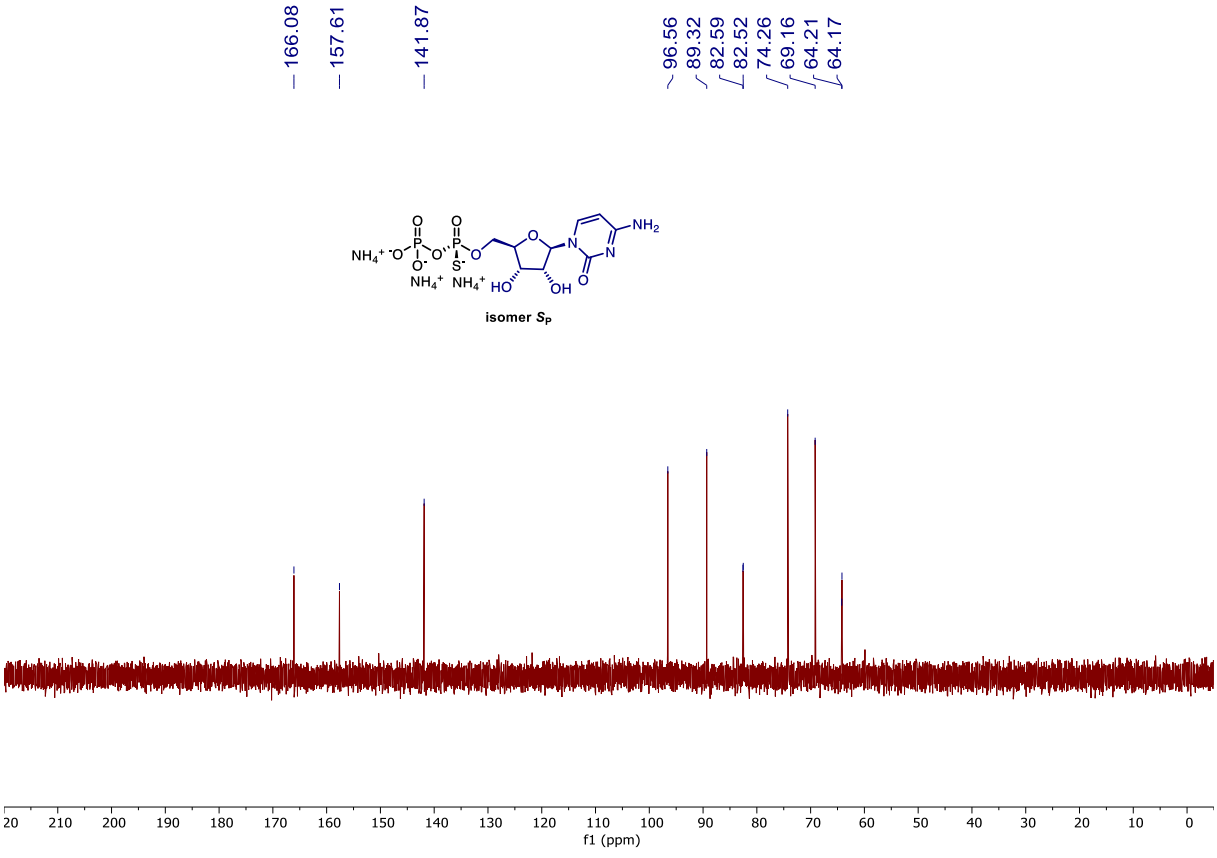
^{31}P NMR of compound (R_P)-16 (162 MHz, D_2O)



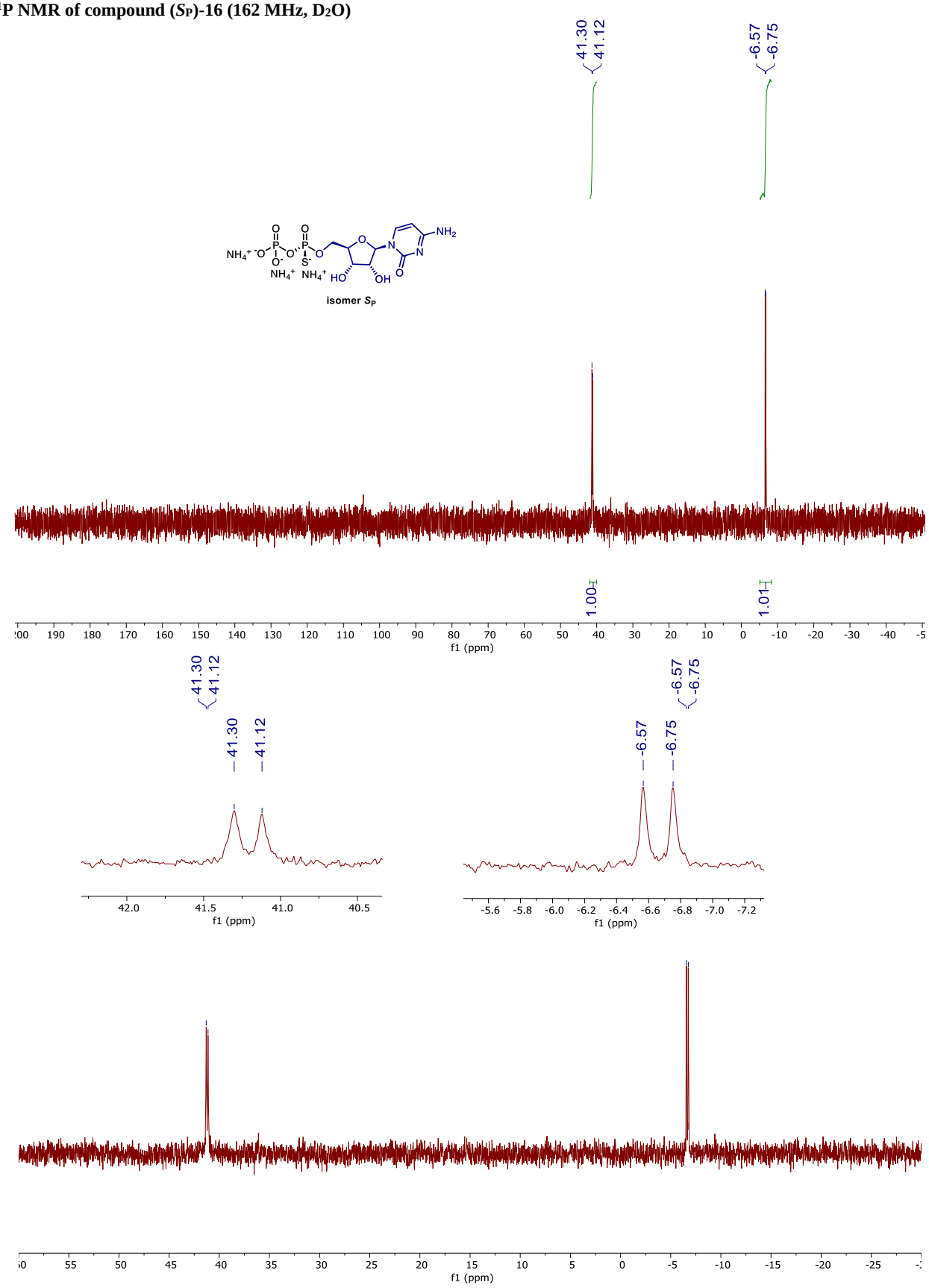
^{1}H NMR of compound (S_P)-16 (600 MHz, D_2O)



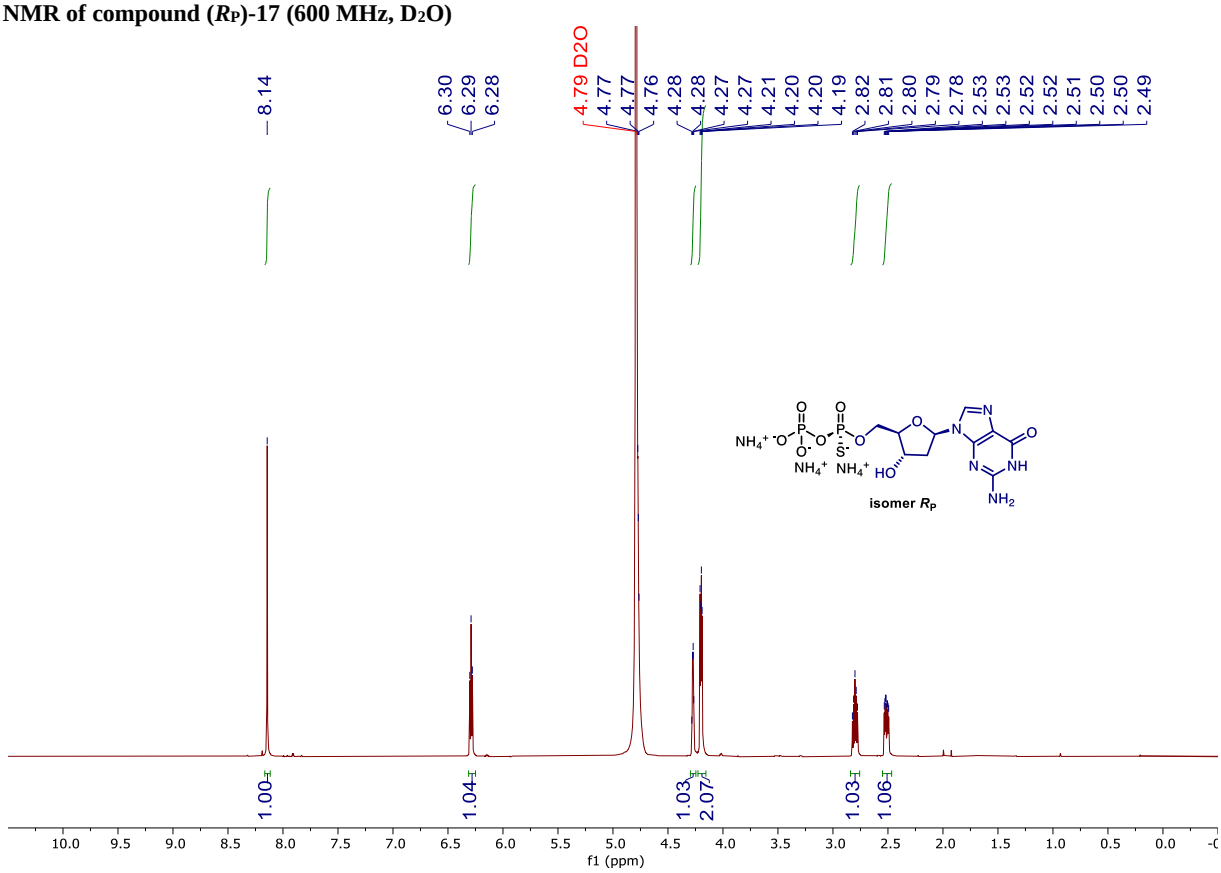
^{13}C NMR of compound (S_P)-16 (150 MHz, D_2O)



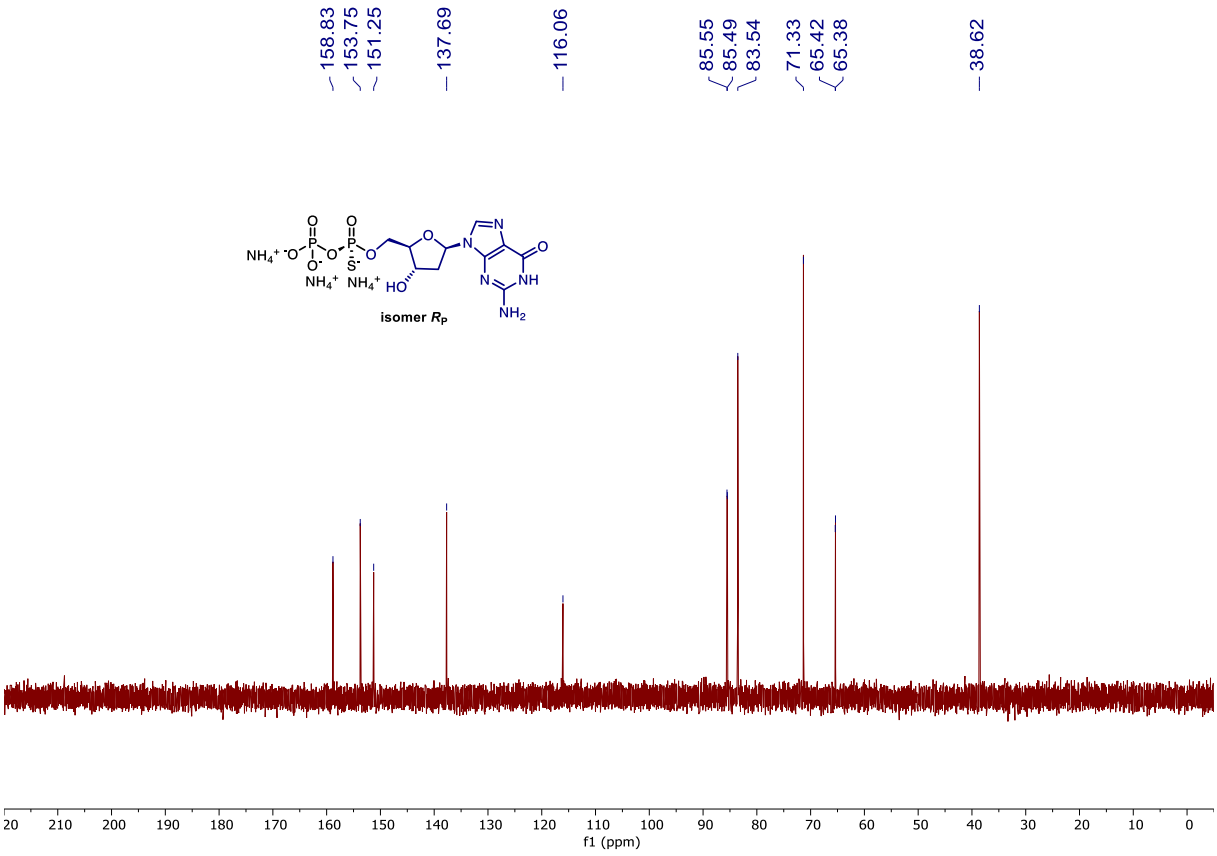
^{31}P NMR of compound (S_P)-16 (162 MHz, D_2O)



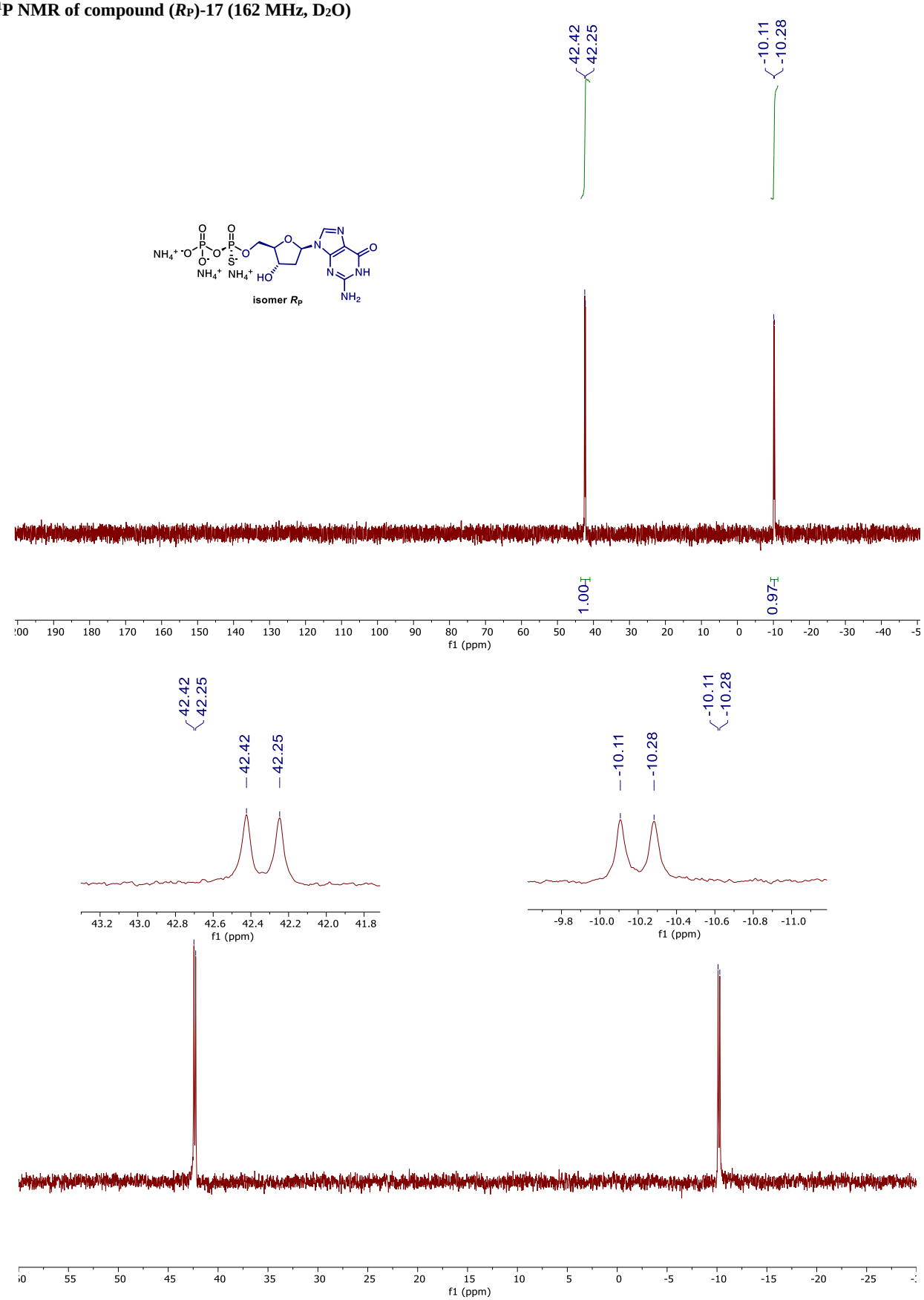
^{1}H NMR of compound (R_P)-17 (600 MHz, D_2O)



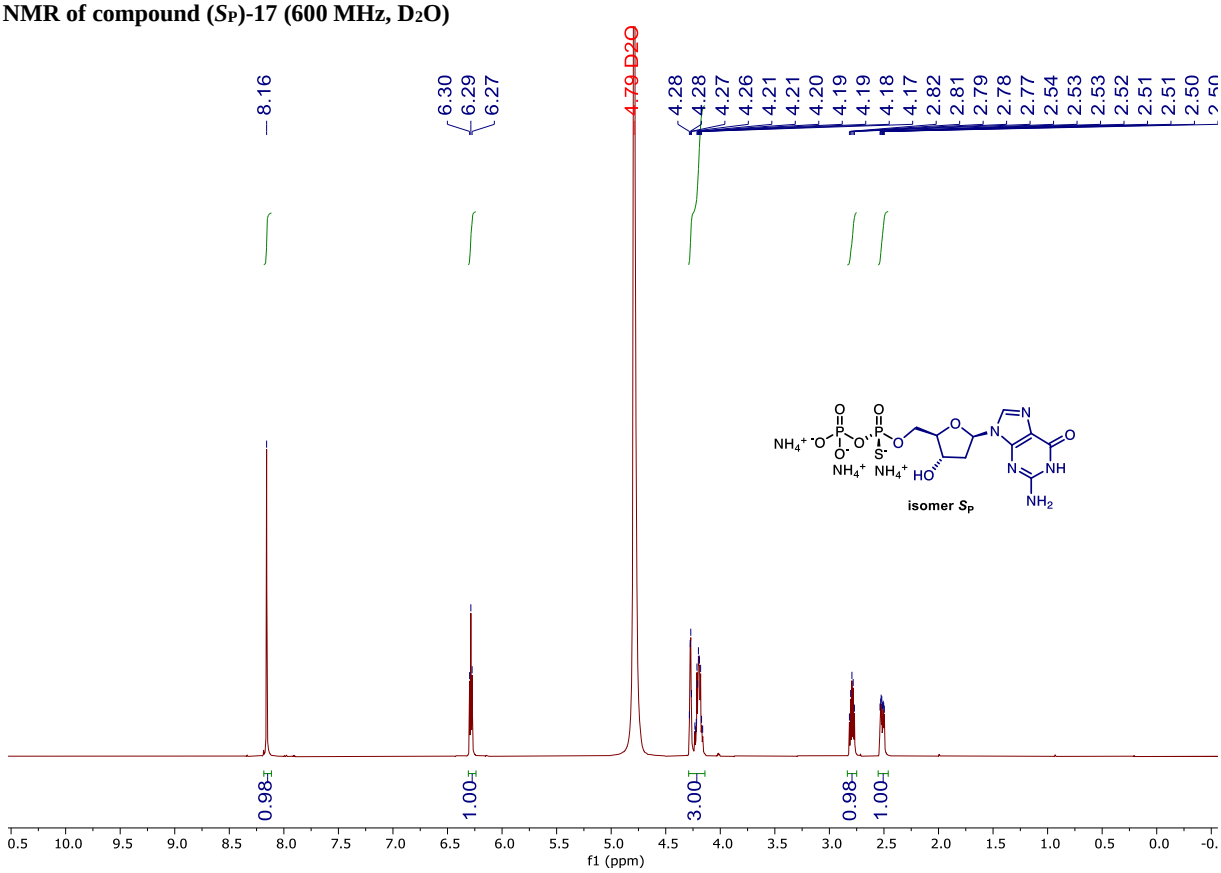
^{13}C NMR of compound (R_P)-17 (150 MHz, D_2O)



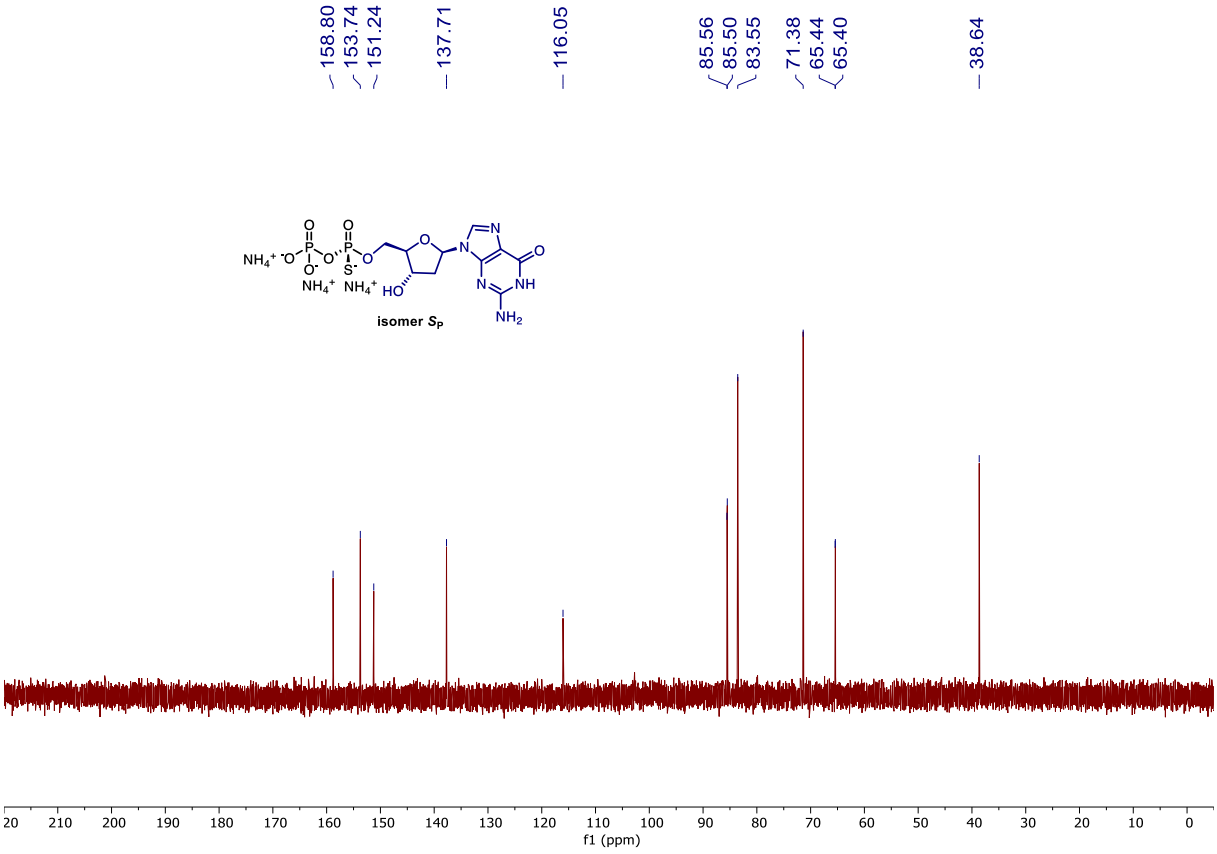
^{31}P NMR of compound (R_P)-17 (162 MHz, D_2O)



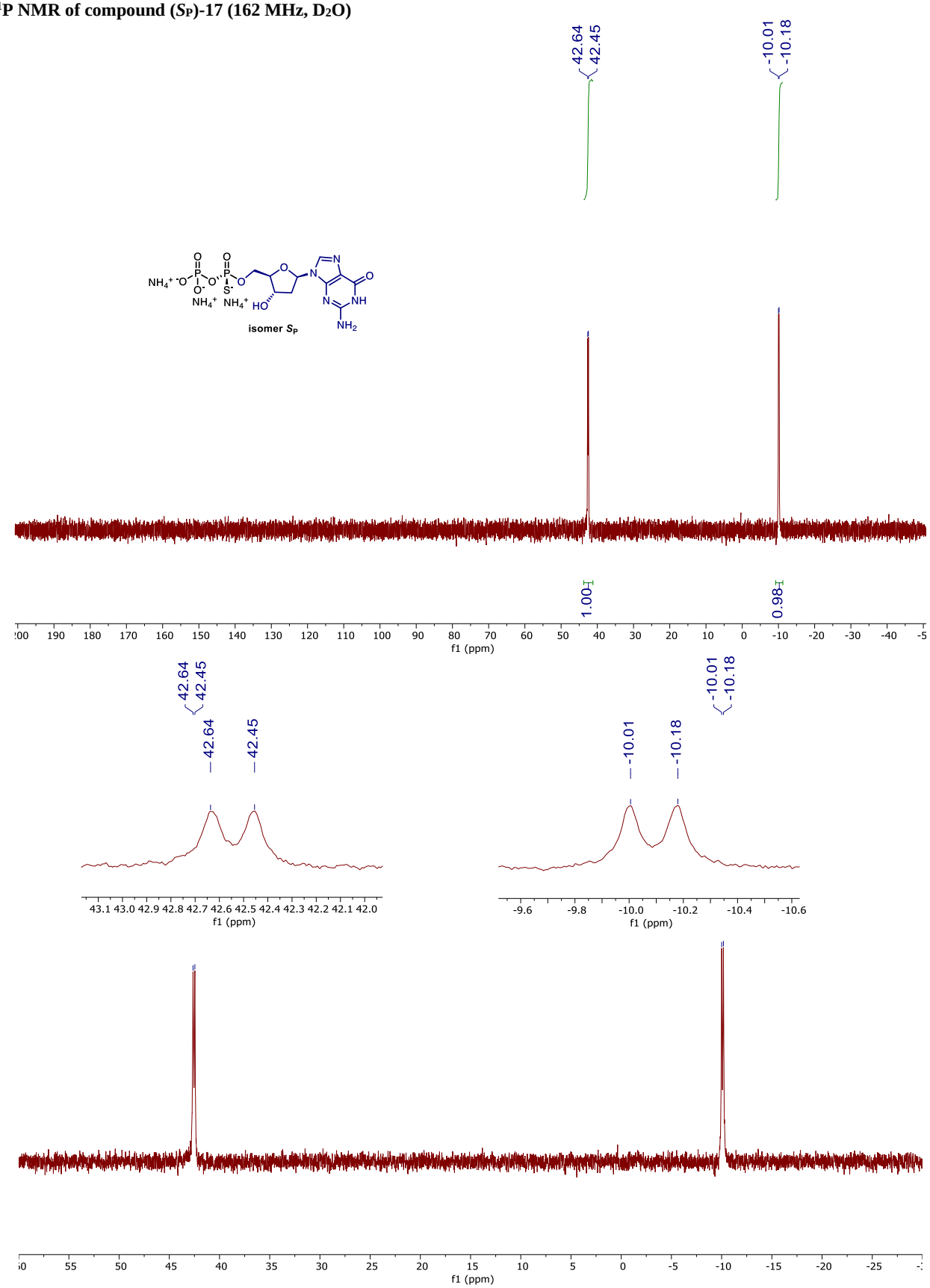
^{1}H NMR of compound (S_P)-17 (600 MHz, D_2O)



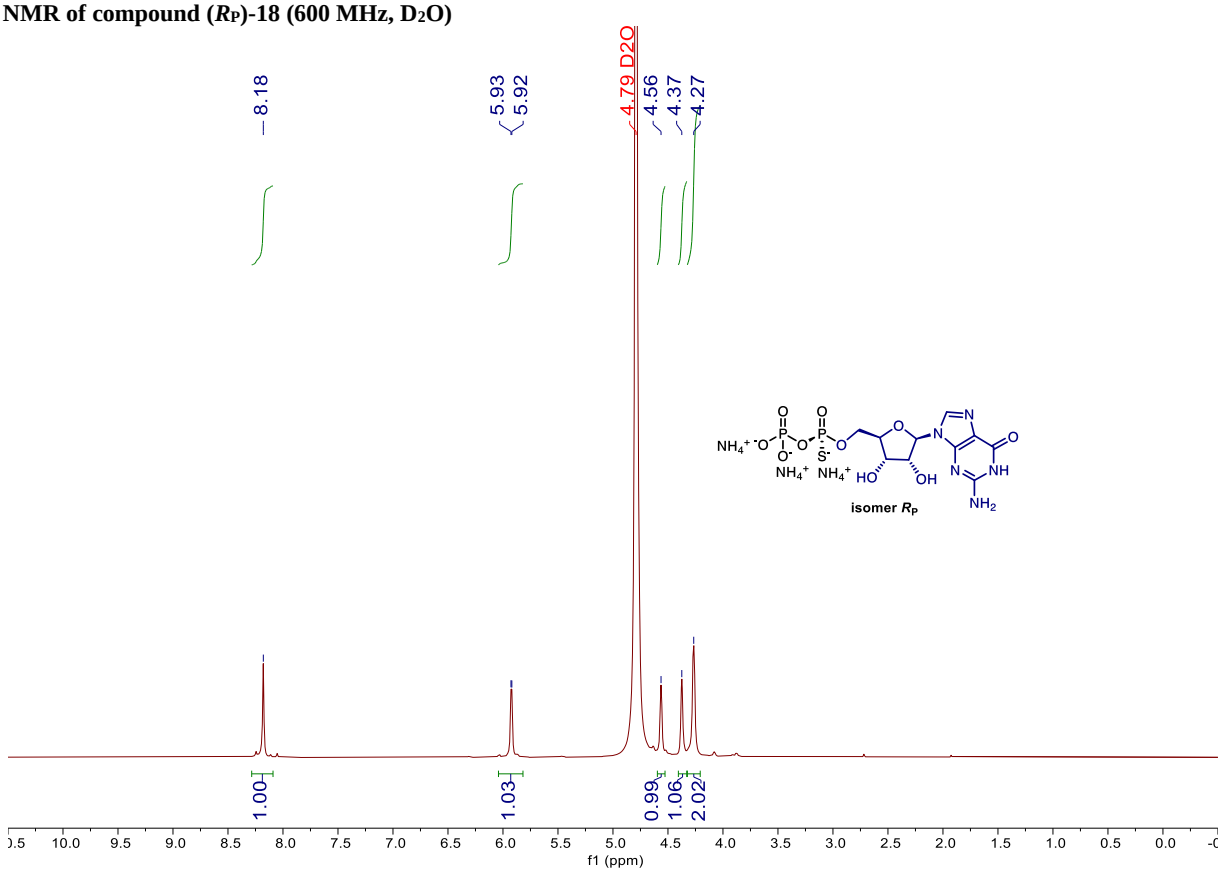
^{13}C NMR of compound (S_P)-17 (150 MHz, D_2O)



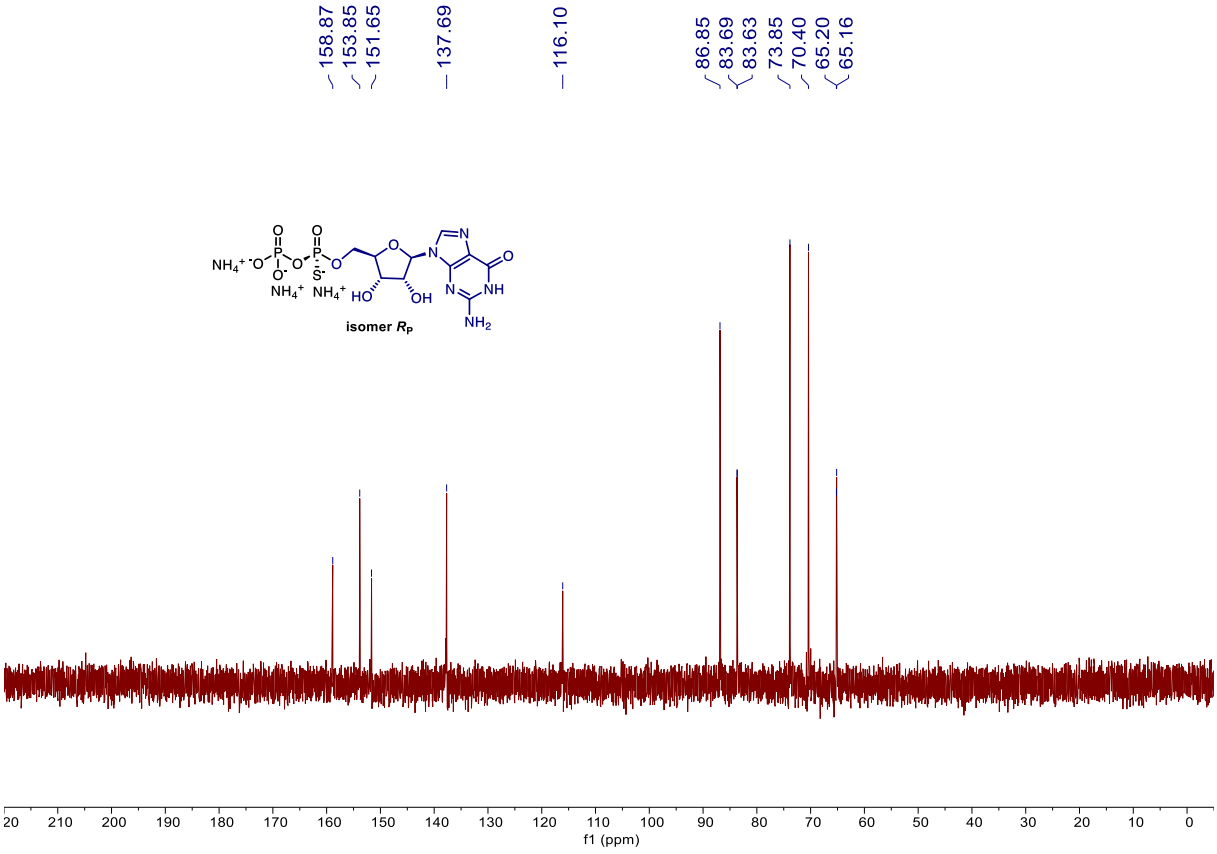
^{31}P NMR of compound (S_P)-17 (162 MHz, D_2O)



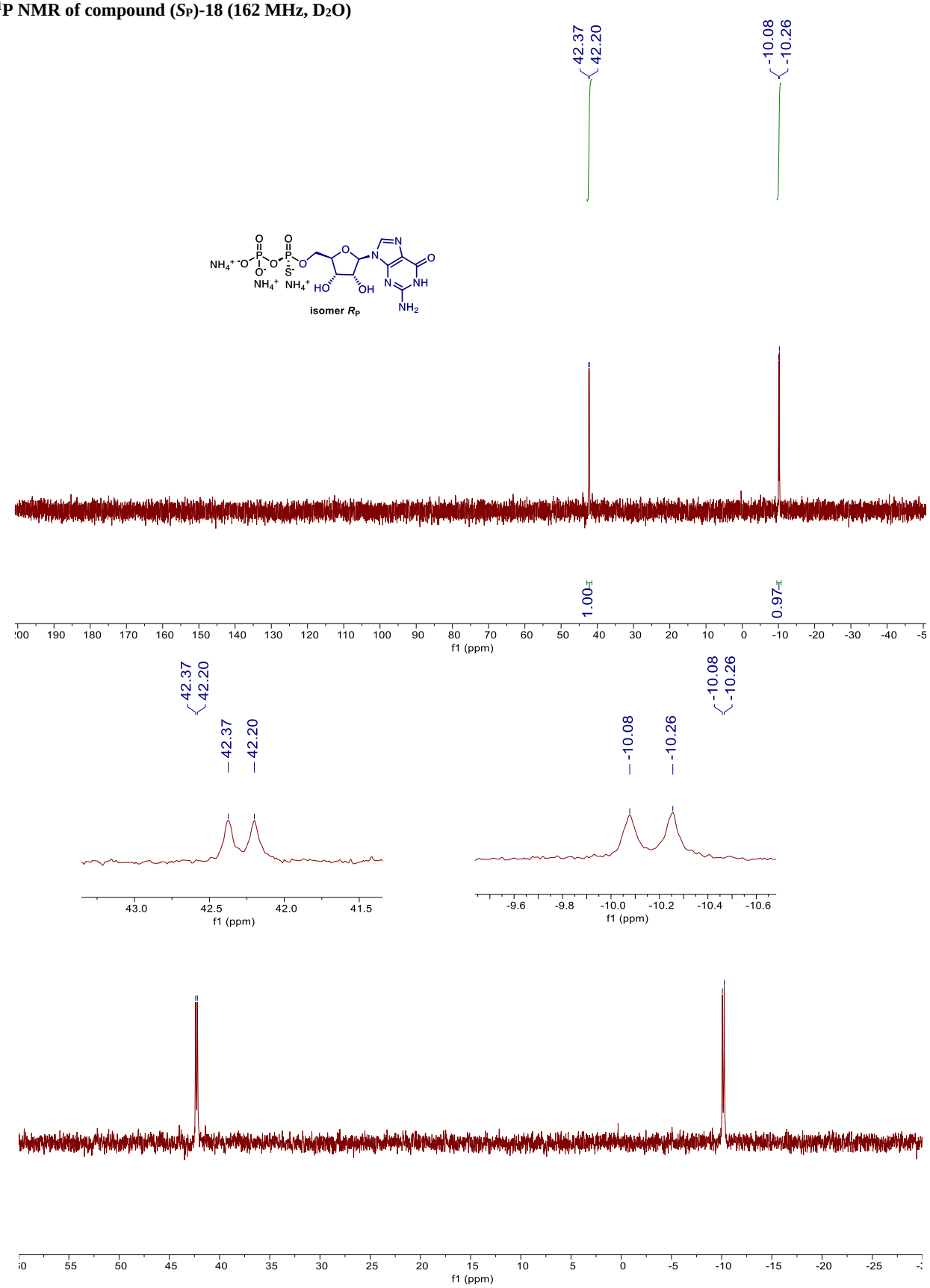
^{1}H NMR of compound (R_P)-18 (600 MHz, D_2O)



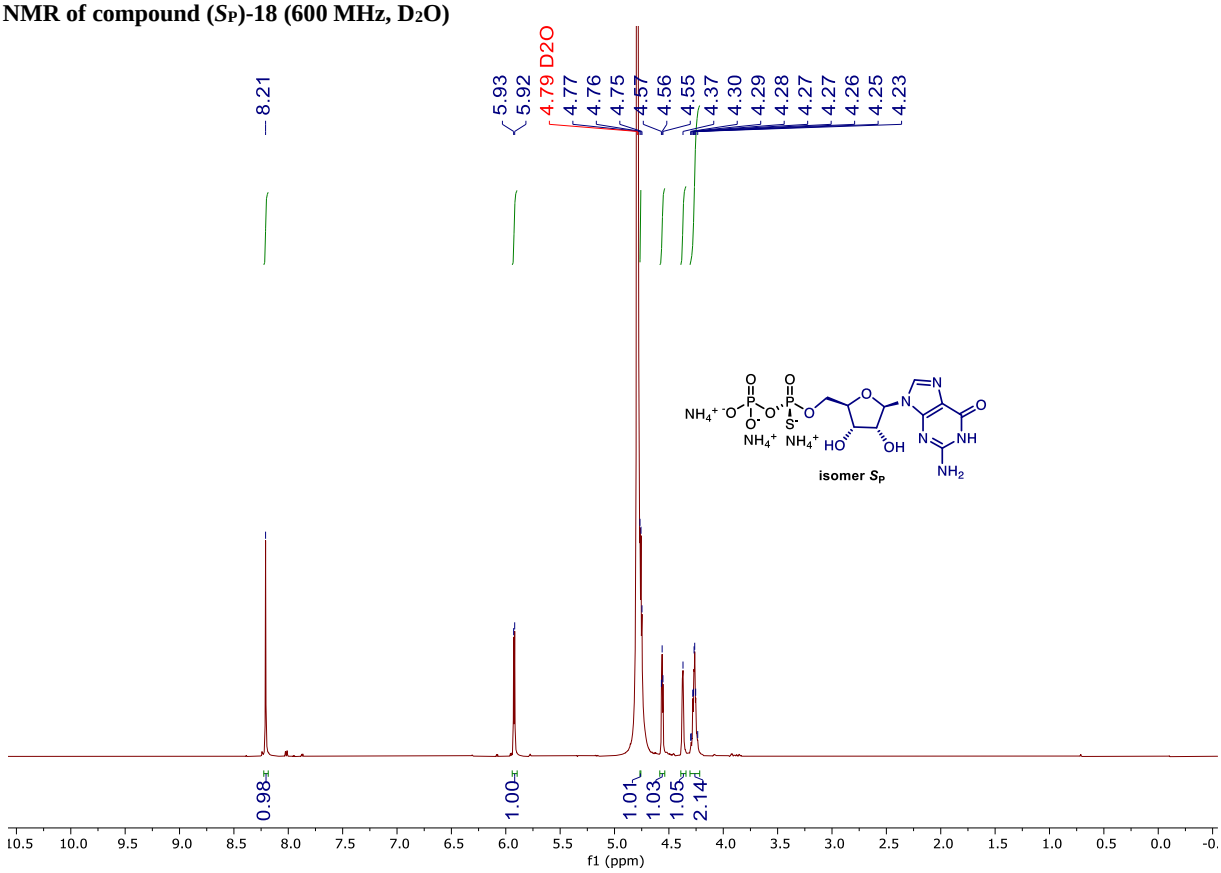
^{13}C NMR of compound (S_P)-18 (150 MHz, D_2O)



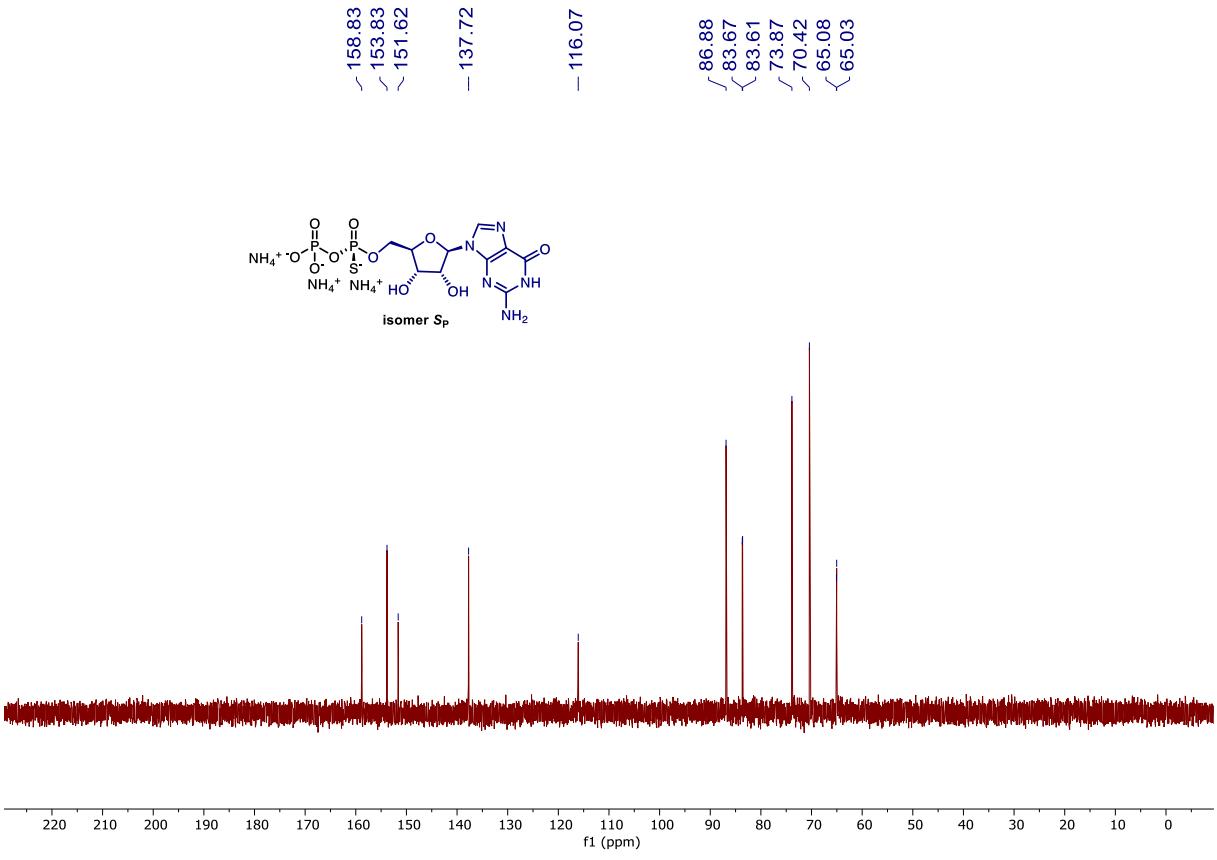
^{31}P NMR of compound (S_P)-18 (162 MHz, D_2O)



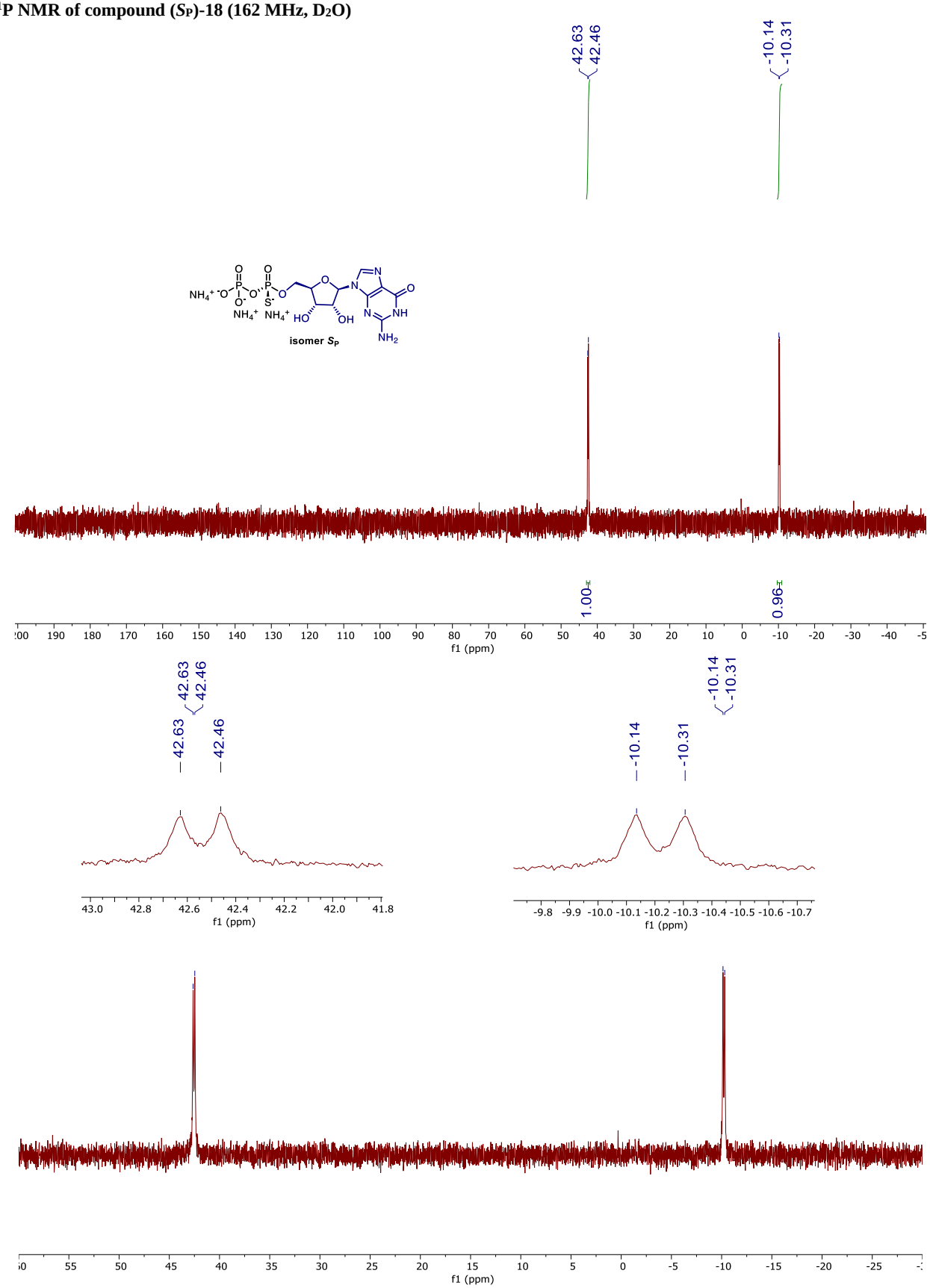
^{1}H NMR of compound (S_P)-18 (600 MHz, D_2O)



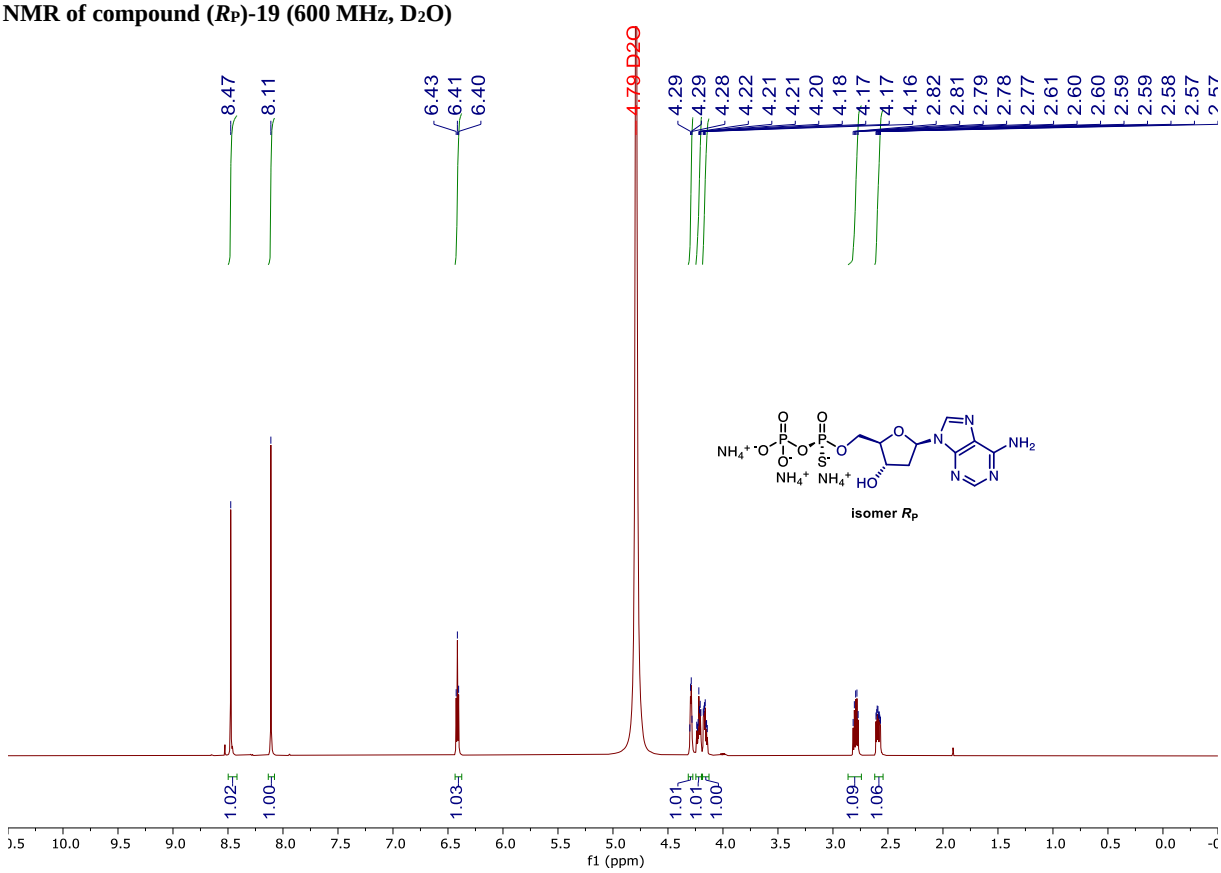
^{13}C NMR of compound (S_P)-18 (150 MHz, D_2O)



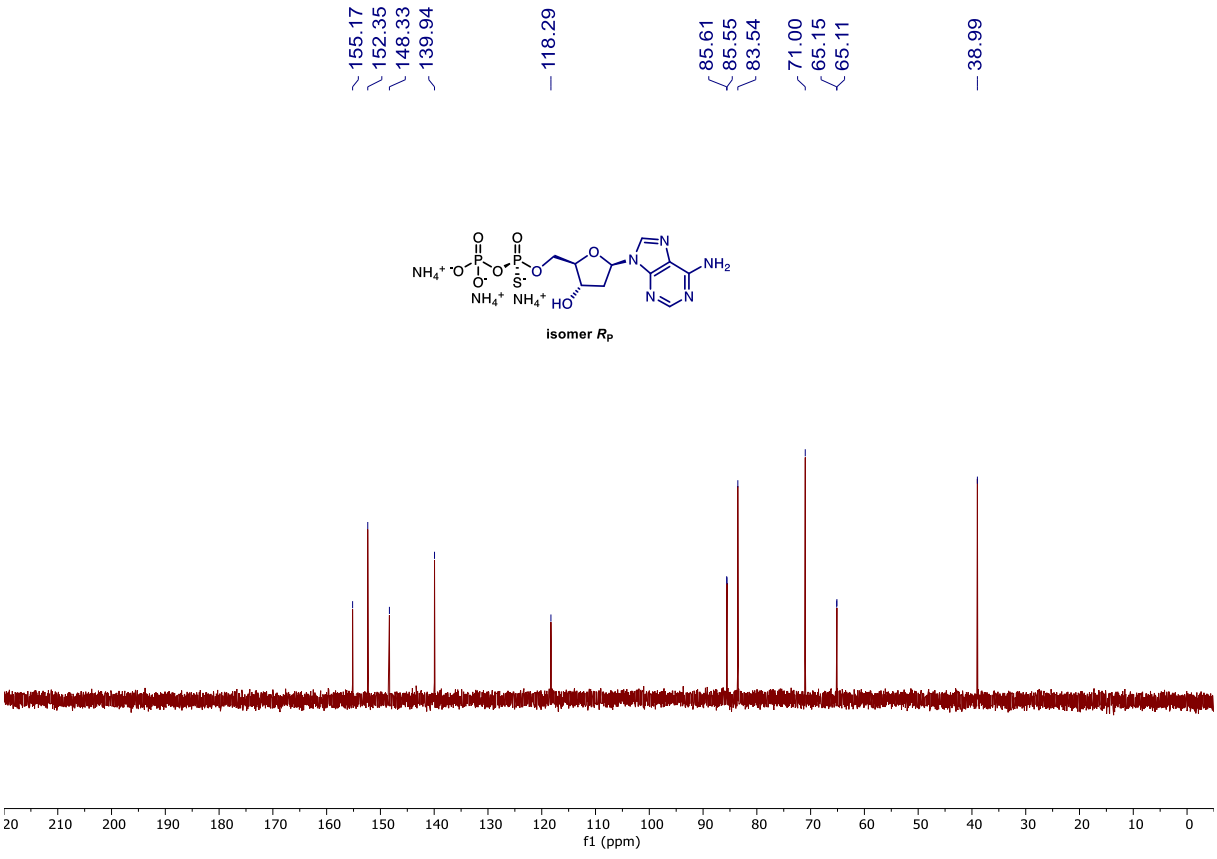
^{31}P NMR of compound (S_P)-18 (162 MHz, D_2O)



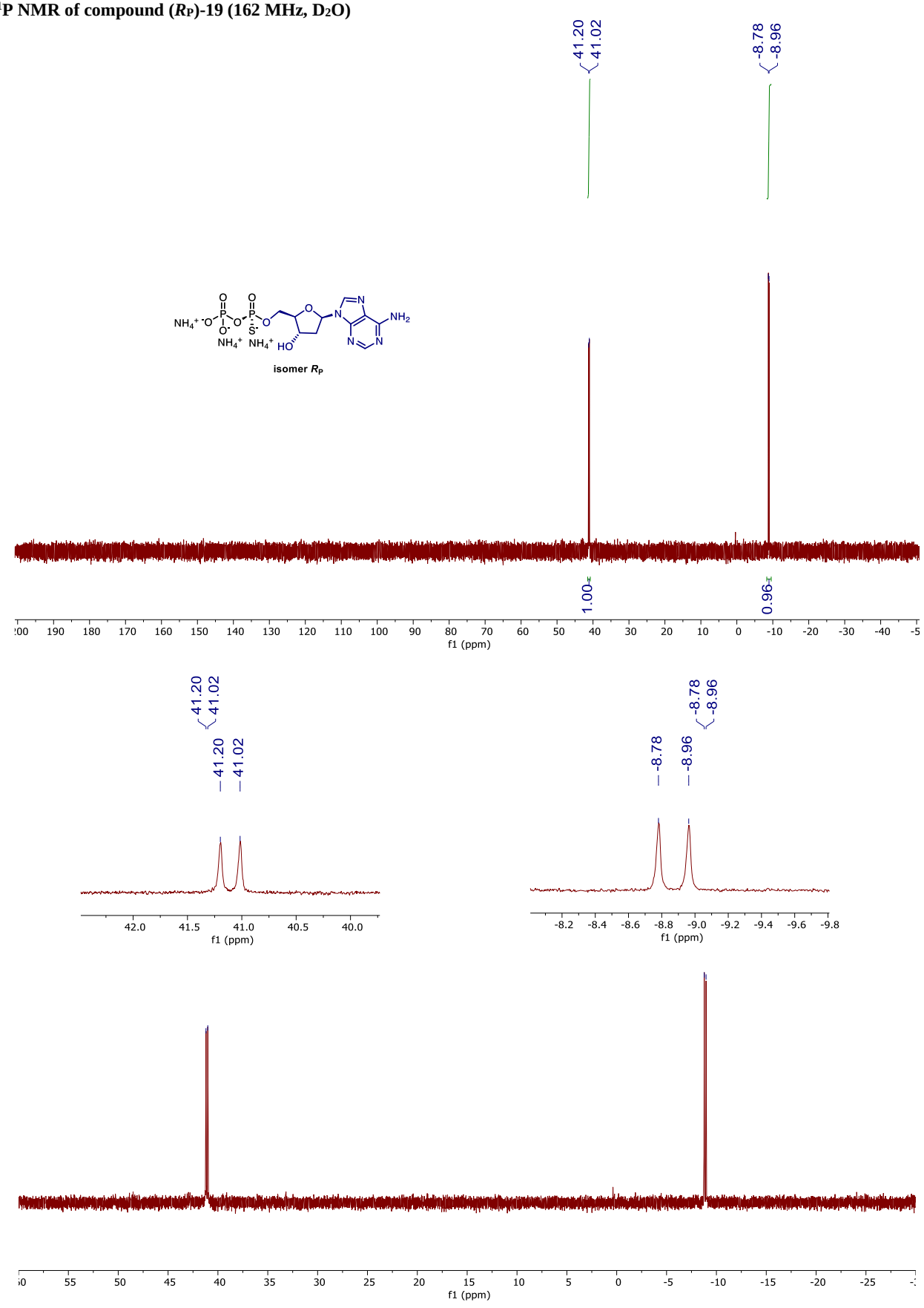
^{1}H NMR of compound (R_P)-19 (600 MHz, D_2O)



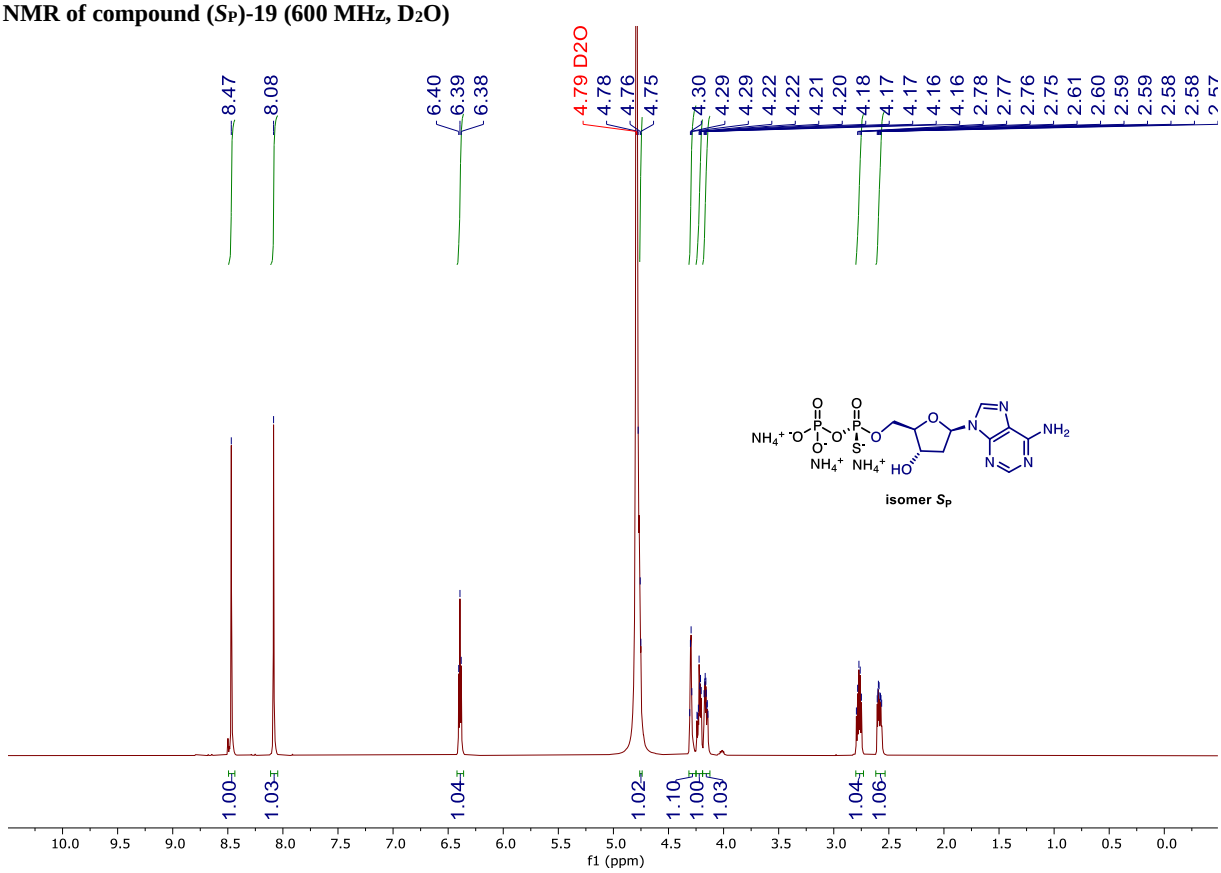
^{13}C NMR of compound (R_P)-19 (150 MHz, D_2O)



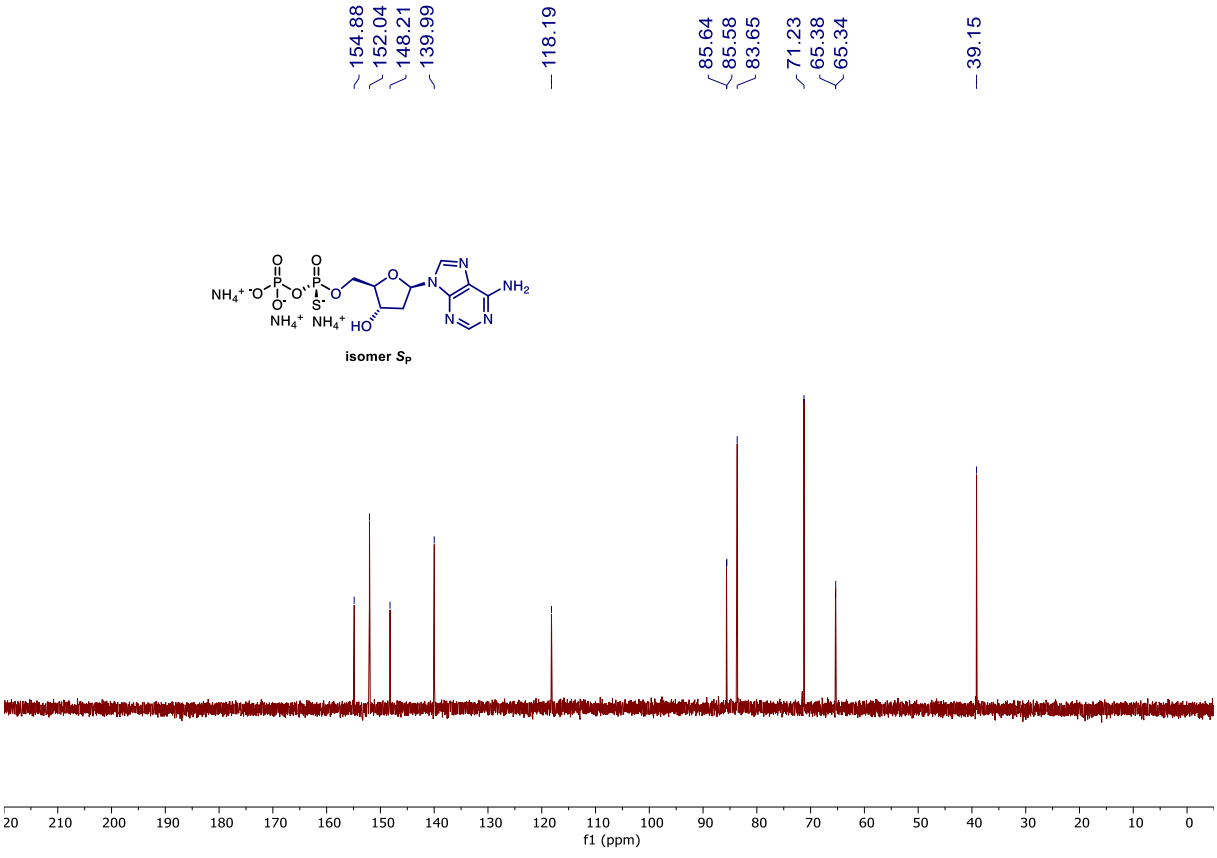
^{31}P NMR of compound (R_P)-19 (162 MHz, D_2O)



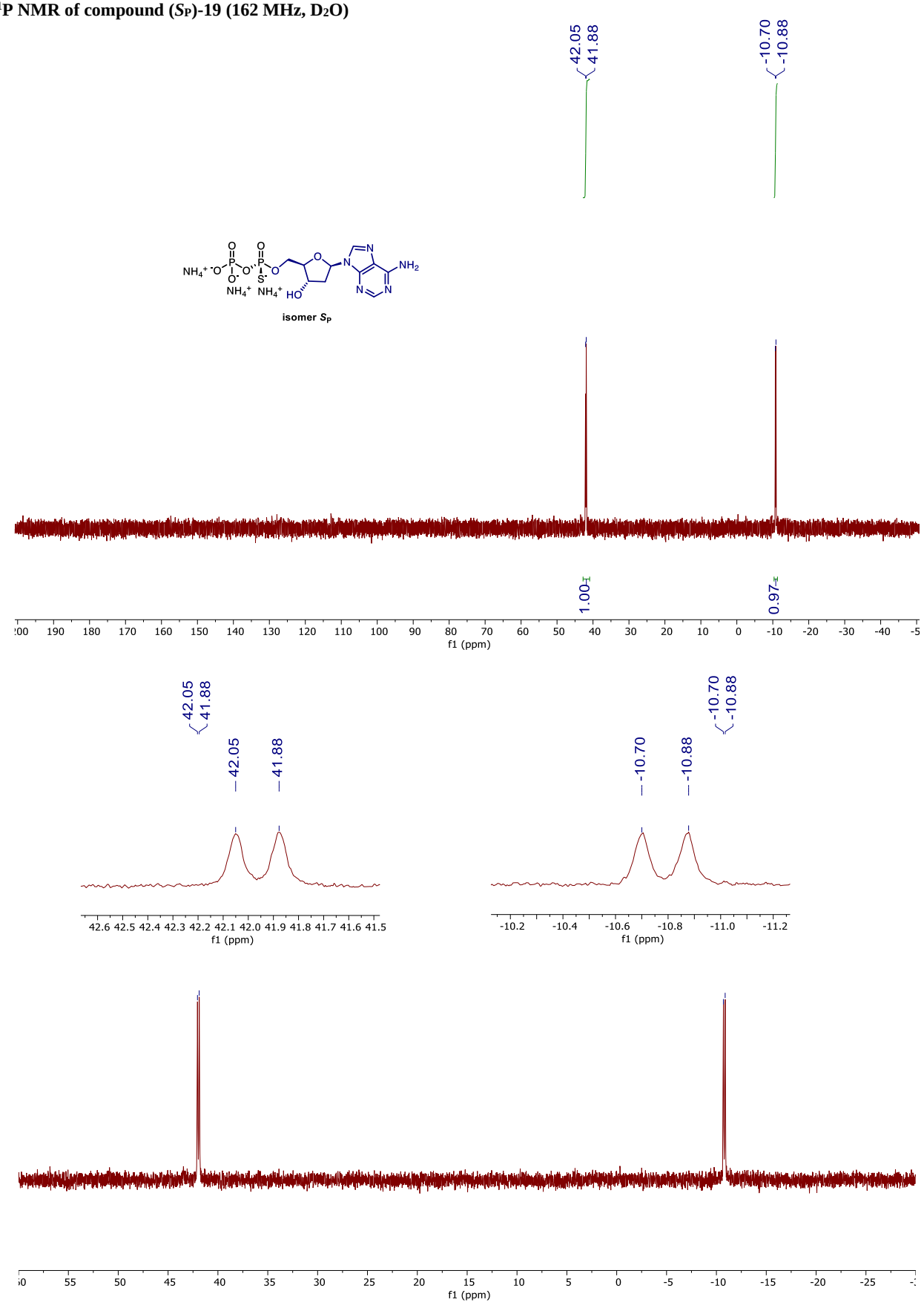
^{1}H NMR of compound (S_P)-19 (600 MHz, D_2O)



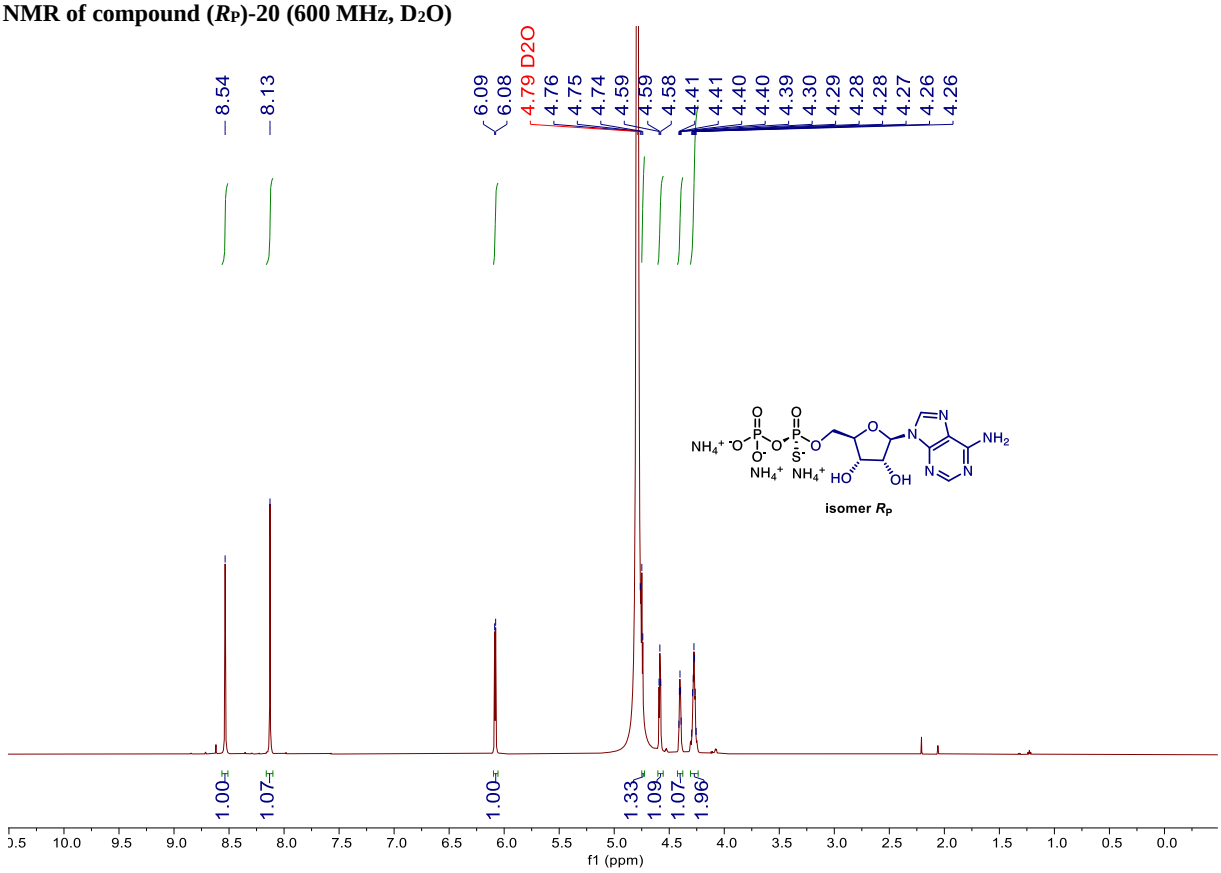
^{13}C NMR of compound (S_P)-19 (150 MHz, D_2O)



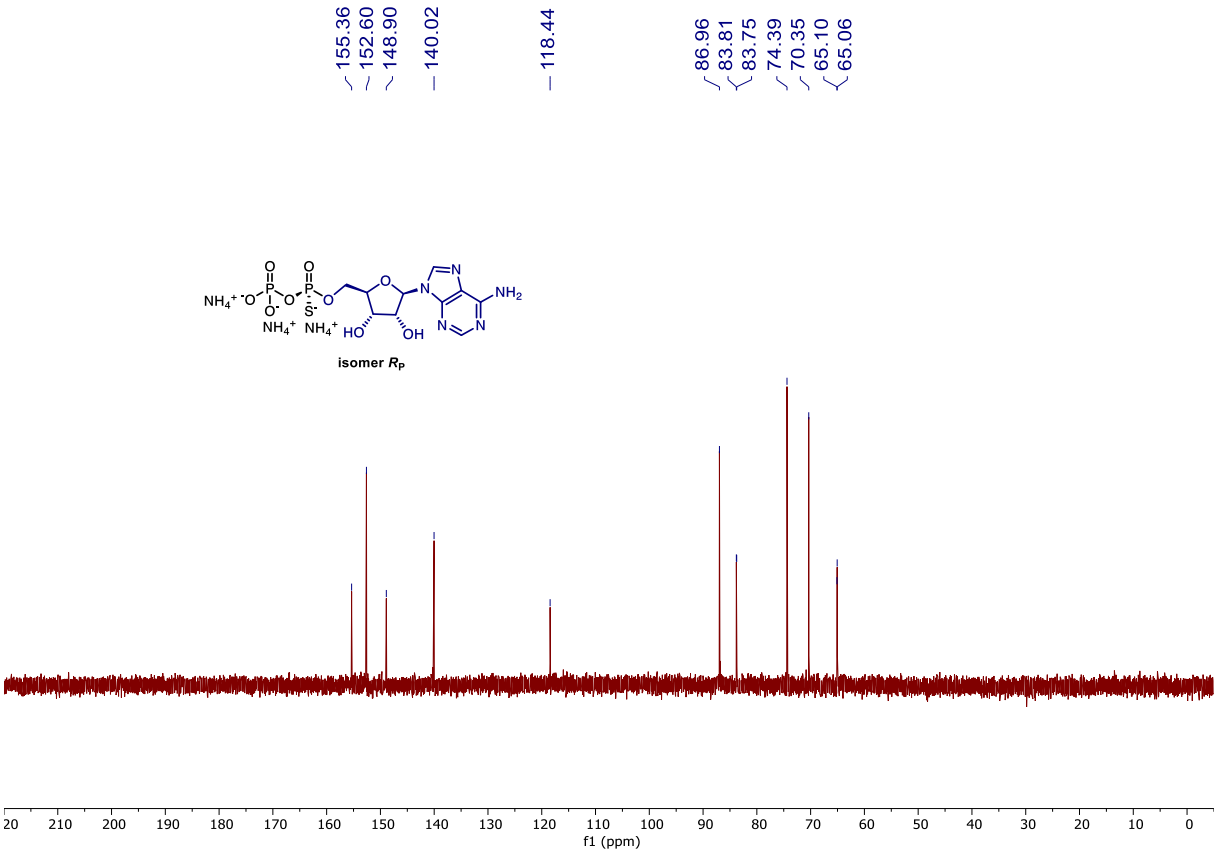
^{31}P NMR of compound (S_P)-19 (162 MHz, D_2O)



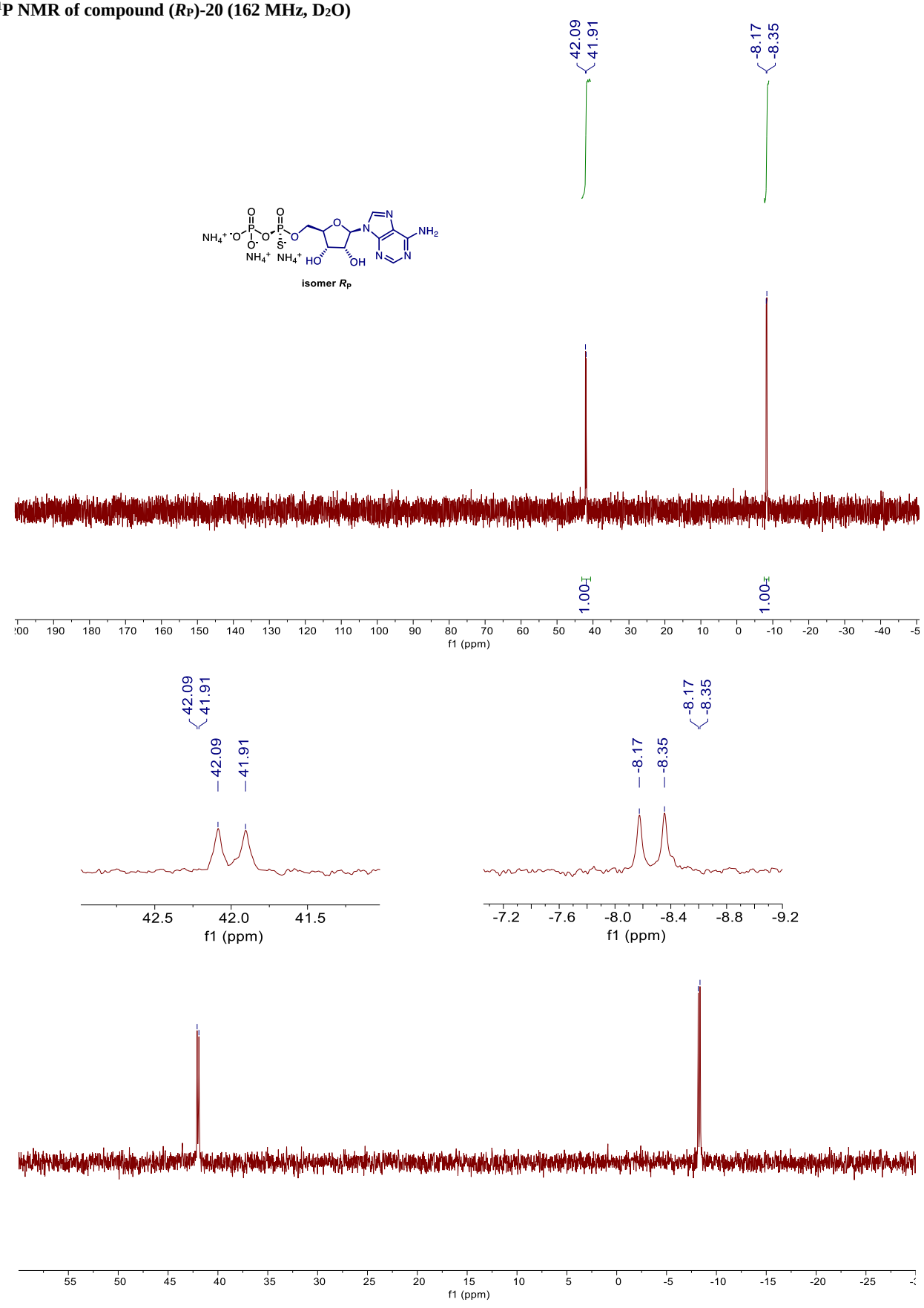
^{1}H NMR of compound (R_P)-20 (600 MHz, D_2O)



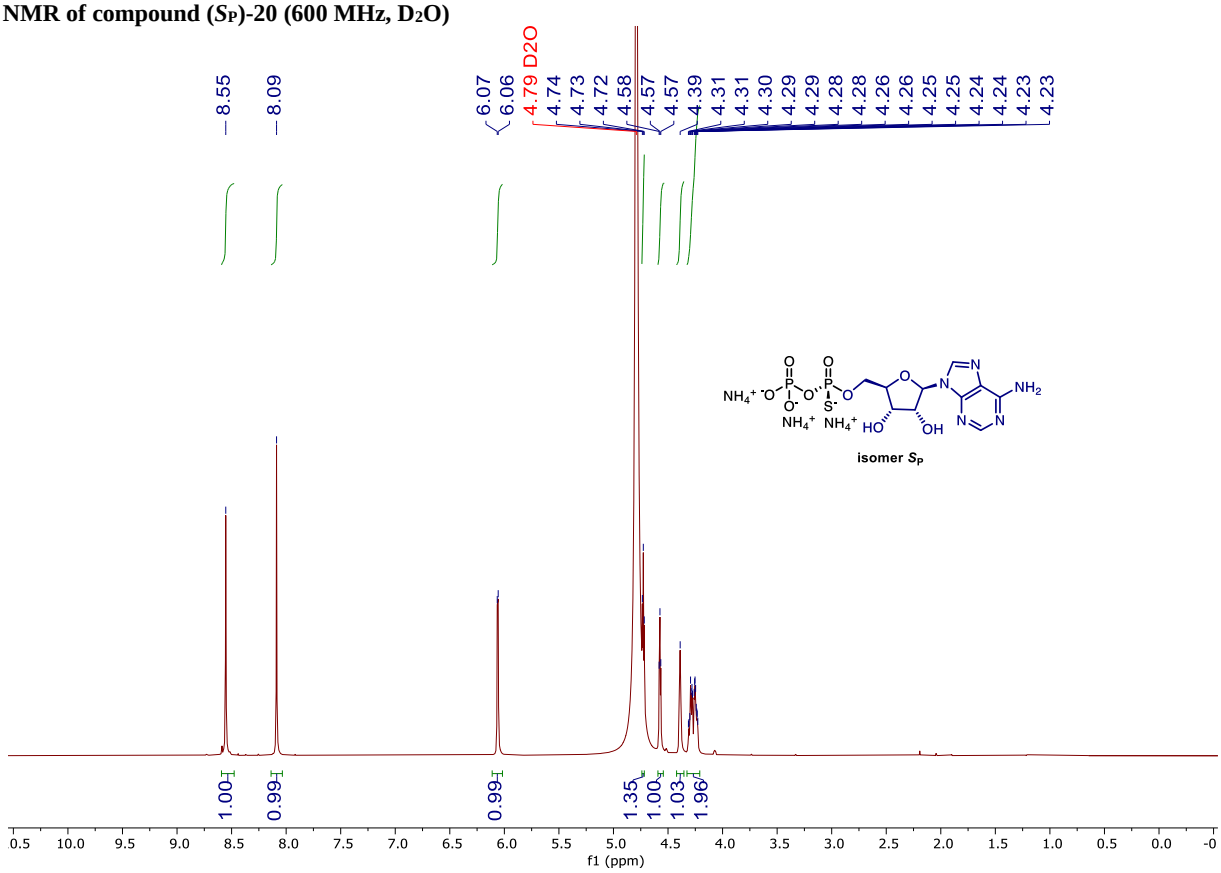
^{13}C NMR of compound (R_P)-20 (150 MHz, D_2O)



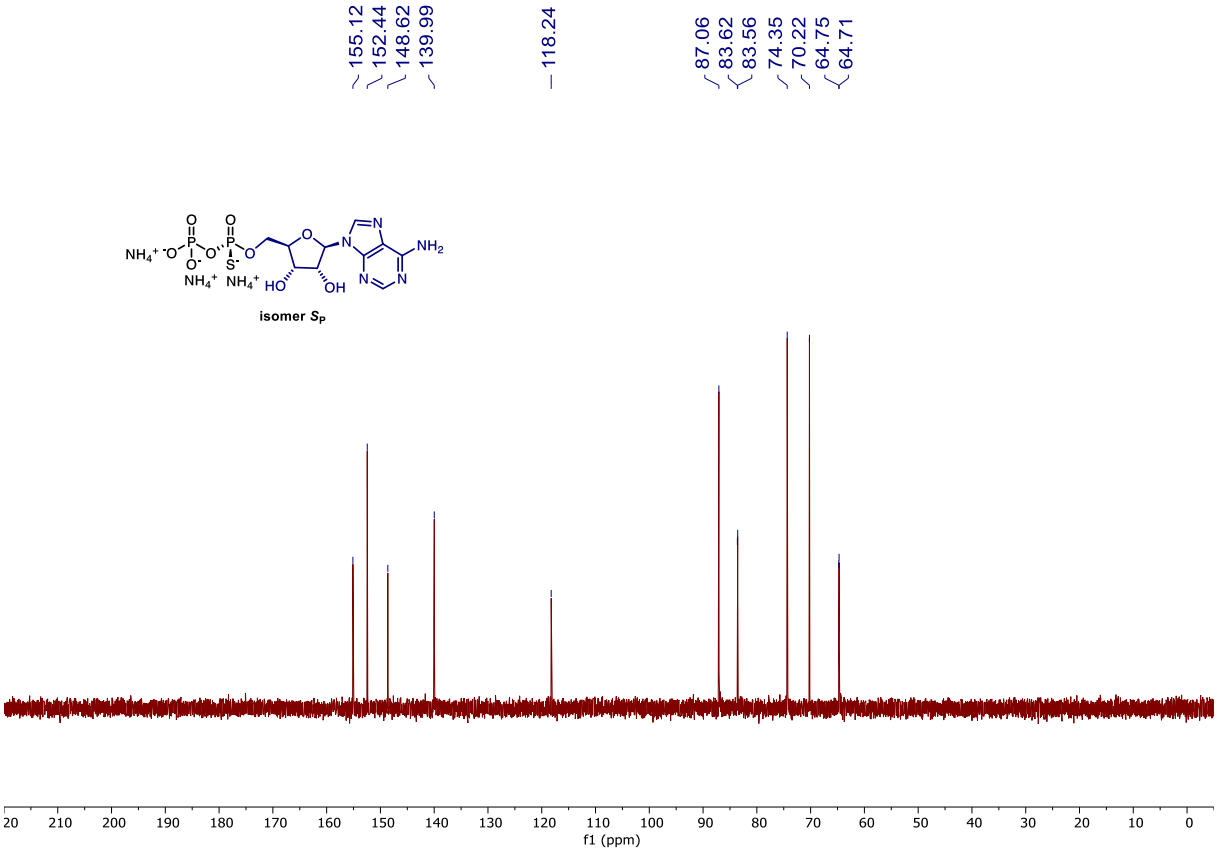
^{31}P NMR of compound (R_P)-20 (162 MHz, D_2O)



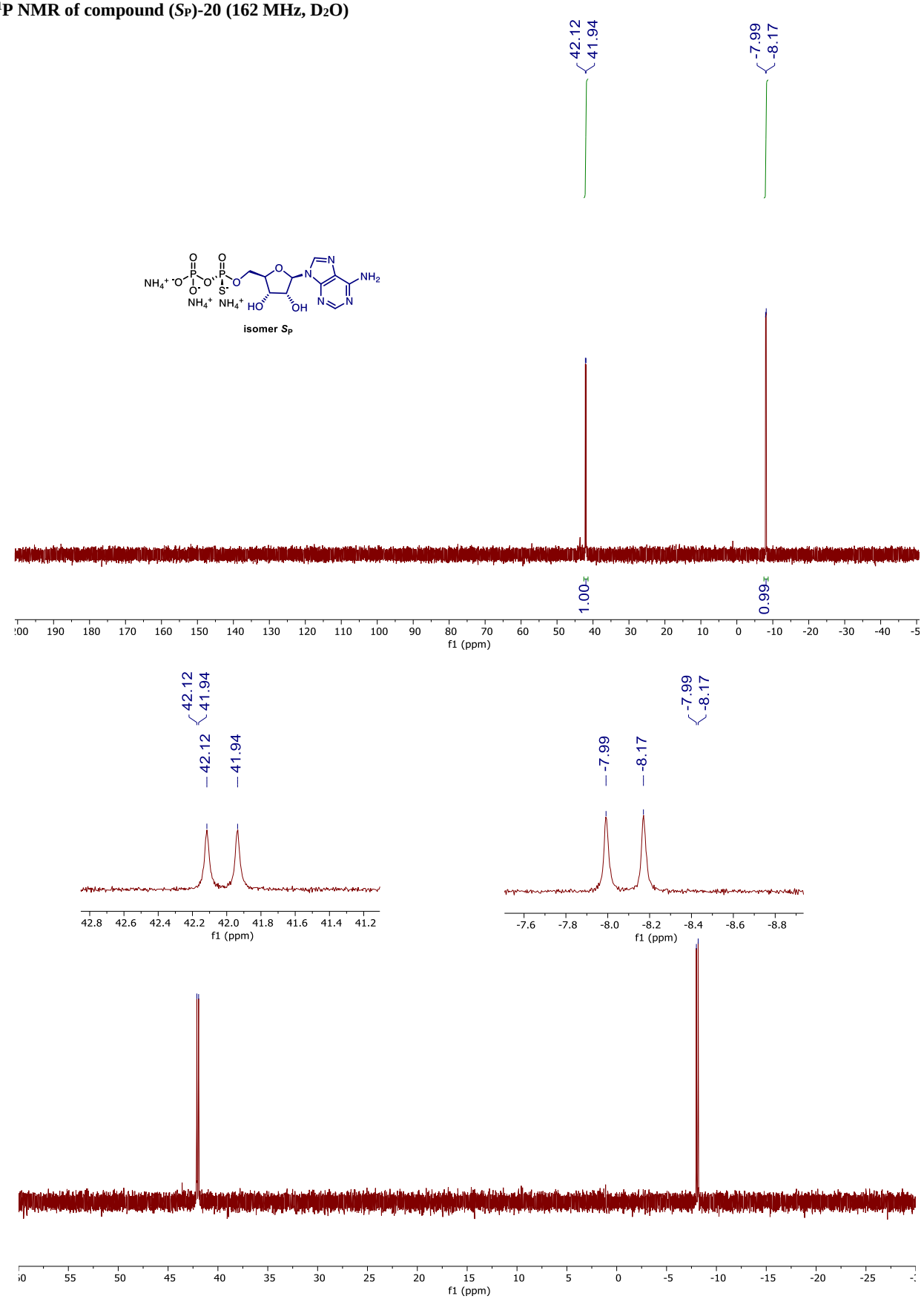
^{1}H NMR of compound (S_P)-20 (600 MHz, D_2O)



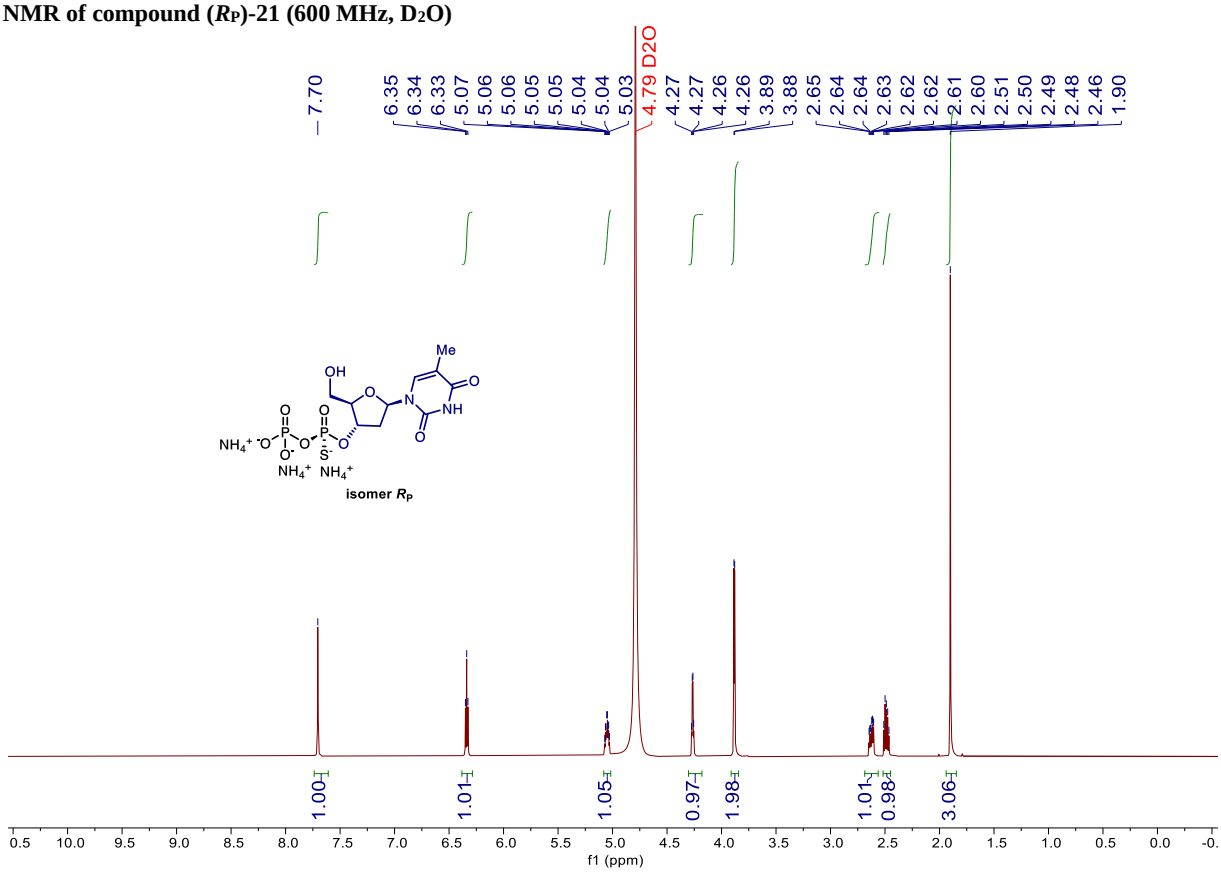
^{13}C NMR of compound (S_P)-20 (150 MHz, D_2O)



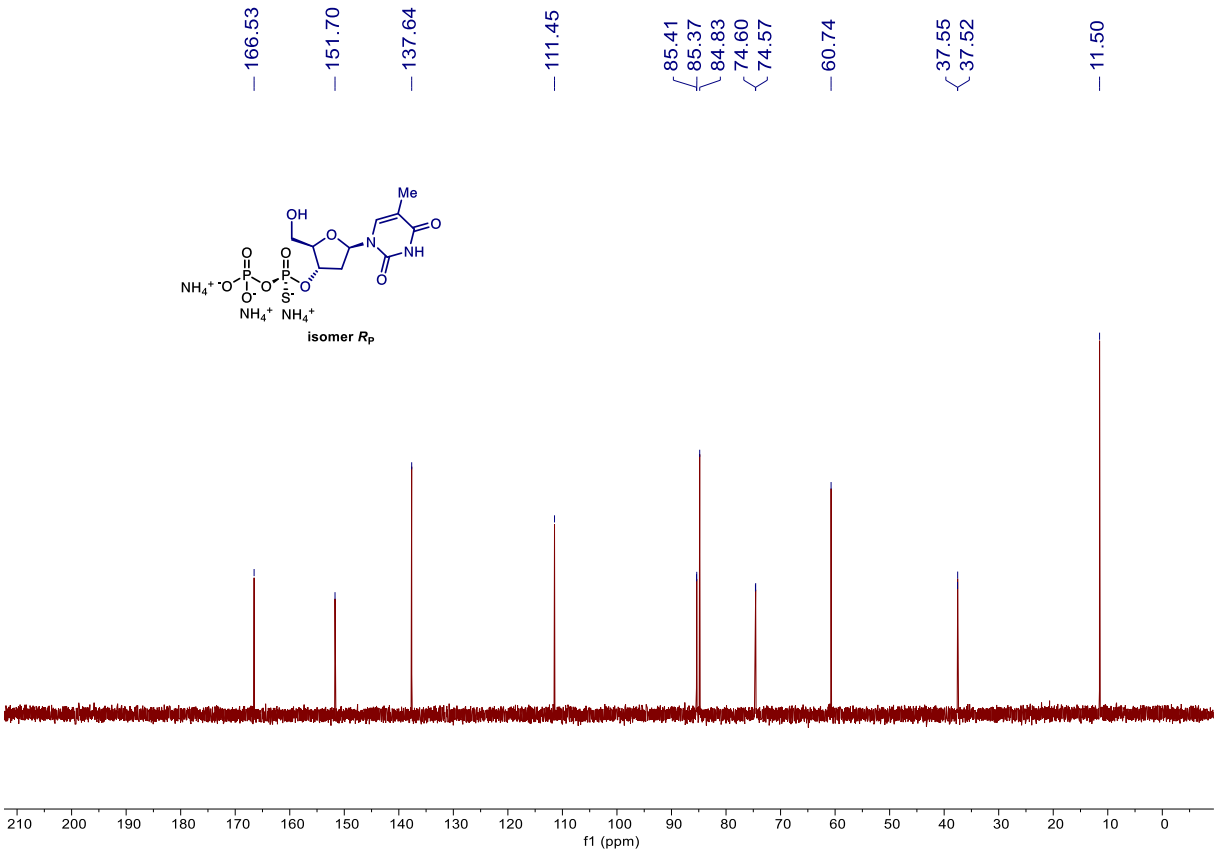
^{31}P NMR of compound (S_P)-20 (162 MHz, D_2O)



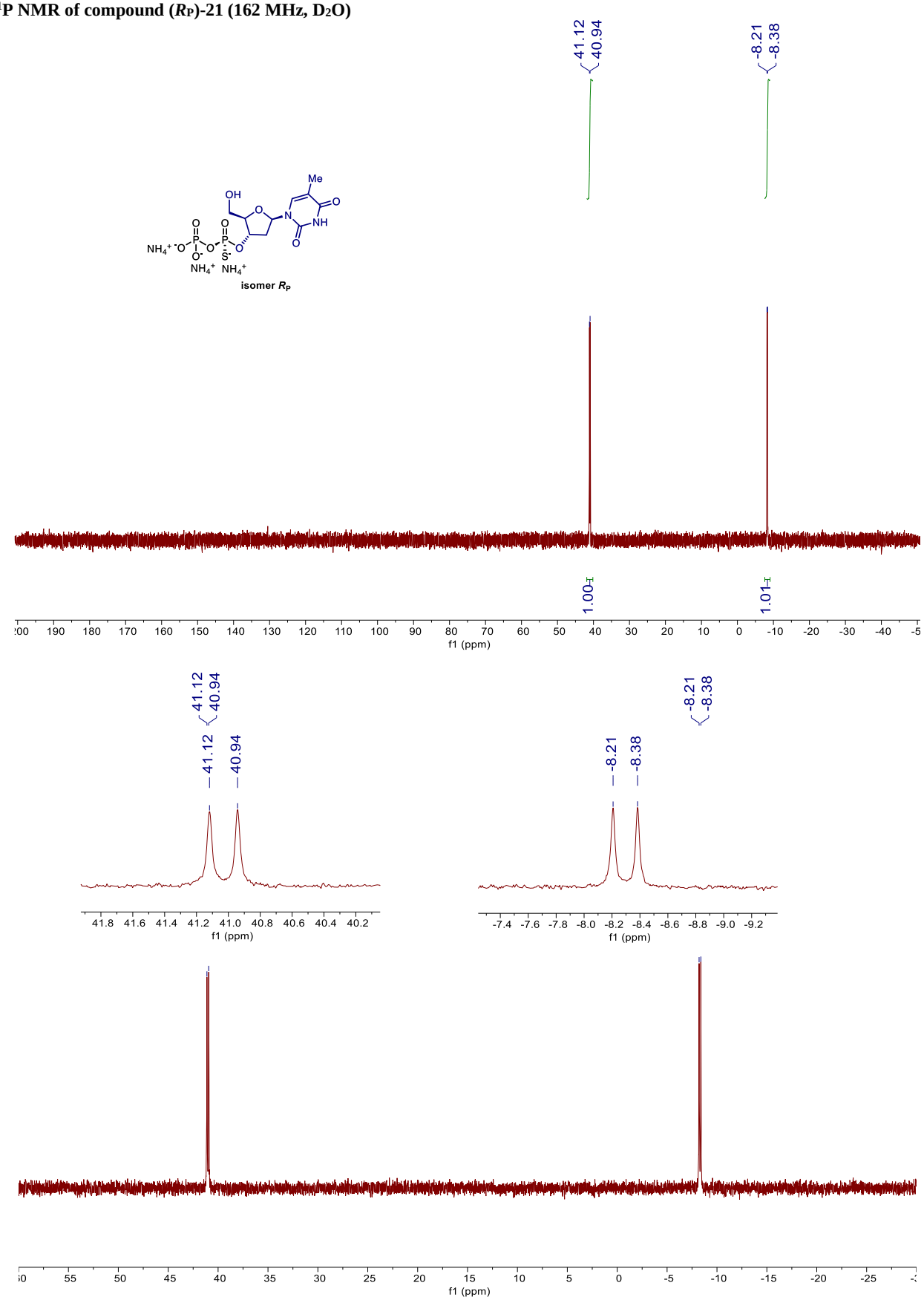
^{1}H NMR of compound (R_P)-21 (600 MHz, D_2O)



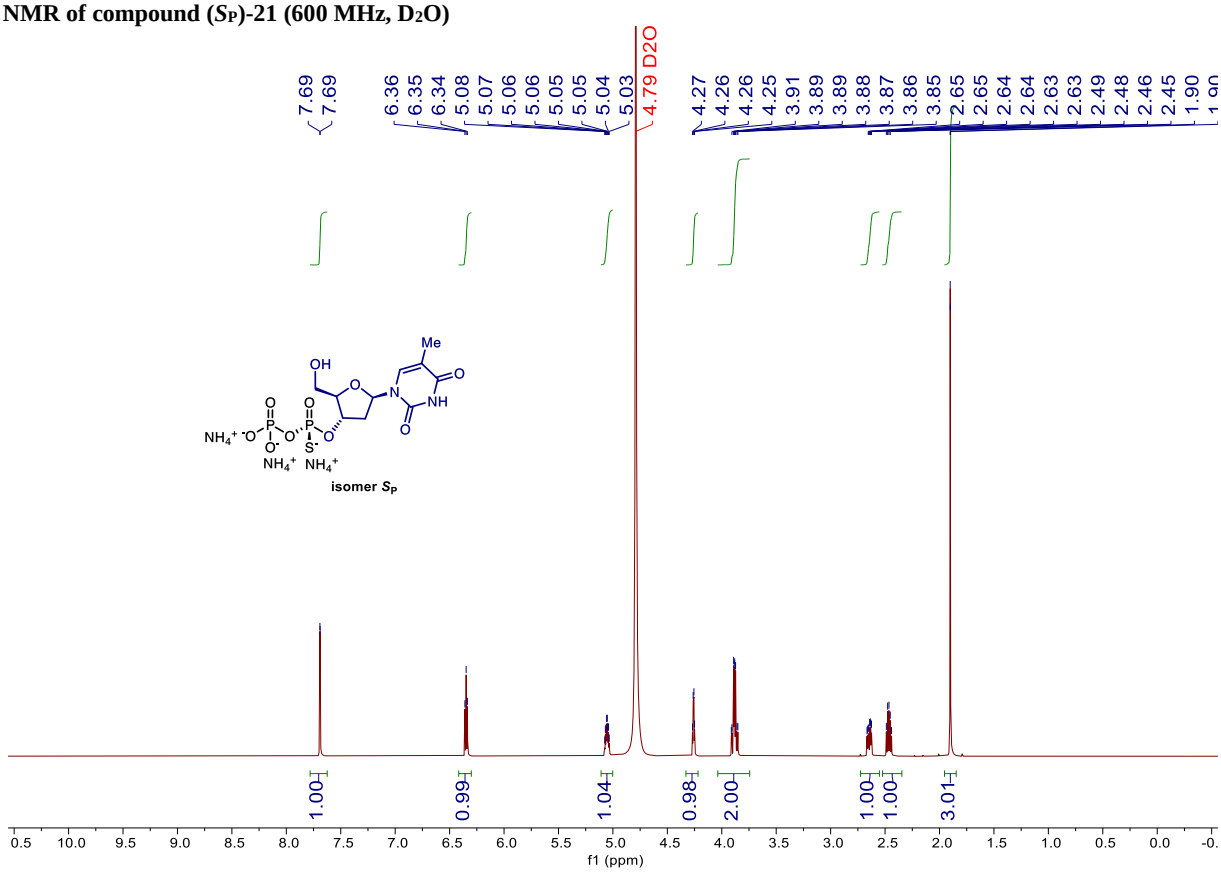
^{13}C NMR of compound (R_P)-21 (150 MHz, D_2O)



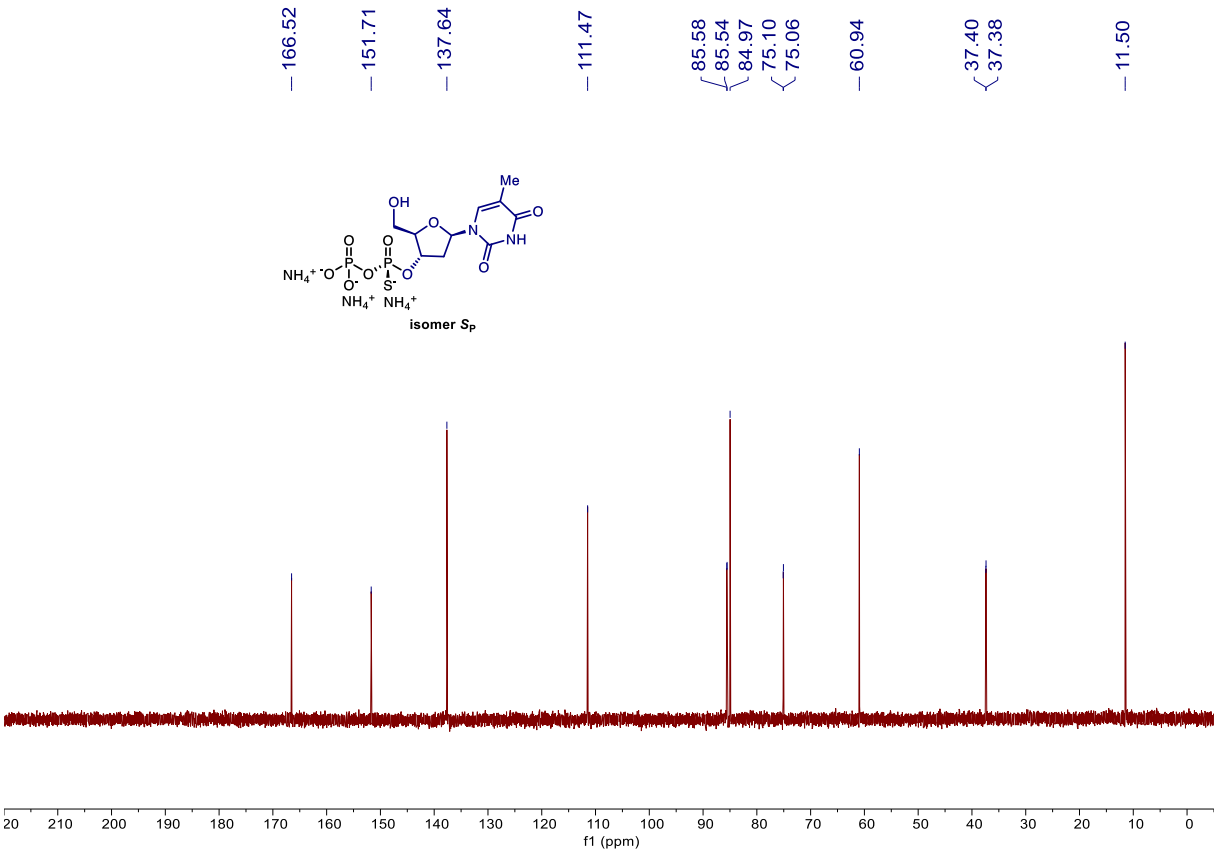
^{31}P NMR of compound (R_P)-21 (162 MHz, D_2O)



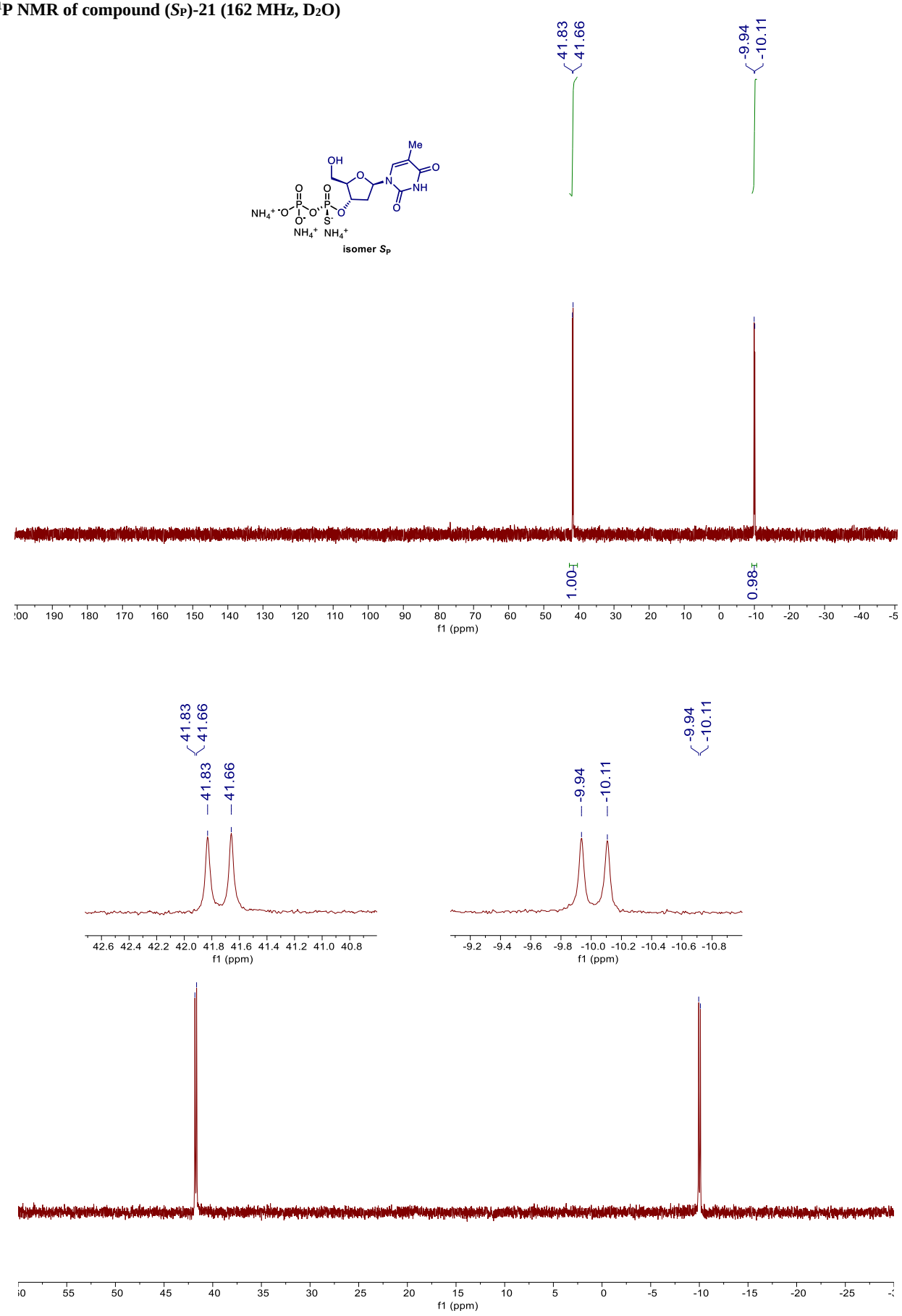
^{1}H NMR of compound (S_P)-21 (600 MHz, D_2O)



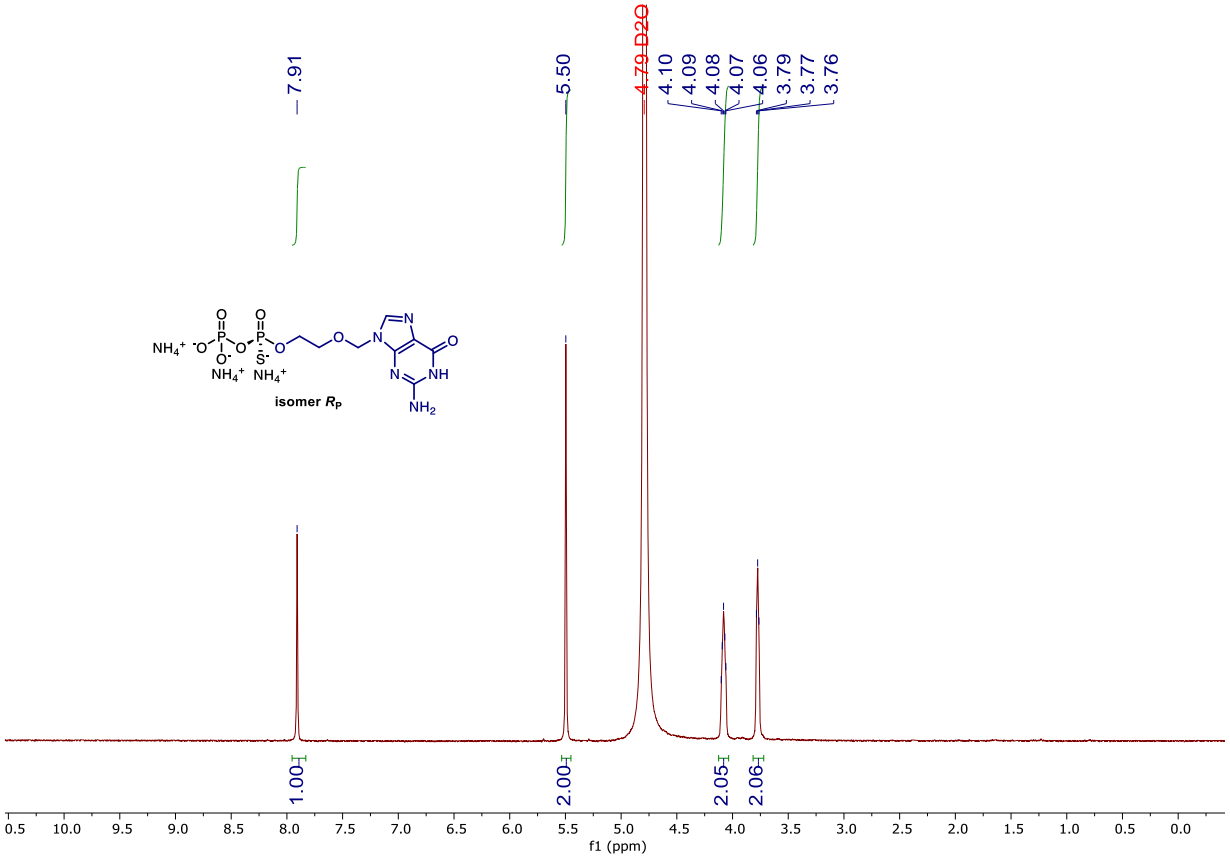
^{13}C NMR of compound (S_P)-21 (150 MHz, D_2O)



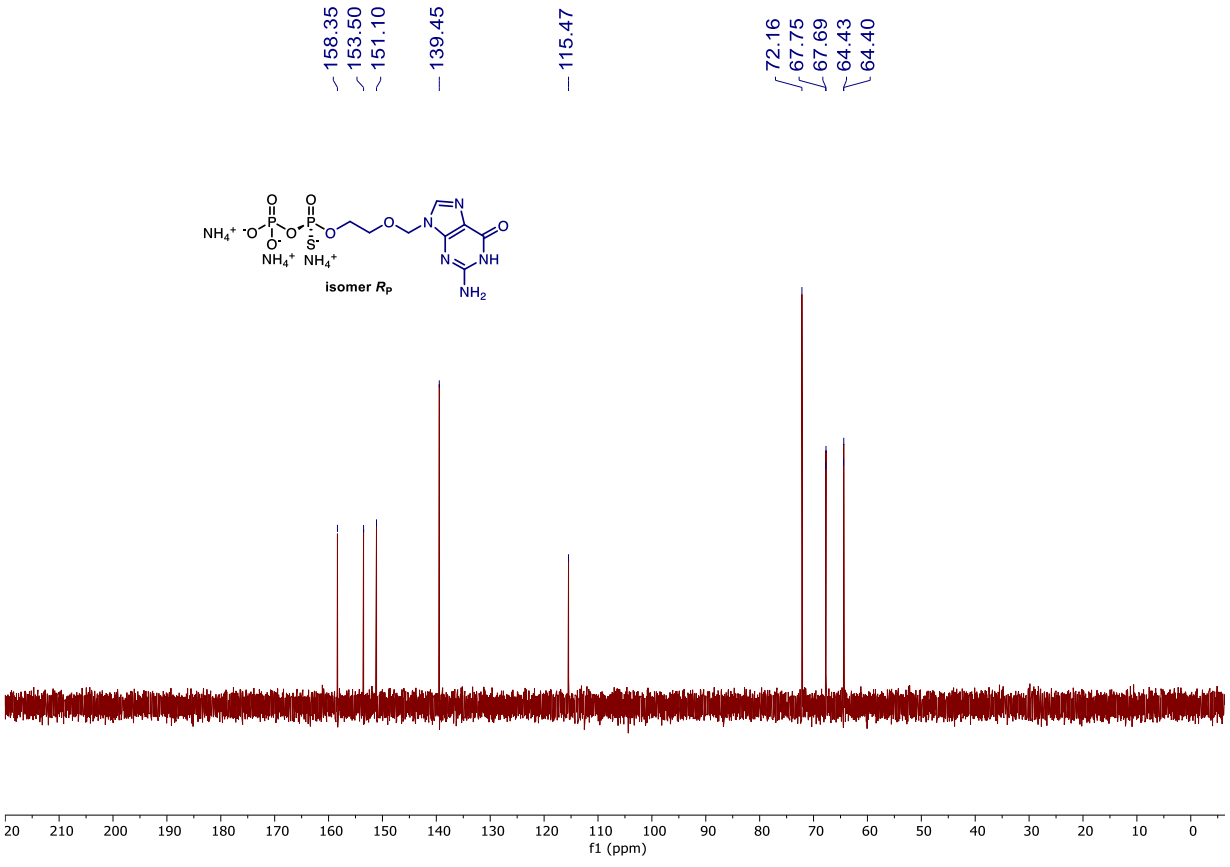
^{31}P NMR of compound (S_P)-21 (162 MHz, D_2O)



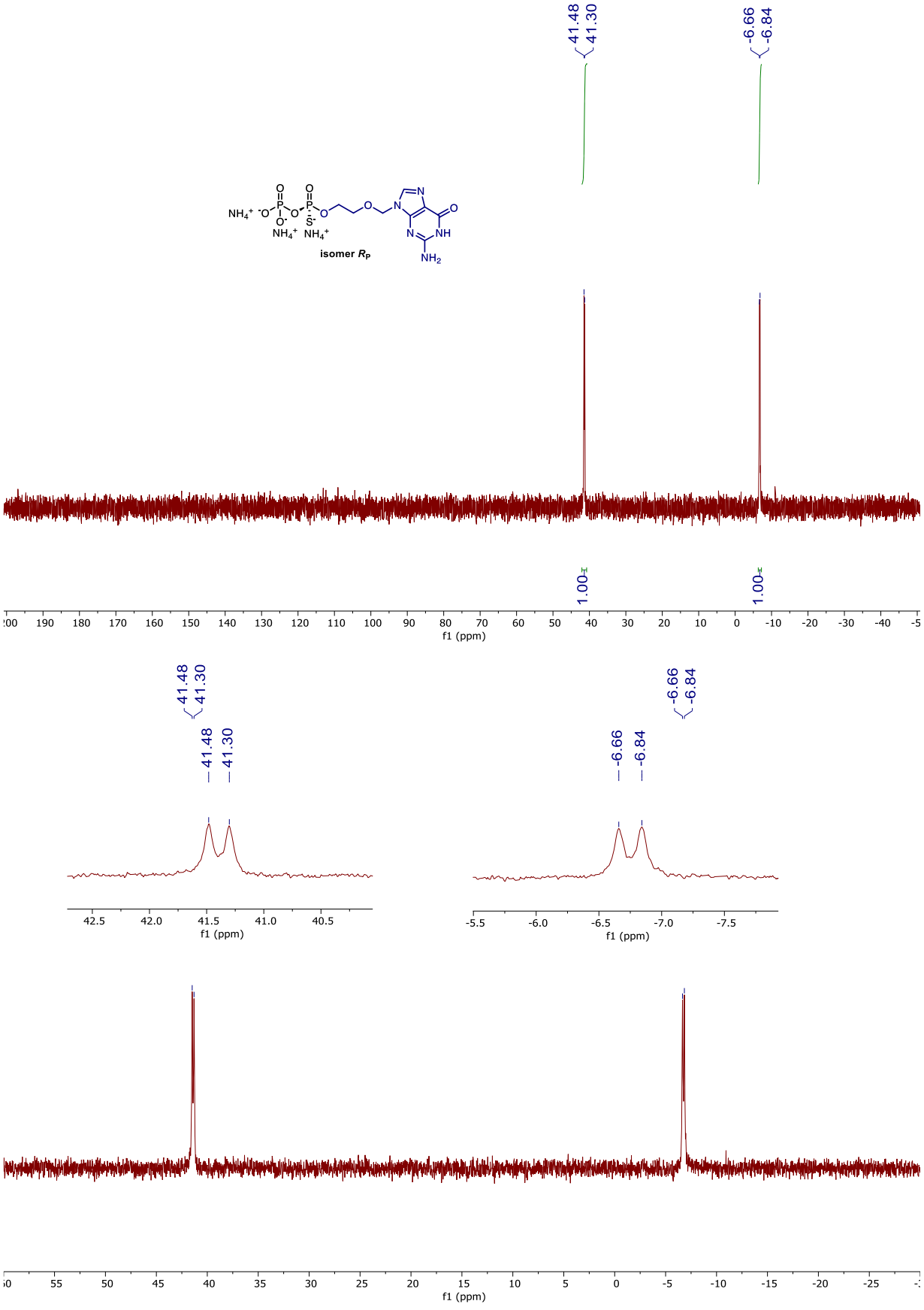
^{1}H NMR of compound (R_P)-22 (600 MHz, D_2O)



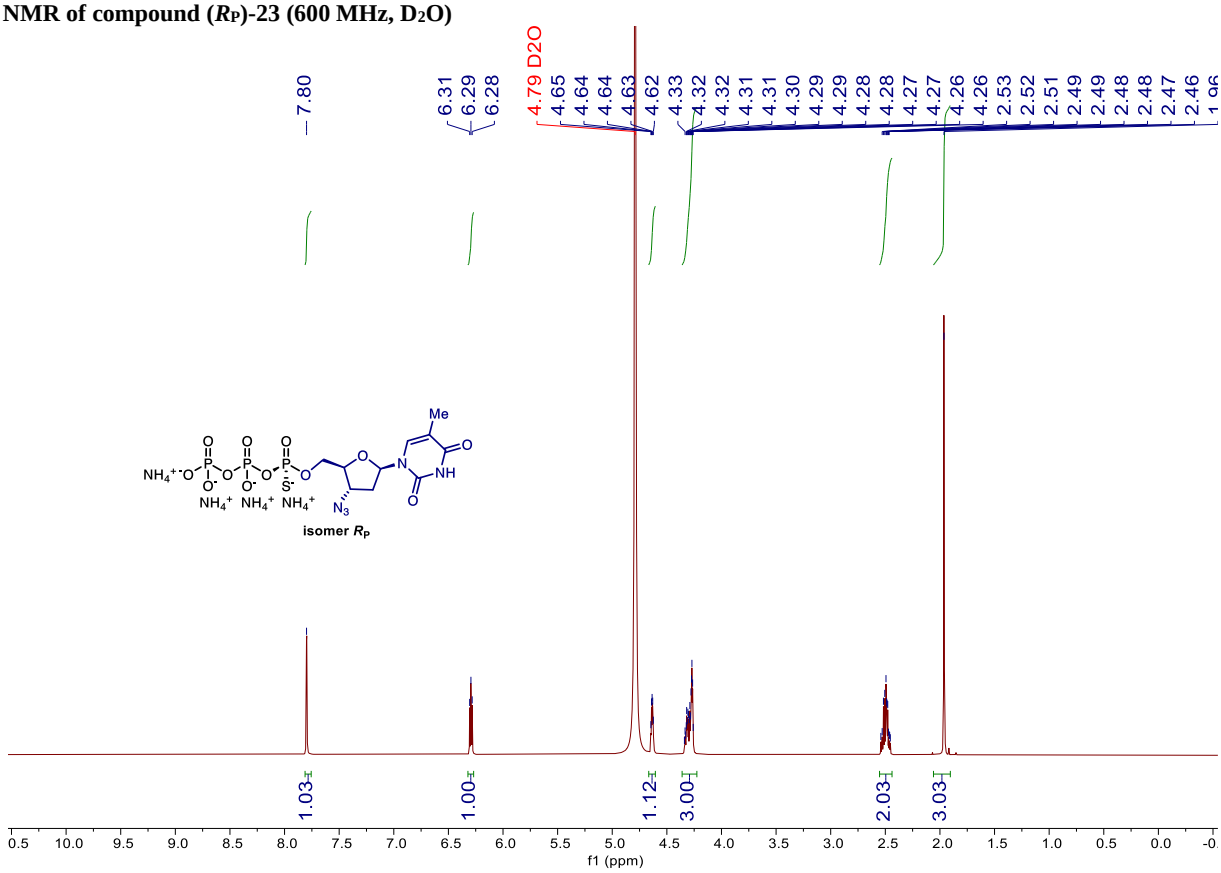
^{13}C NMR of compound (R_P)-22 (150 MHz, D_2O)



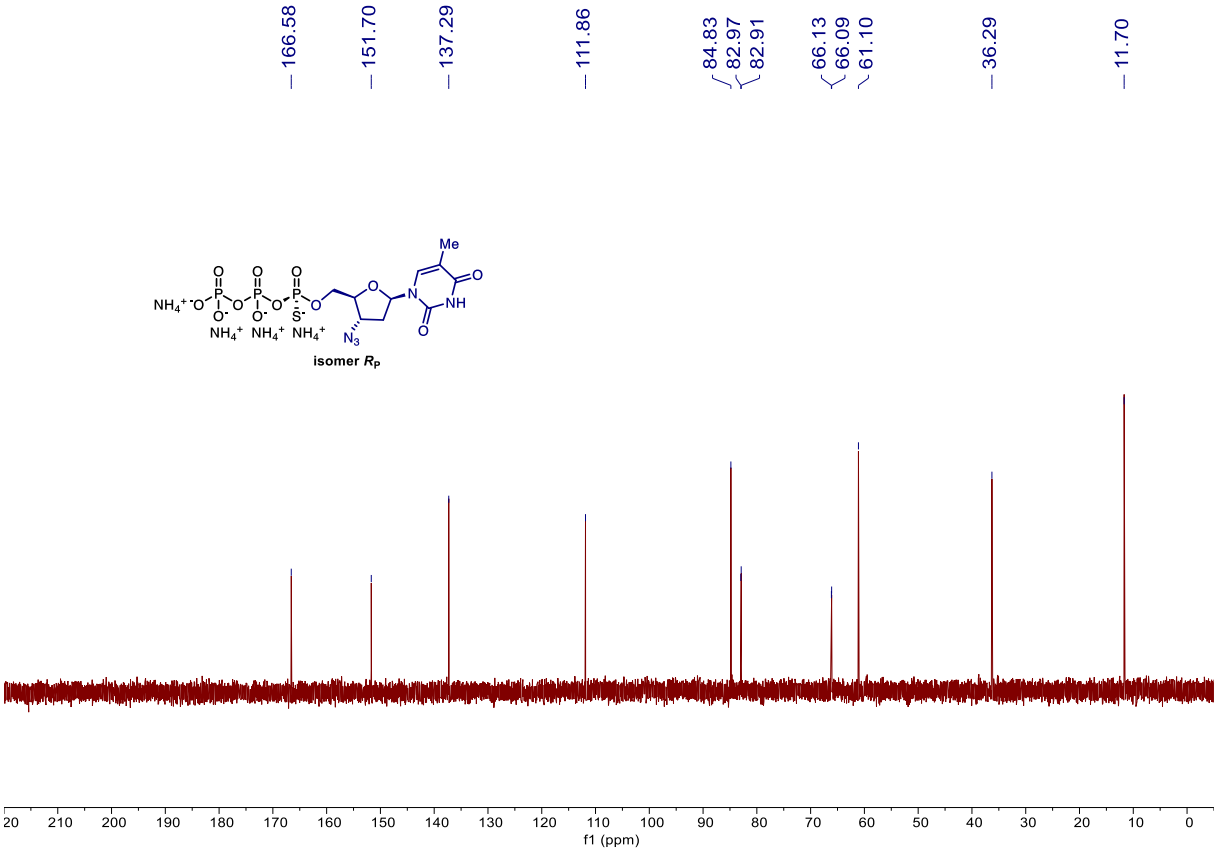
^{31}P NMR of compound (R_P)-22 (162 MHz, D_2O)



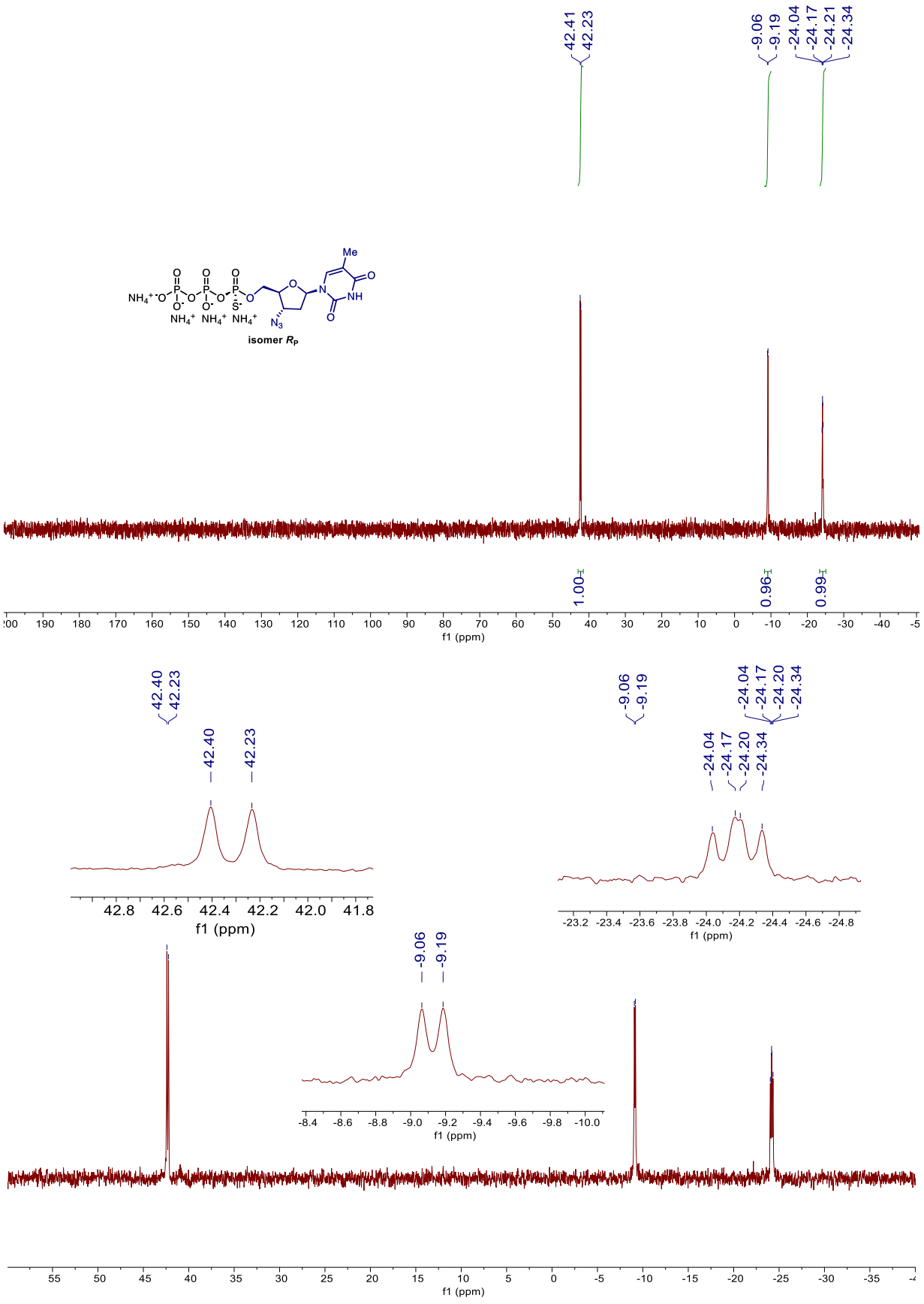
^{1}H NMR of compound (R_P)-23 (600 MHz, D_2O)



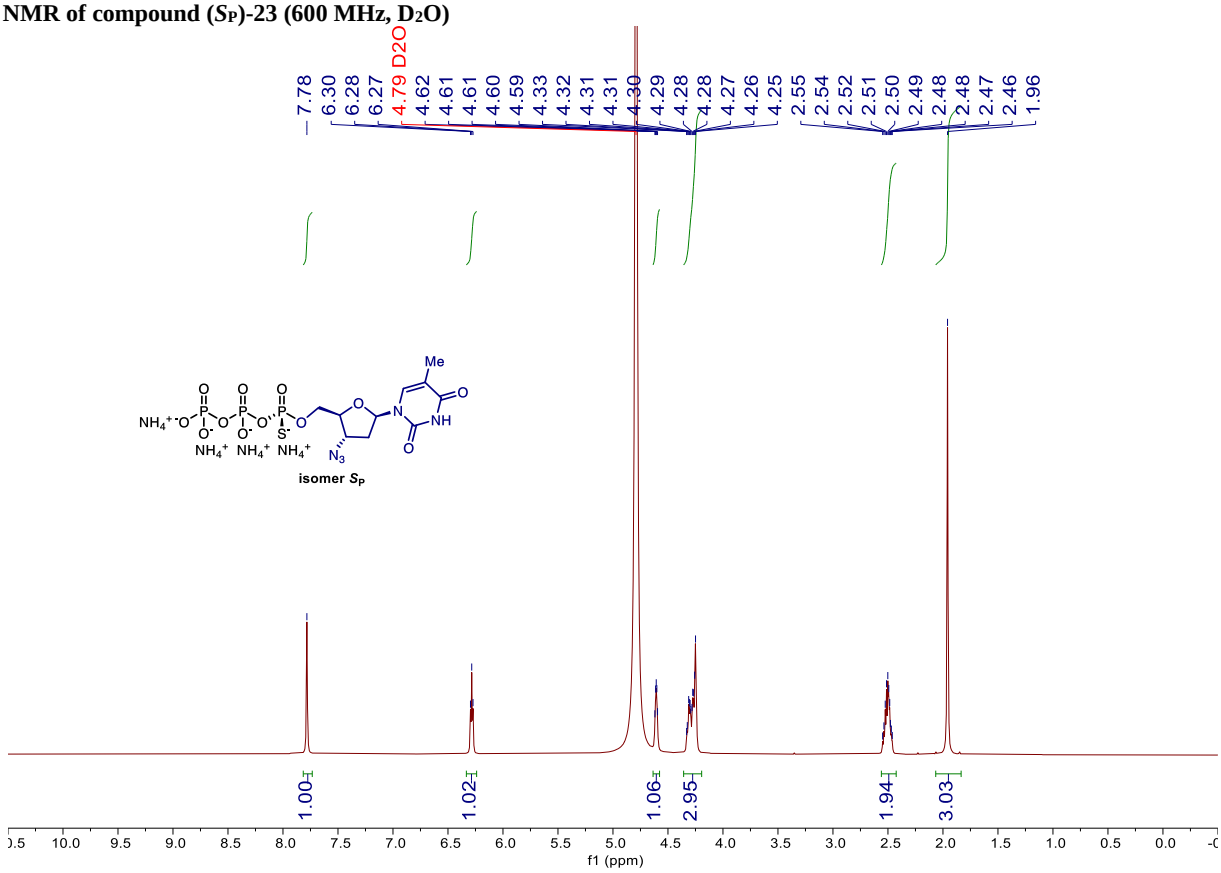
^{13}C NMR of compound (R_P)-23 (150 MHz, D_2O)



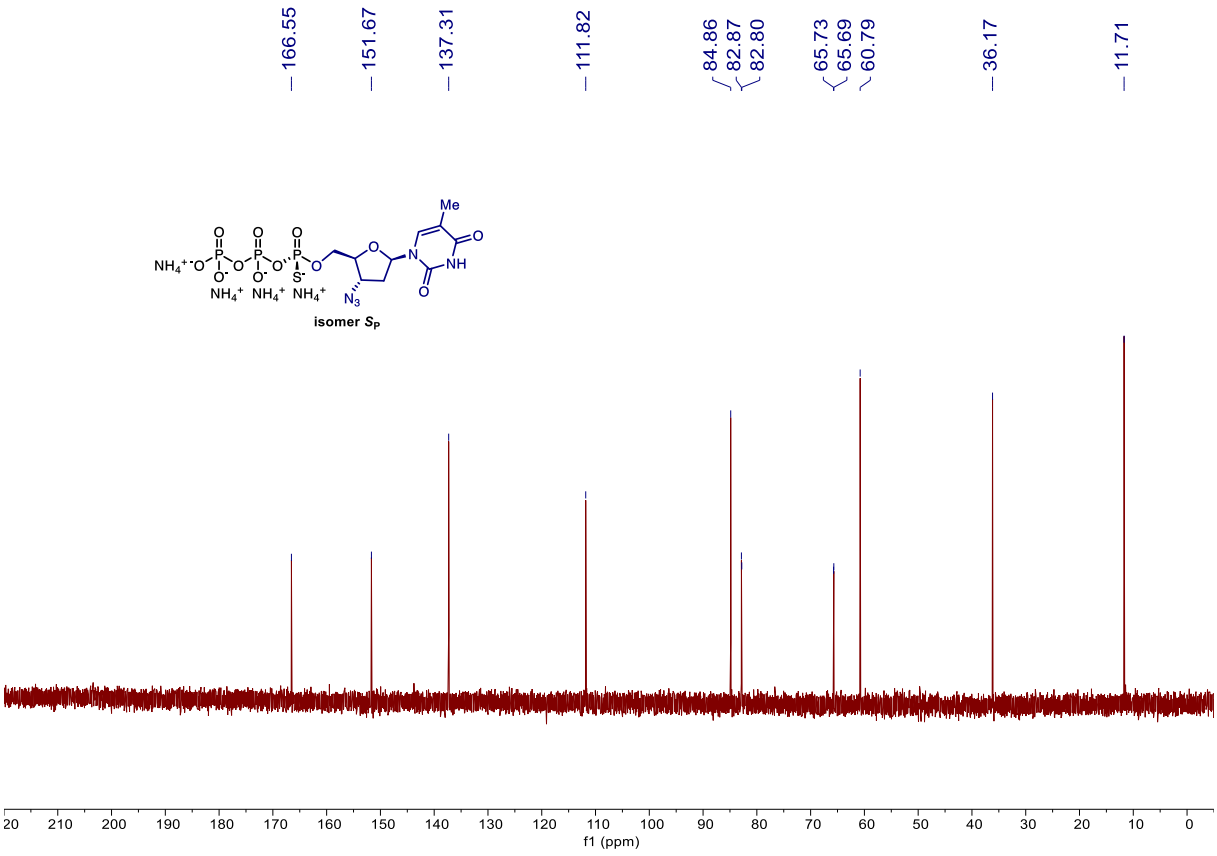
^{31}P NMR of compound (R_P)-23 (162 MHz, D_2O)



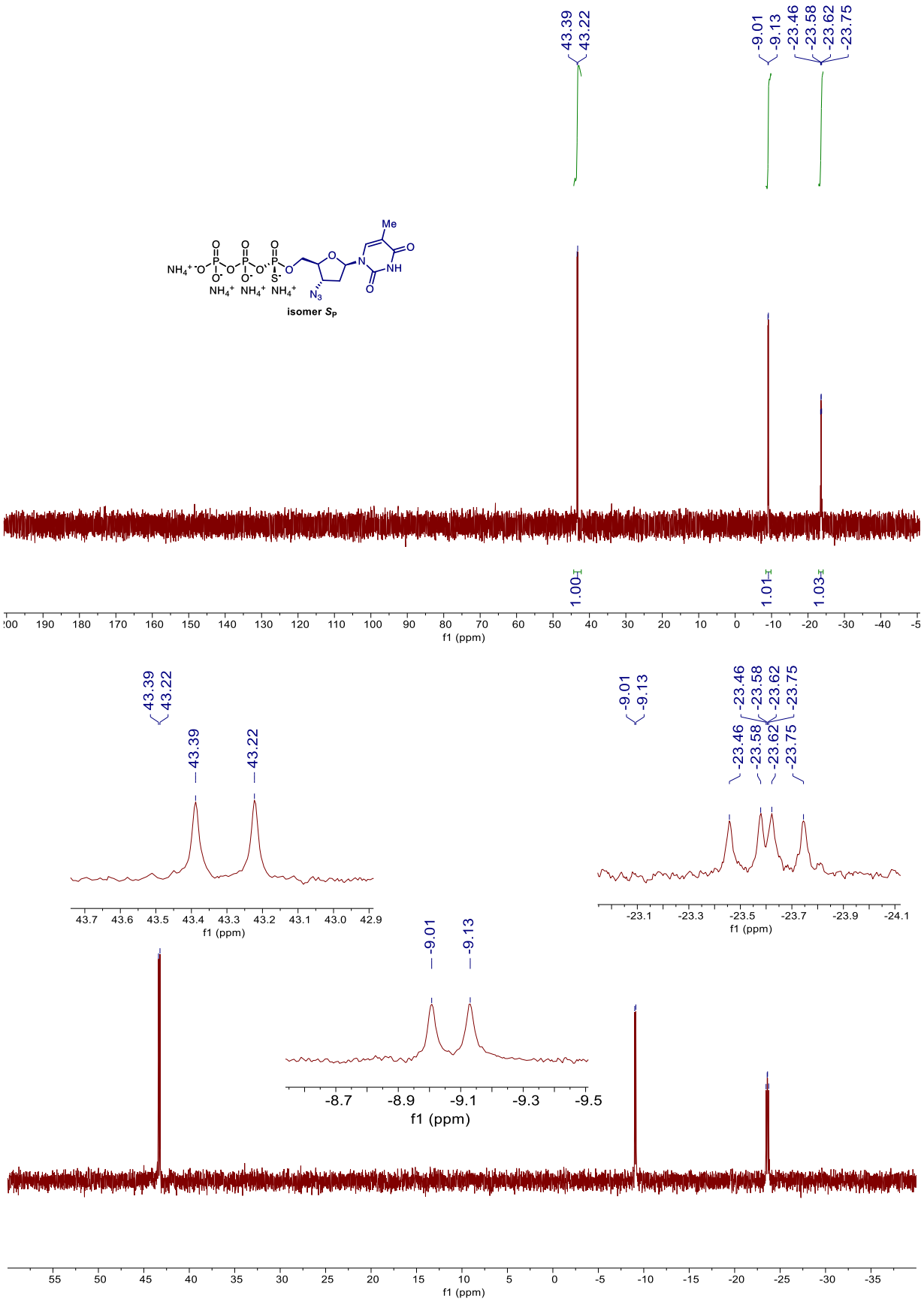
^{1}H NMR of compound (S_P)-23 (600 MHz, D_2O)



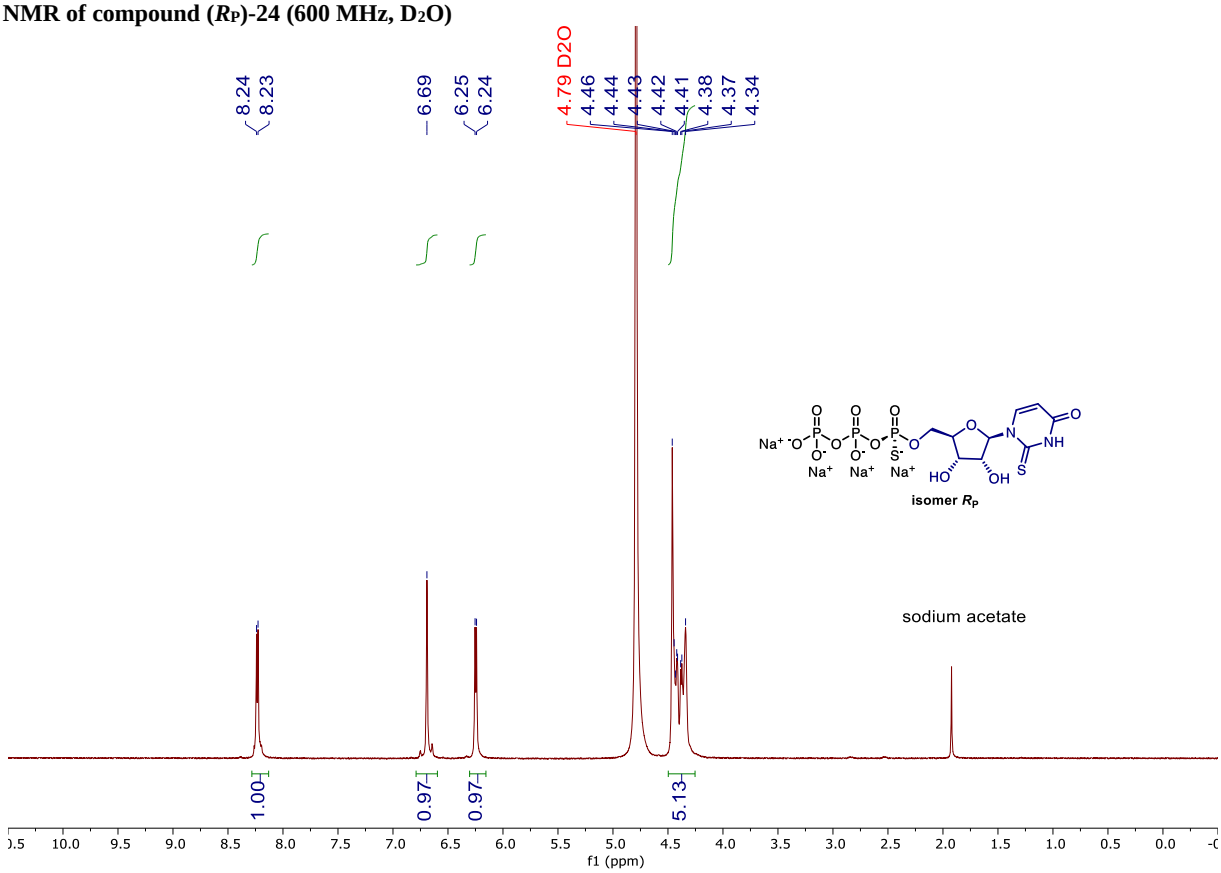
^{13}C NMR of compound (S_P)-23 (150 MHz, D_2O)



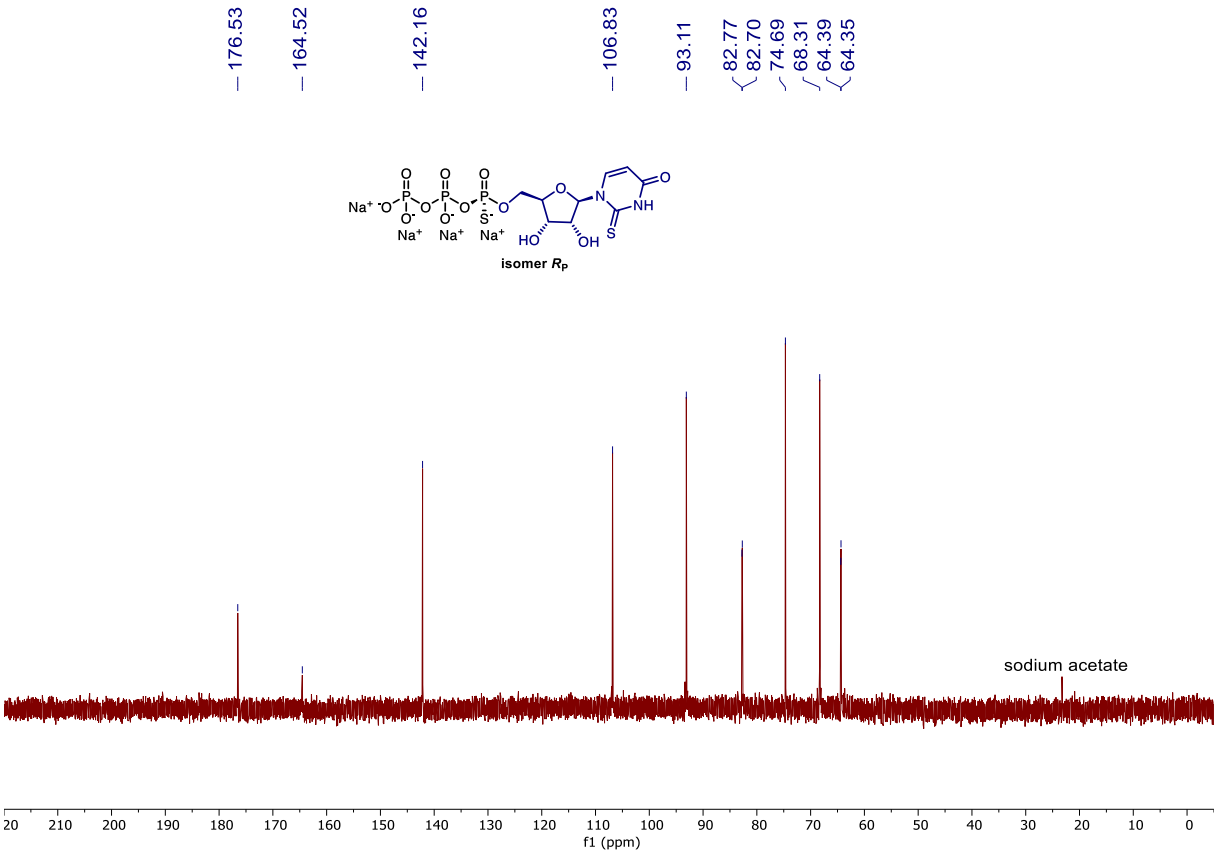
^{31}P NMR of compound (S_P)-23 (162 MHz, D_2O)



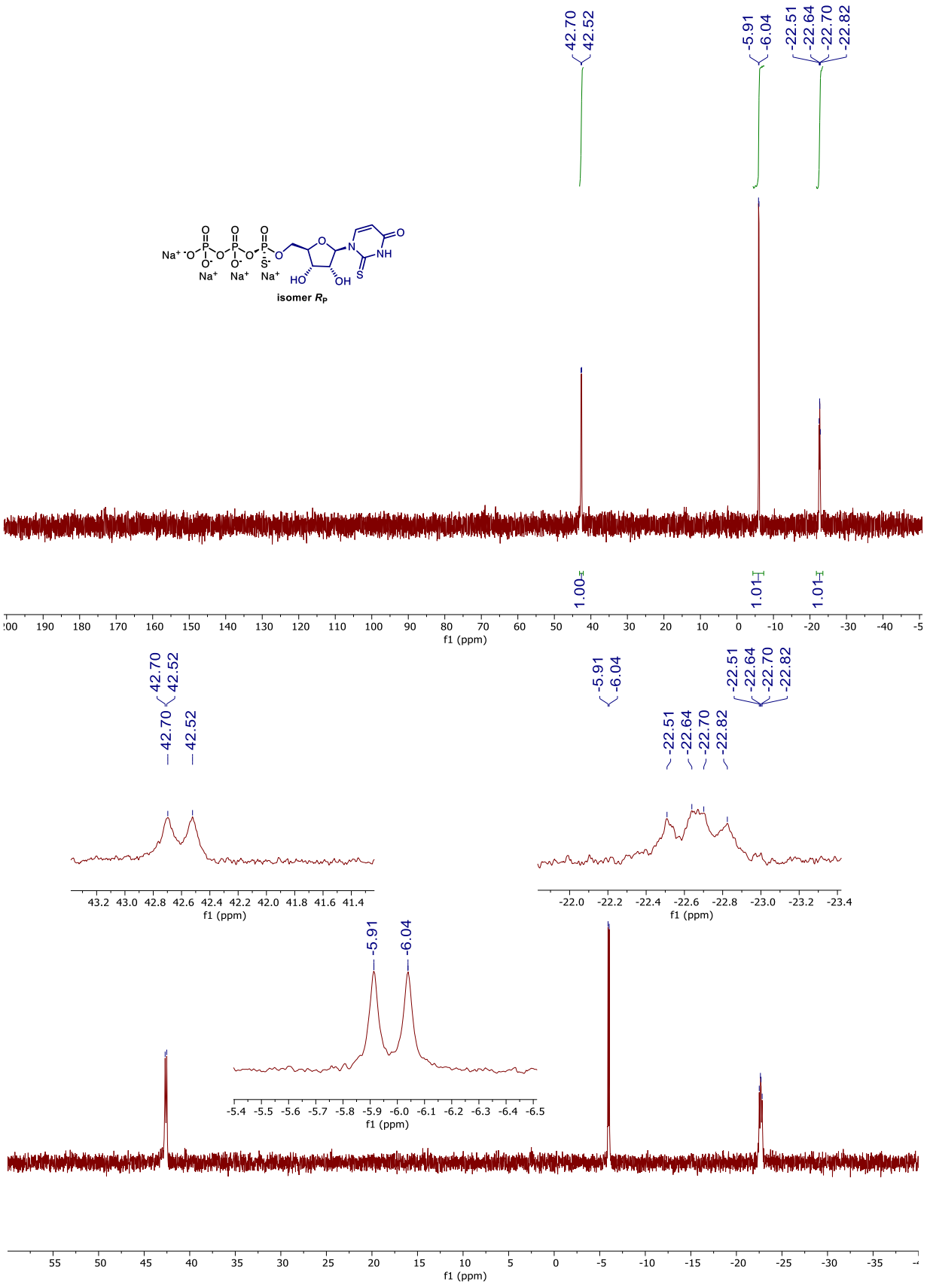
^{1}H NMR of compound (R_P)-24 (600 MHz, D_2O)



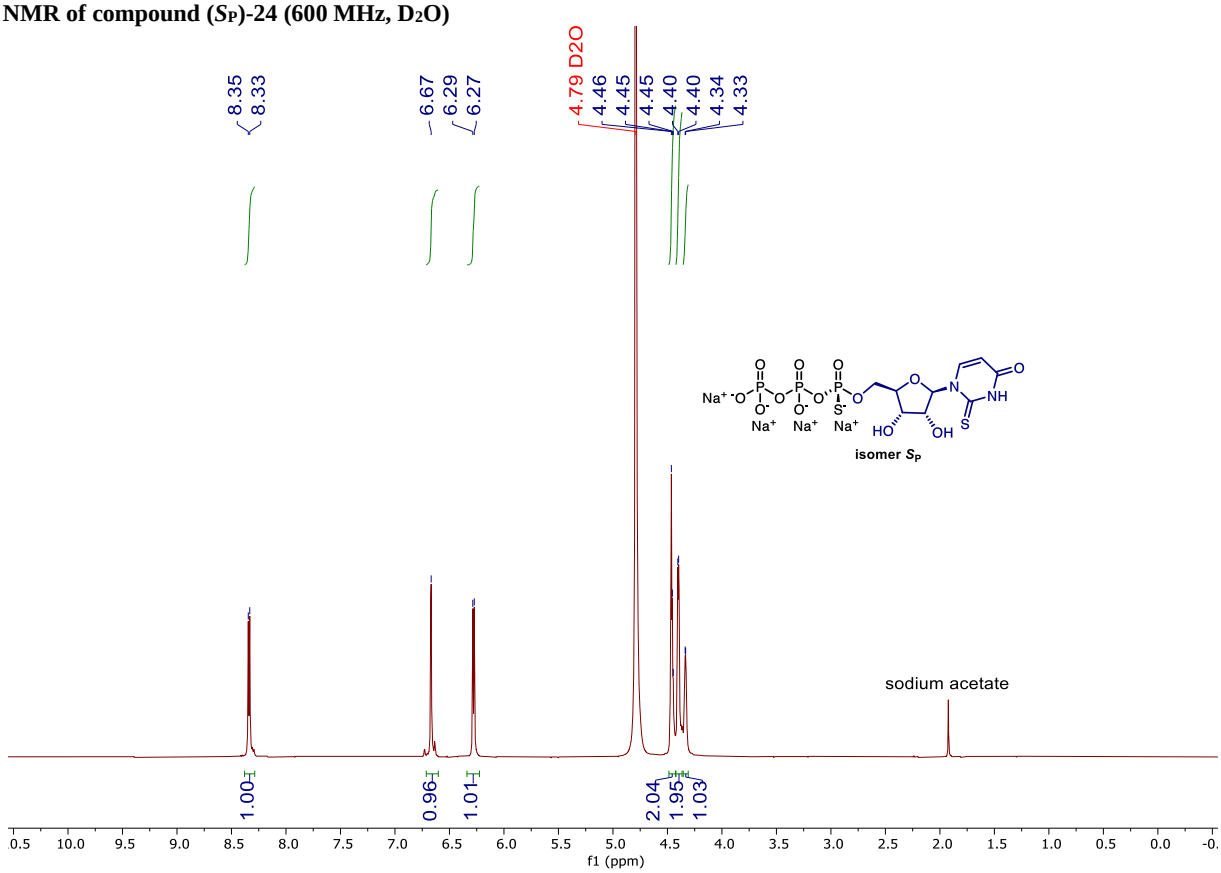
^{13}C NMR of compound (R_P)-24 (150 MHz, D_2O)



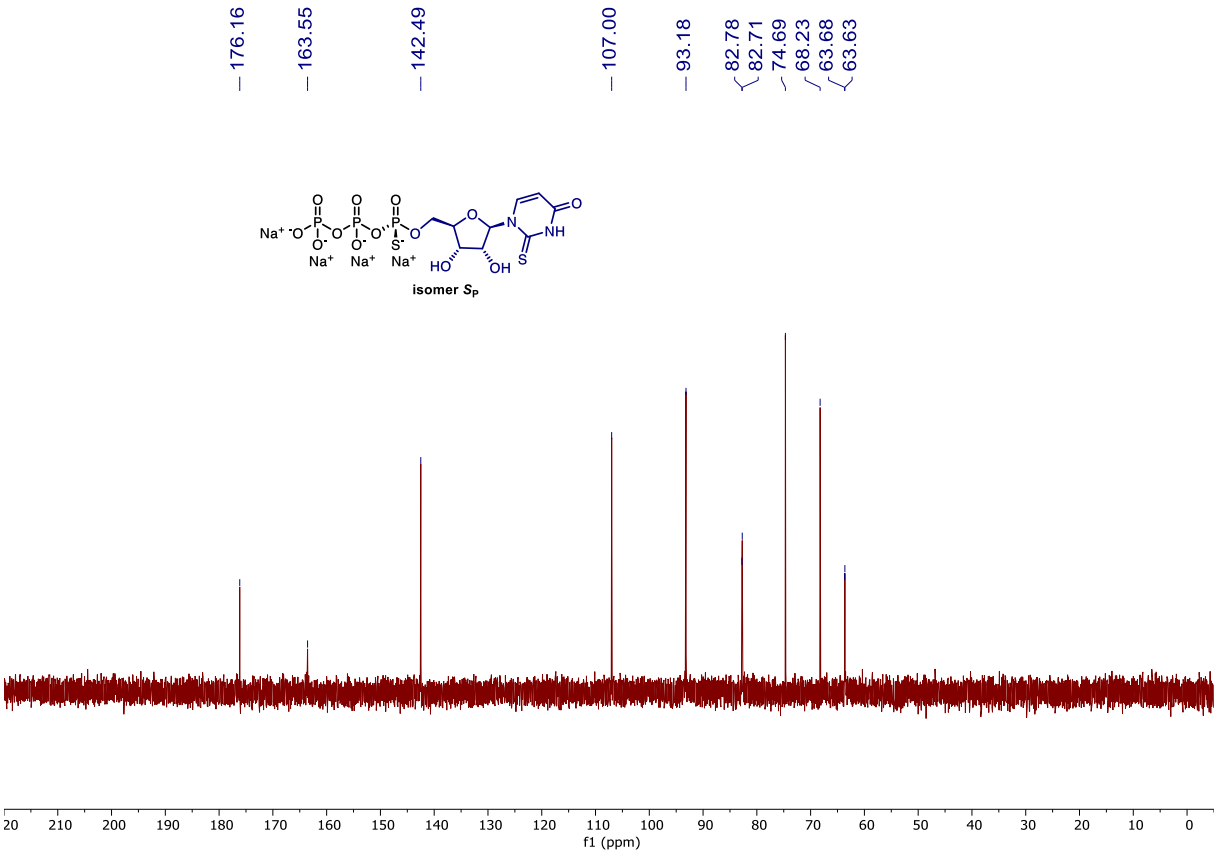
^{31}P NMR of compound (R_P)-24 (162 MHz, D_2O)



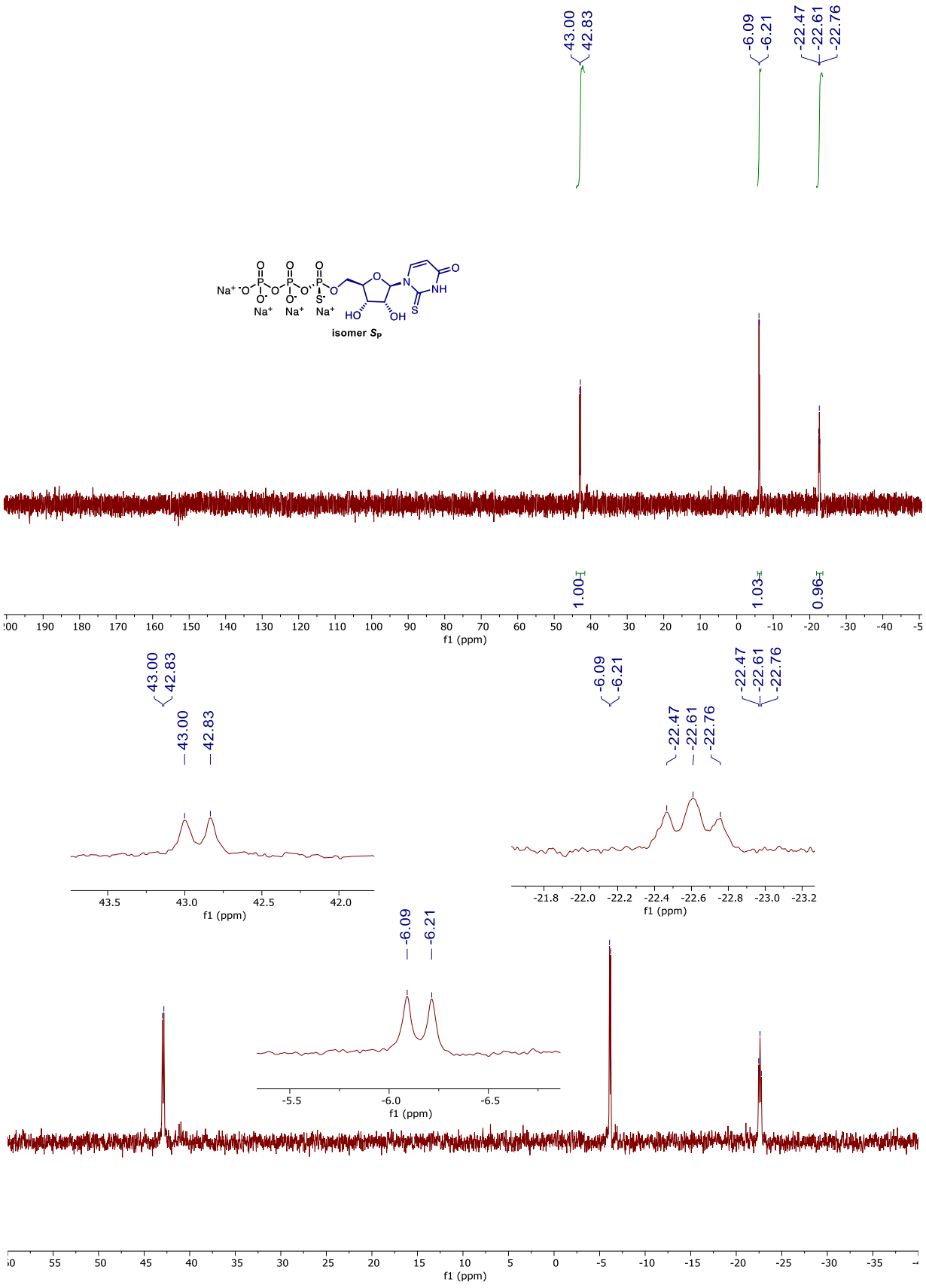
^{1}H NMR of compound (S_P)-24 (600 MHz, D_2O)



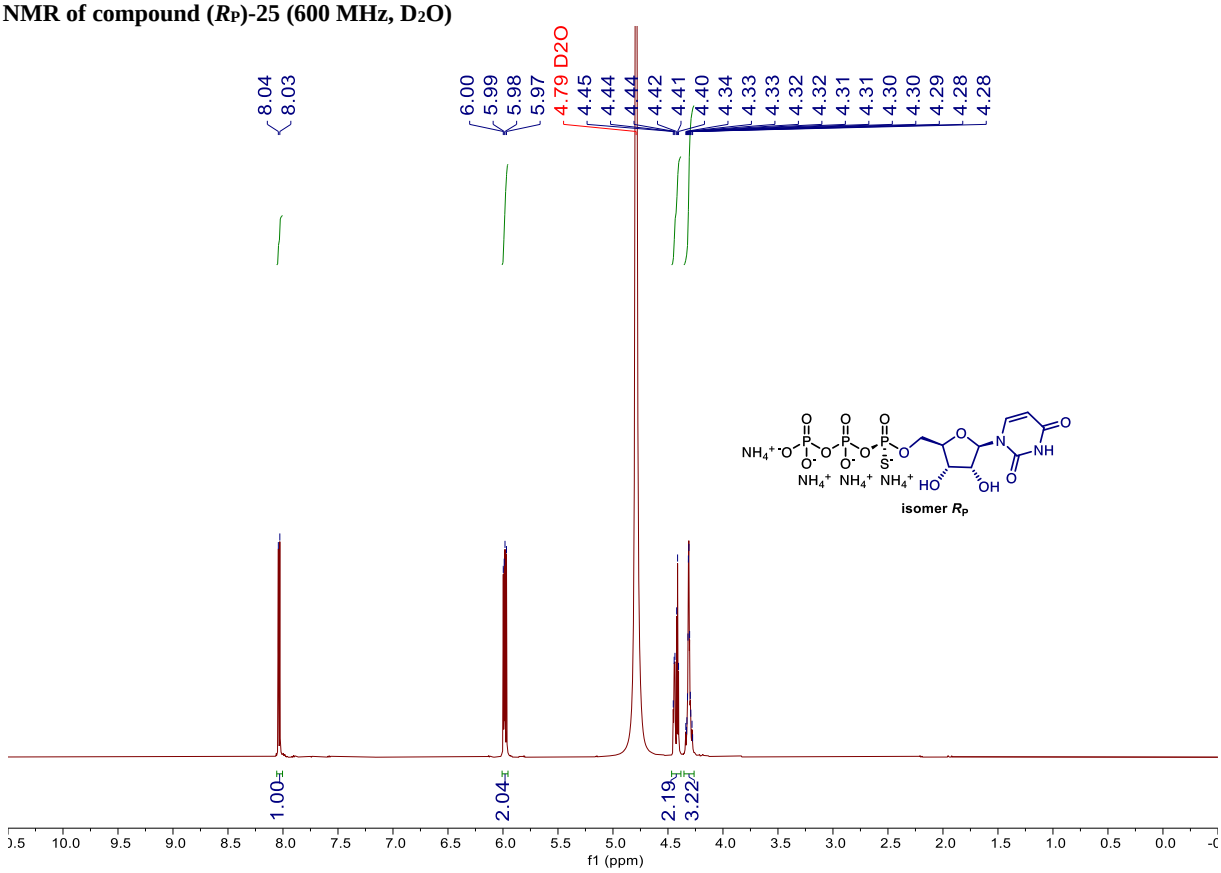
^{13}C NMR of compound (S_P)-24 (150 MHz, D_2O)



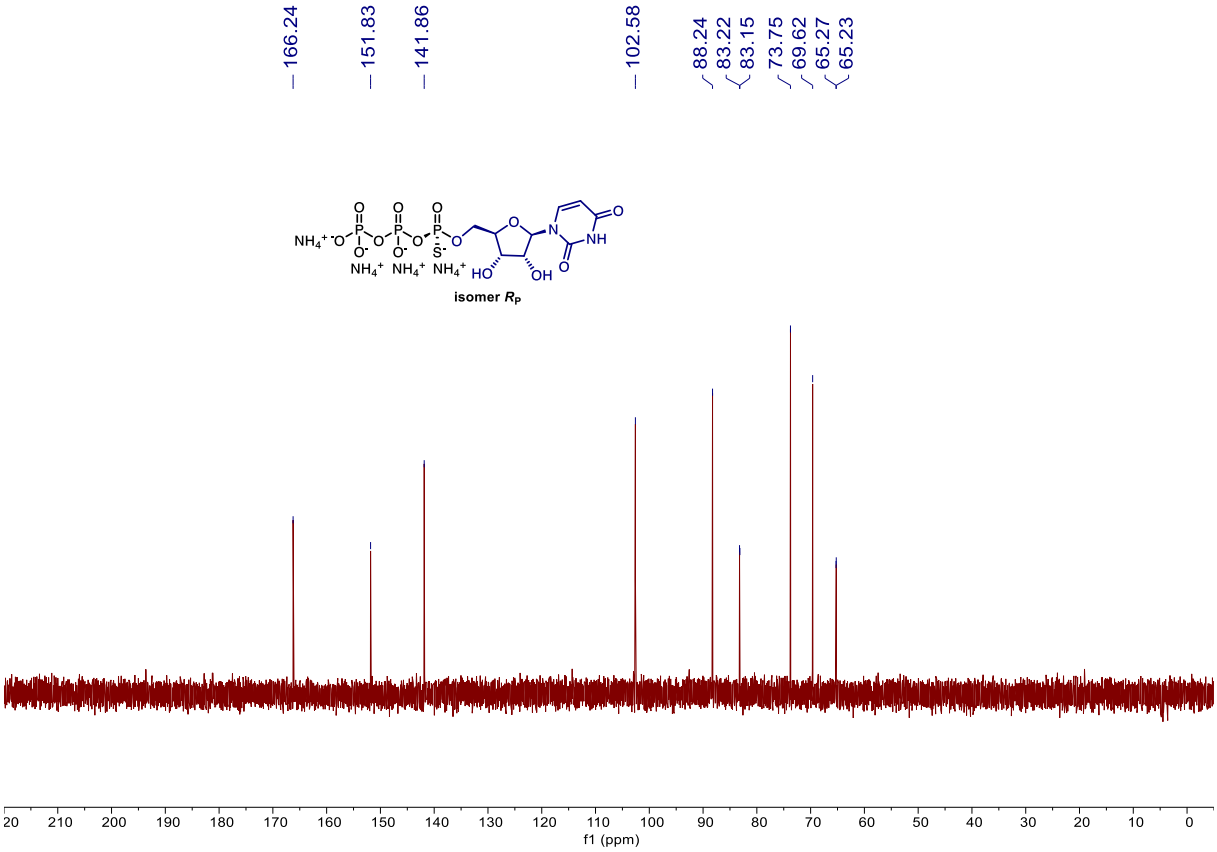
^{31}P NMR of compound (S_P)-24 (162 MHz, D_2O)



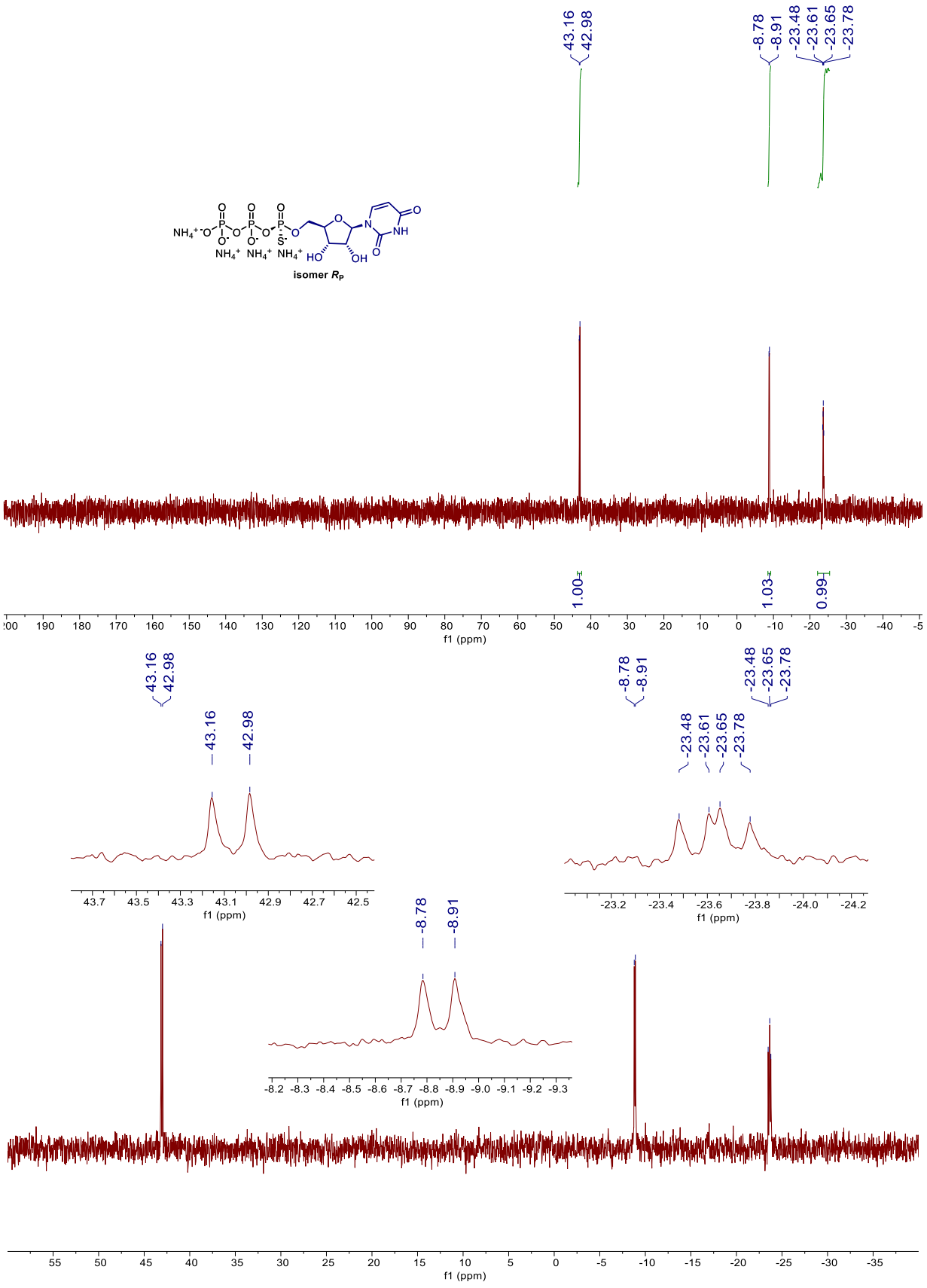
^{1}H NMR of compound (R_P)-25 (600 MHz, D_2O)



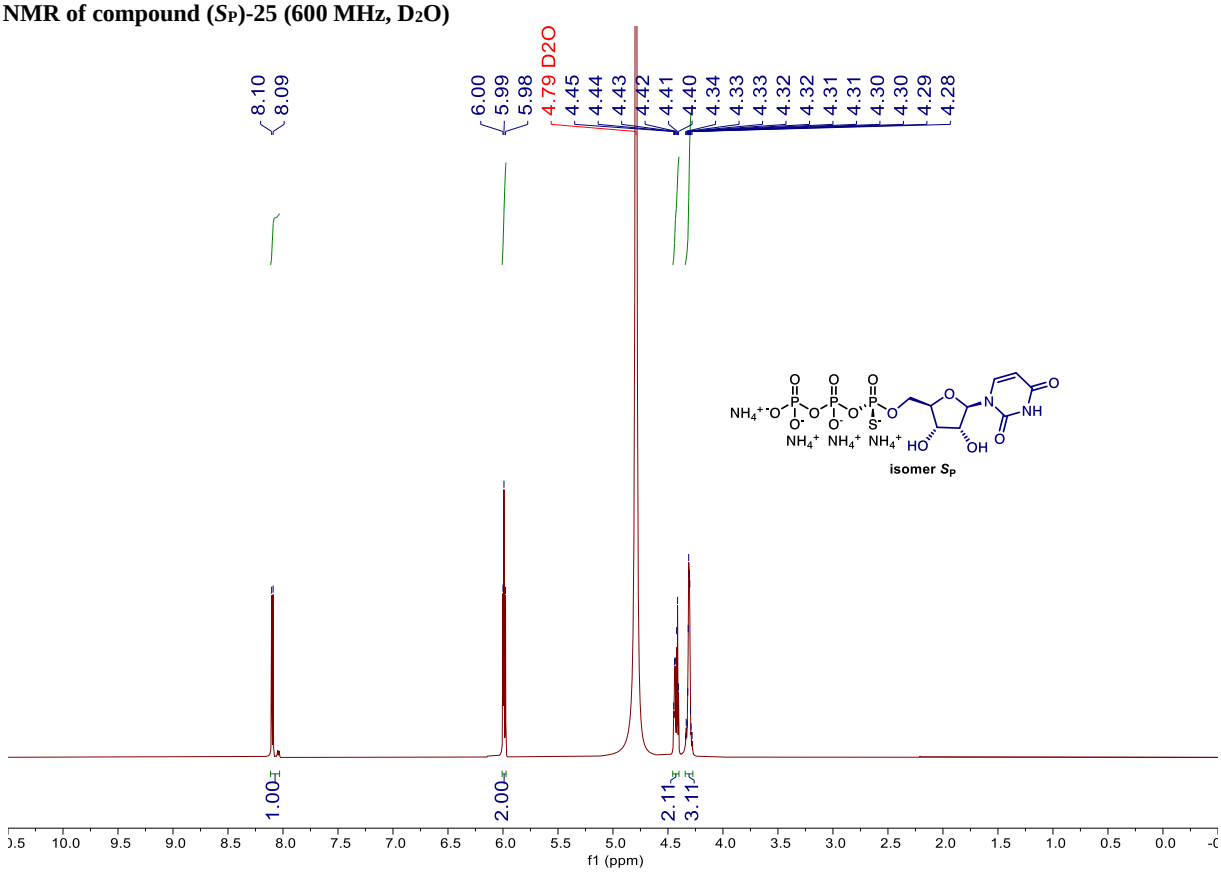
^{13}C NMR of compound (R_P)-25 (150 MHz, D_2O)



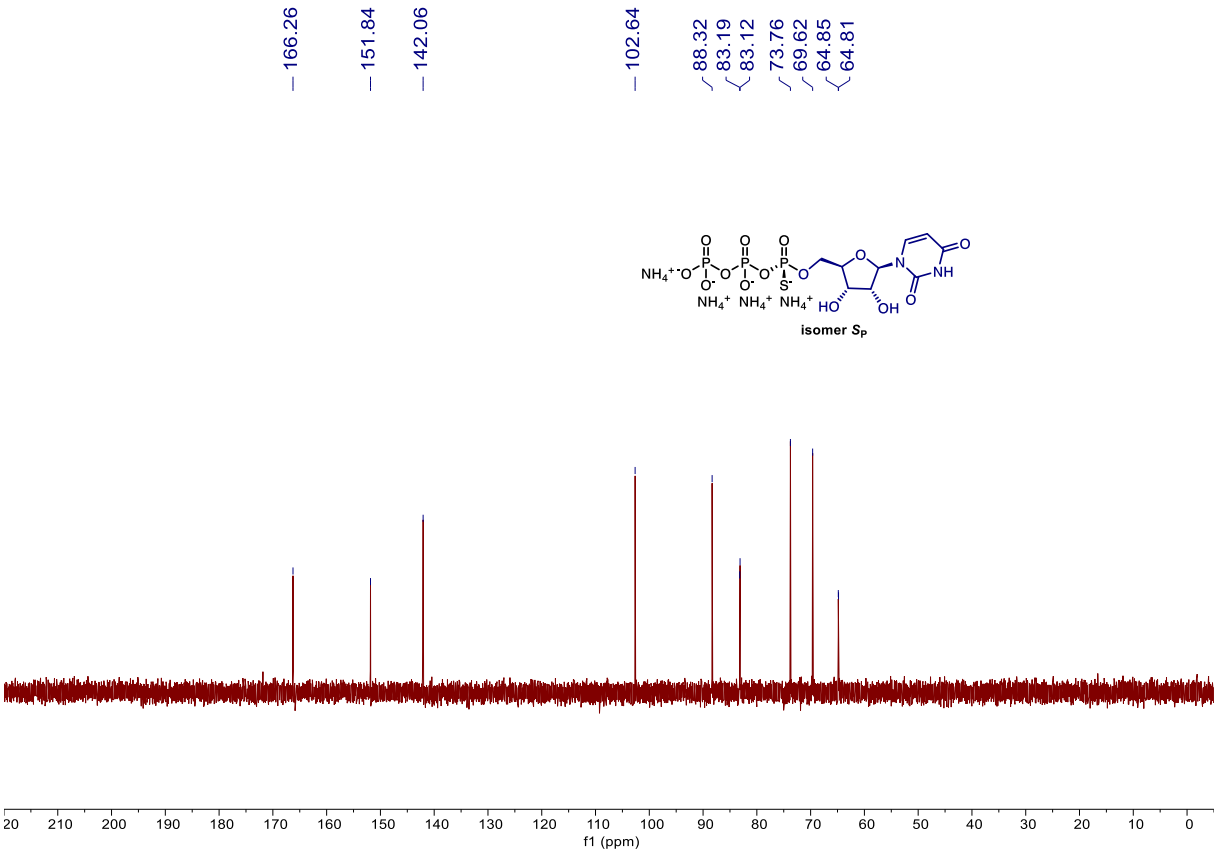
^{31}P NMR of compound (R_P)-25 (162 MHz, D_2O)



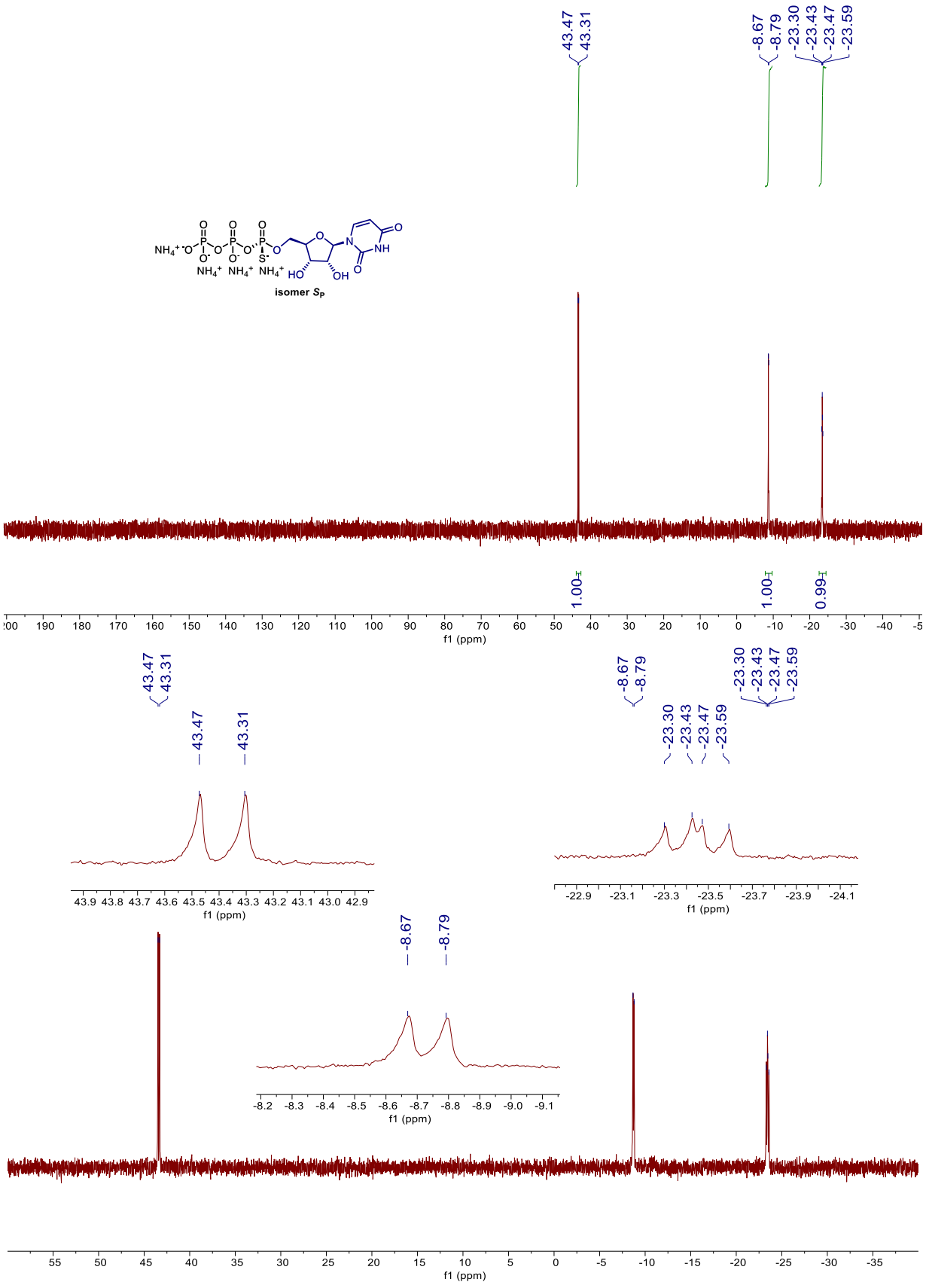
^{1}H NMR of compound (S_P)-25 (600 MHz, D_2O)



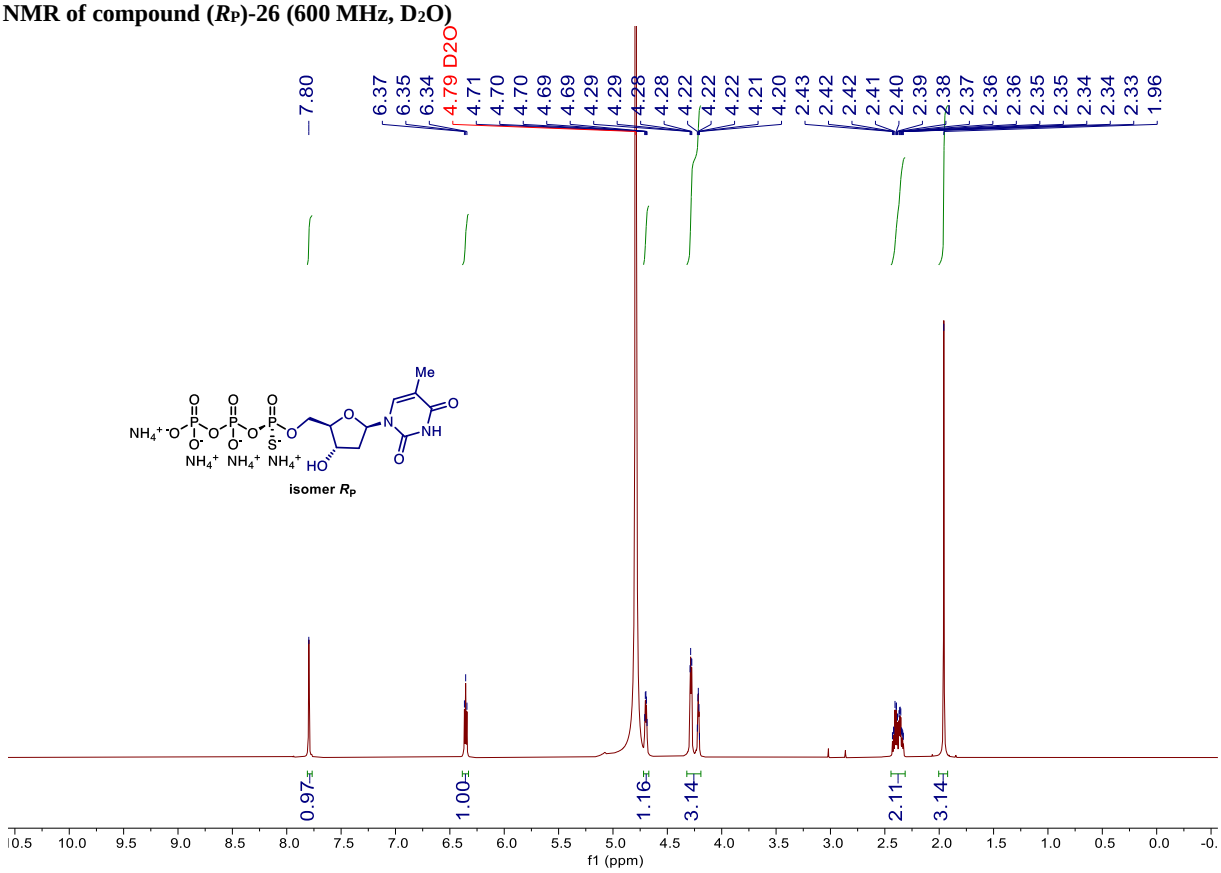
^{13}C NMR of compound (S_P)-25 (150 MHz, D_2O)



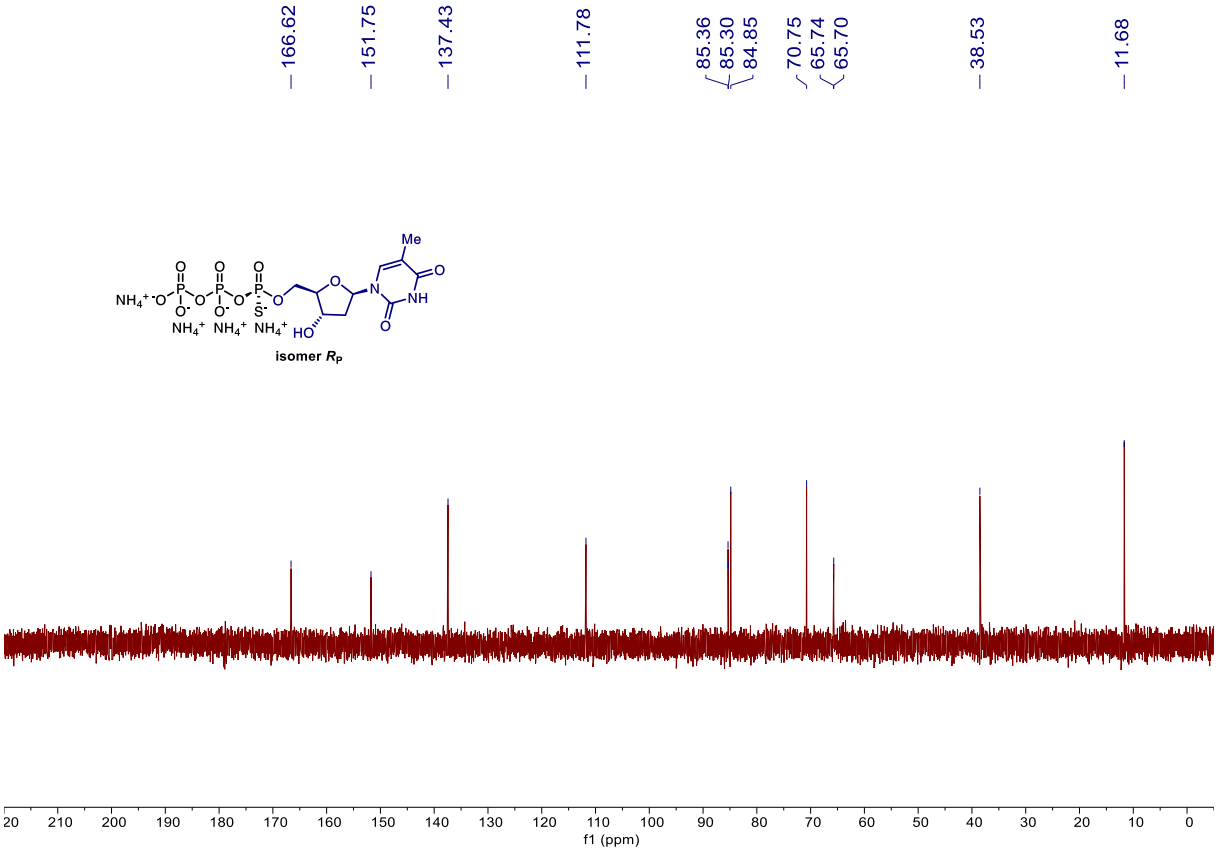
^{31}P NMR of compound (S_P)-25 (162 MHz, D_2O)



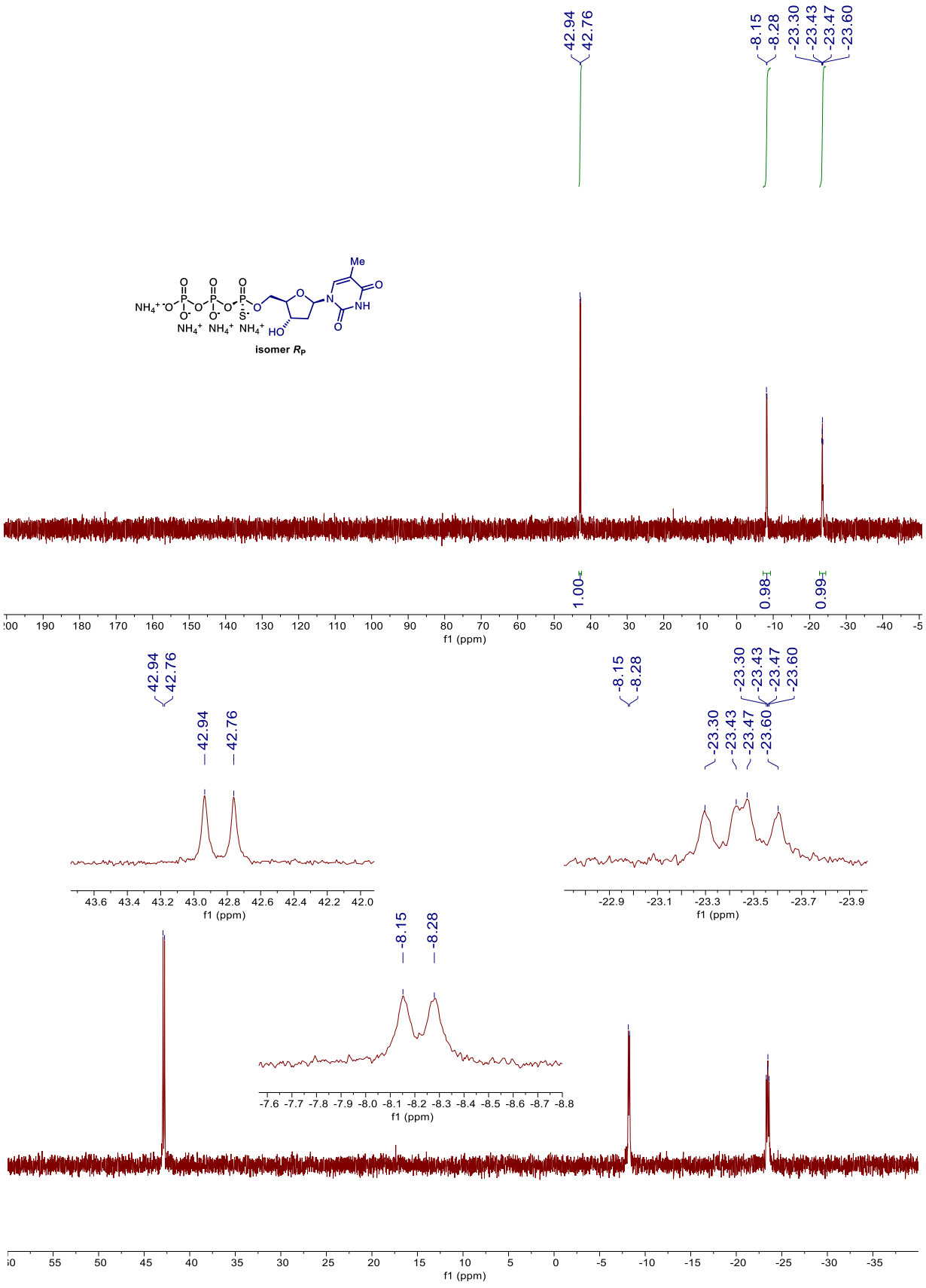
^{1}H NMR of compound (R_P)-26 (600 MHz, D_2O)



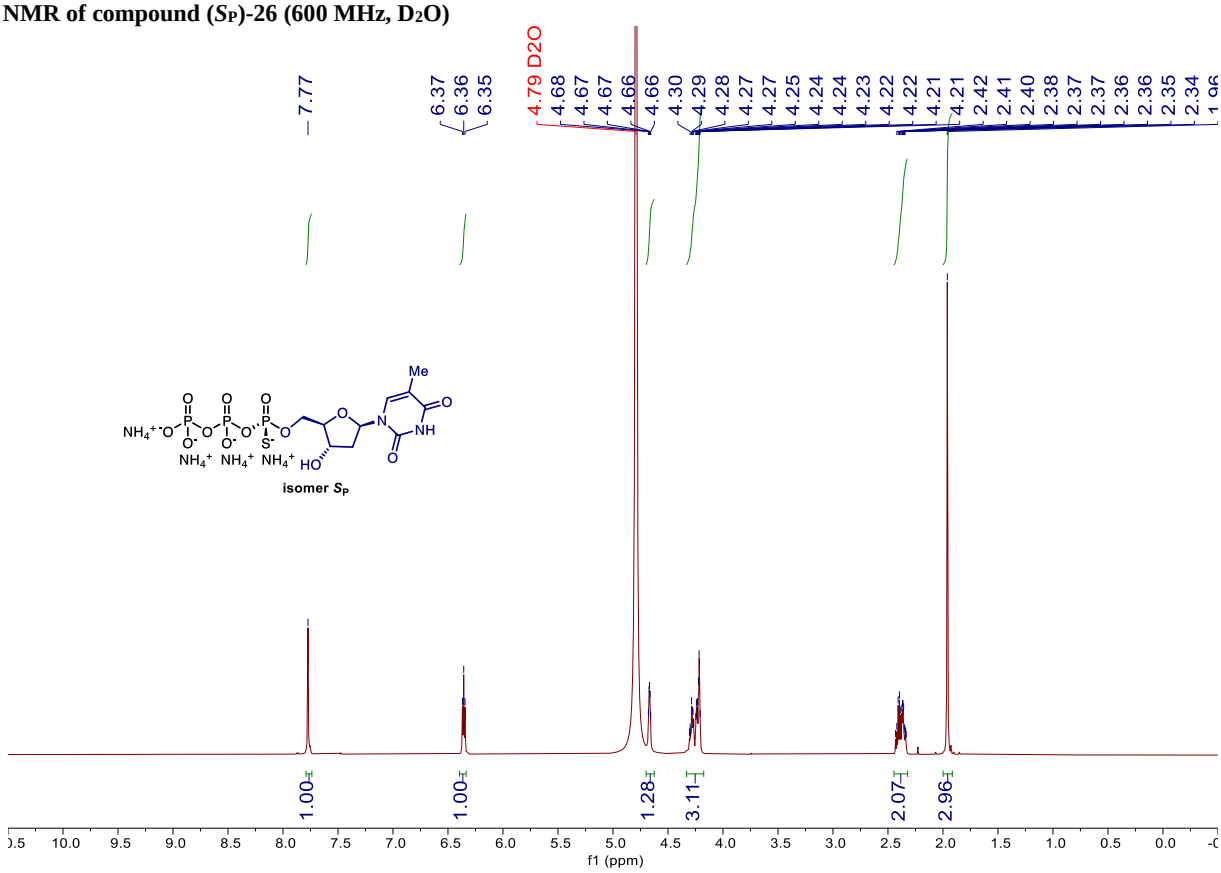
^{13}C NMR of compound (R_P)-26 (150 MHz, D_2O)



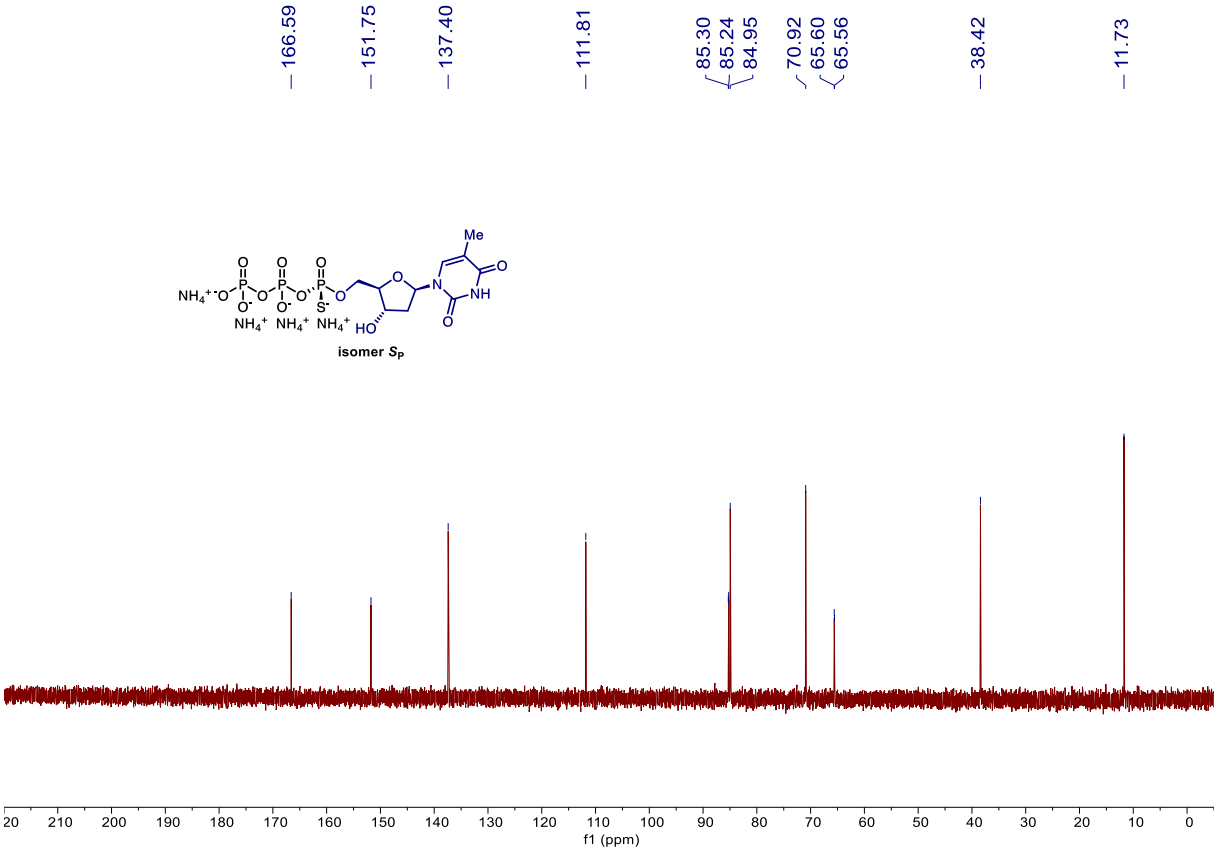
^{31}P NMR of compound (R_P)-26 (162 MHz, D_2O)



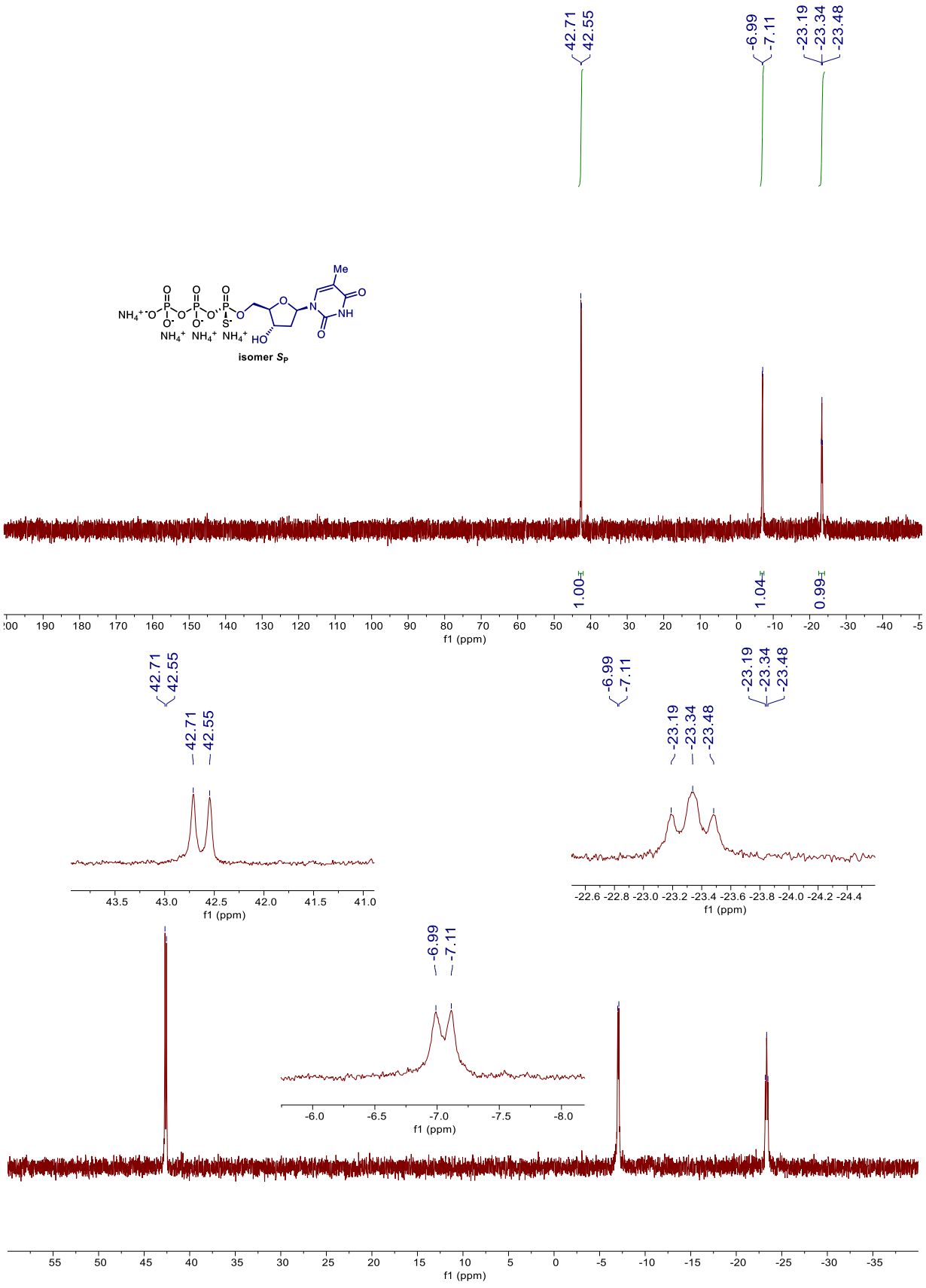
^{1}H NMR of compound (S_P)-26 (600 MHz, D_2O)



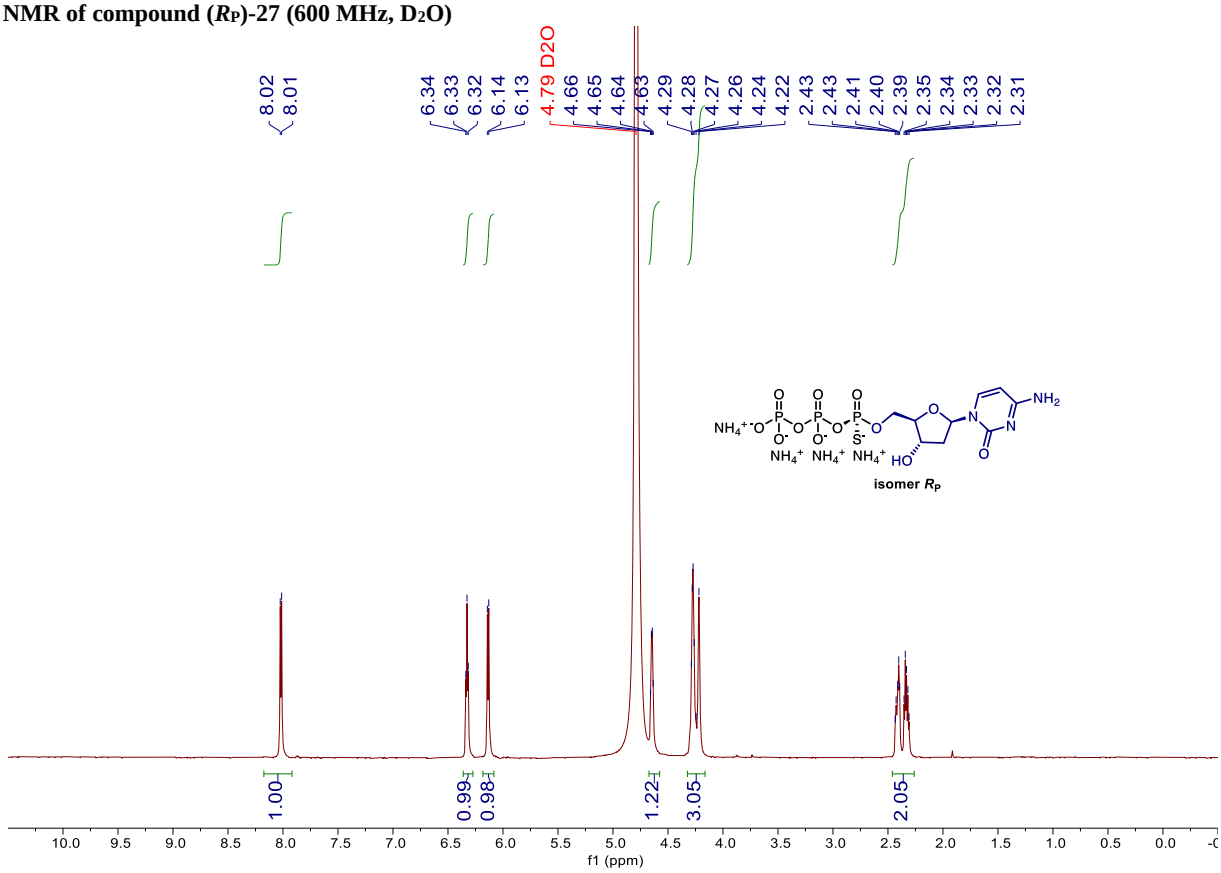
^{13}C NMR of compound (S_P)-26 (150 MHz, D_2O)



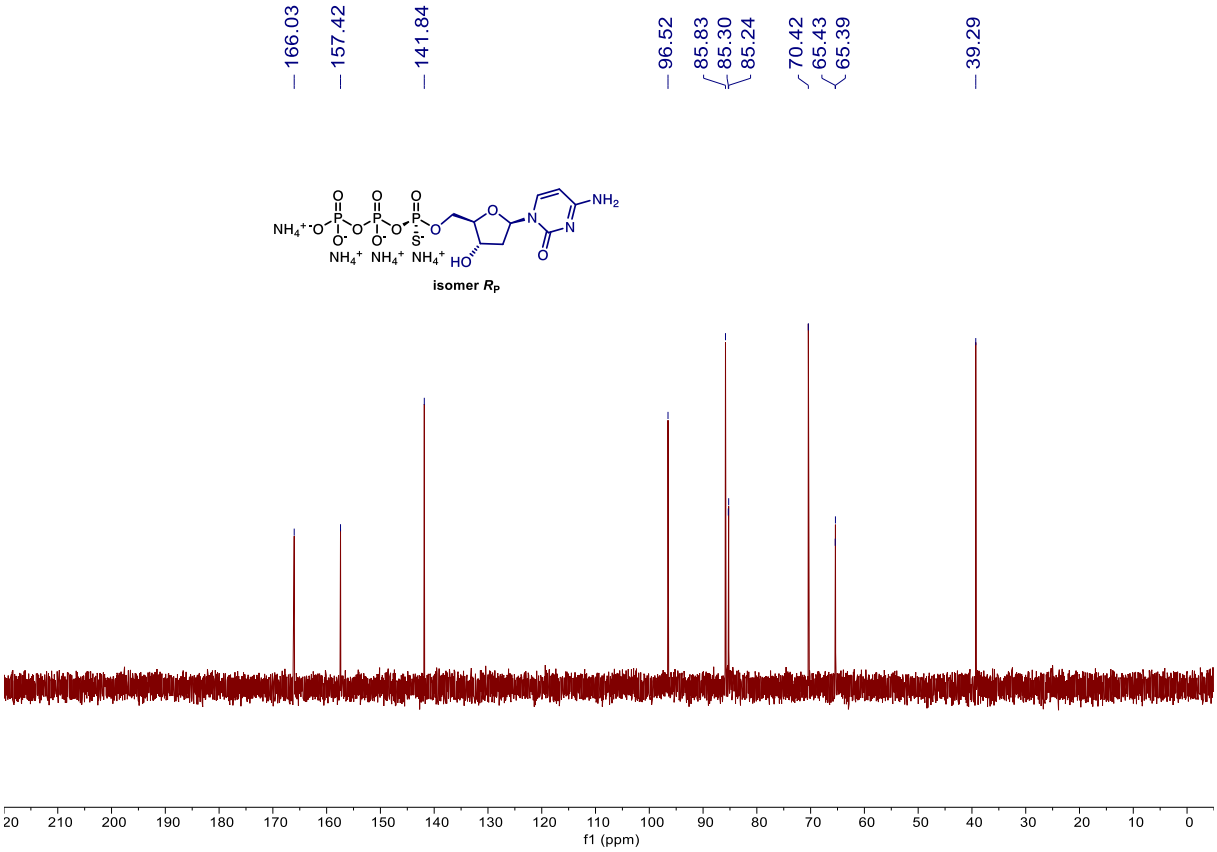
^{31}P NMR of compound (S_P)-26 (162 MHz, D_2O)



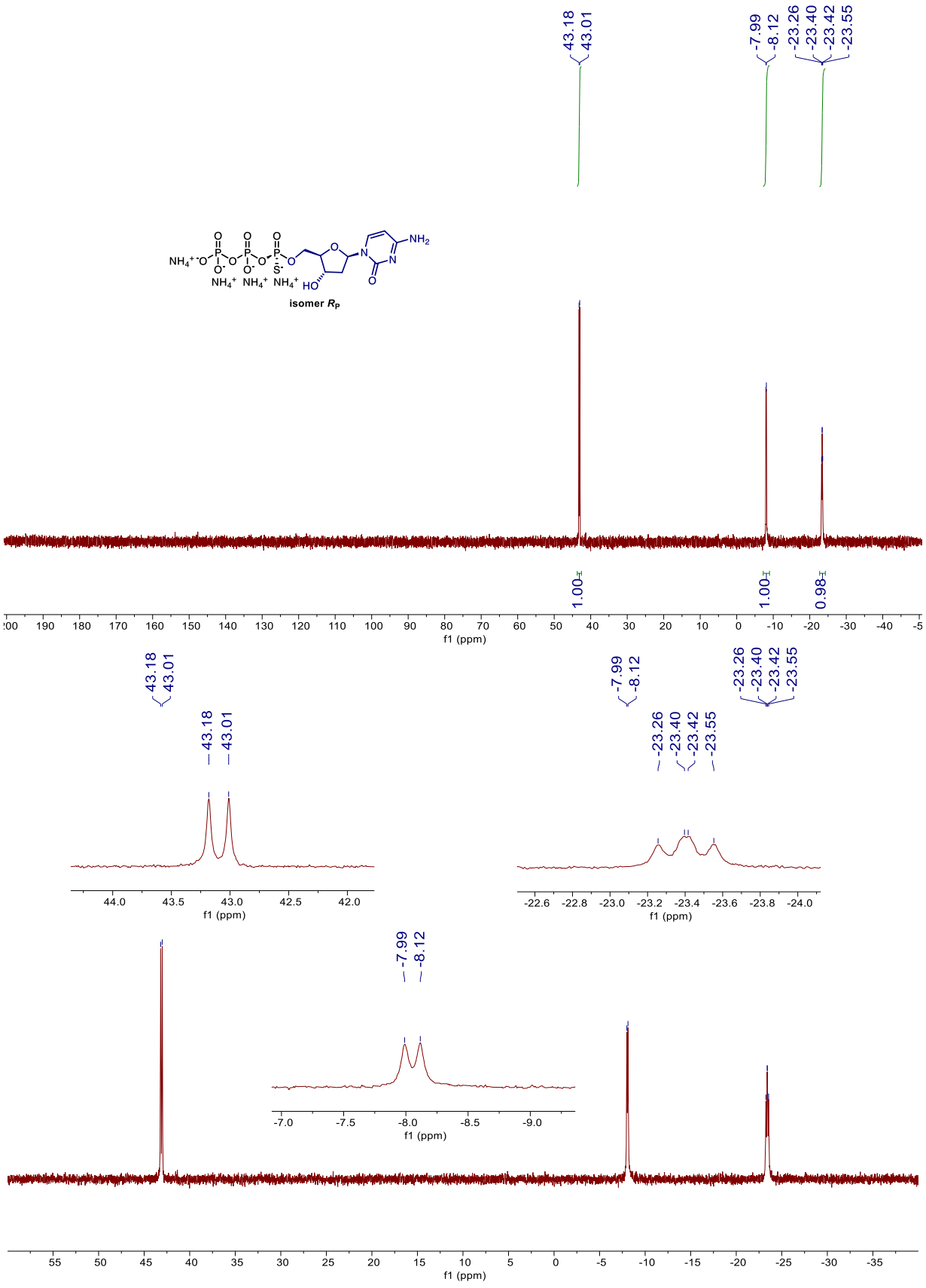
^{1}H NMR of compound (R_P)-27 (600 MHz, D_2O)



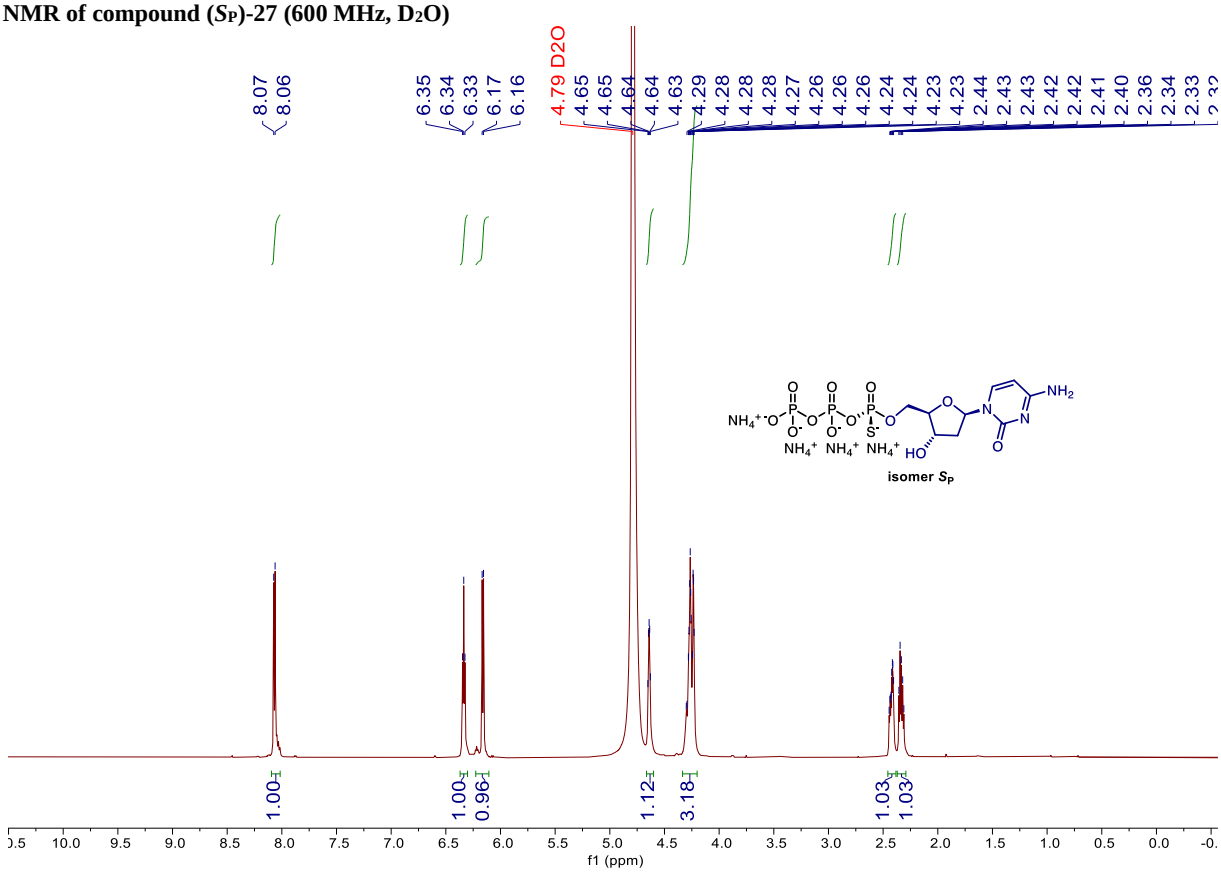
^{13}C NMR of compound (R_P)-27 (150 MHz, D_2O)



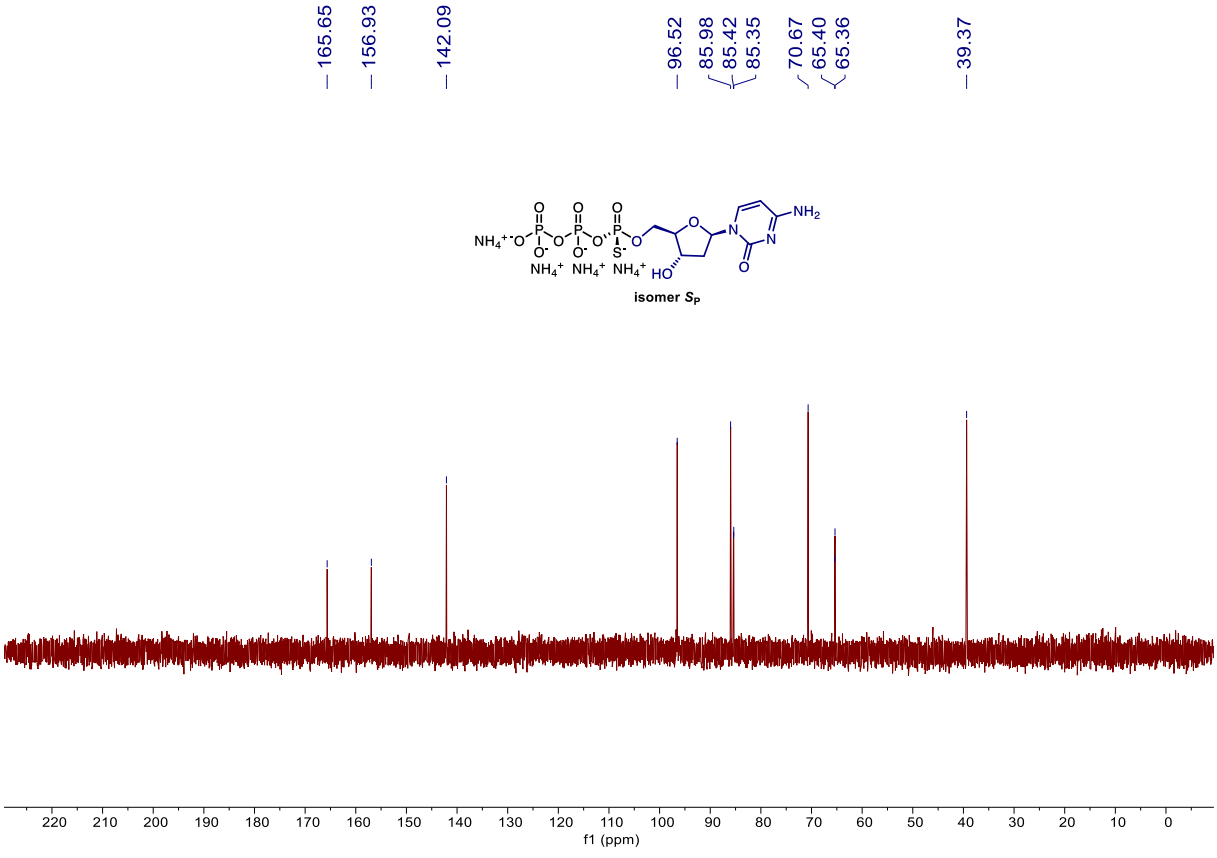
^{31}P NMR of compound (R_P)-27 (162 MHz, D_2O)



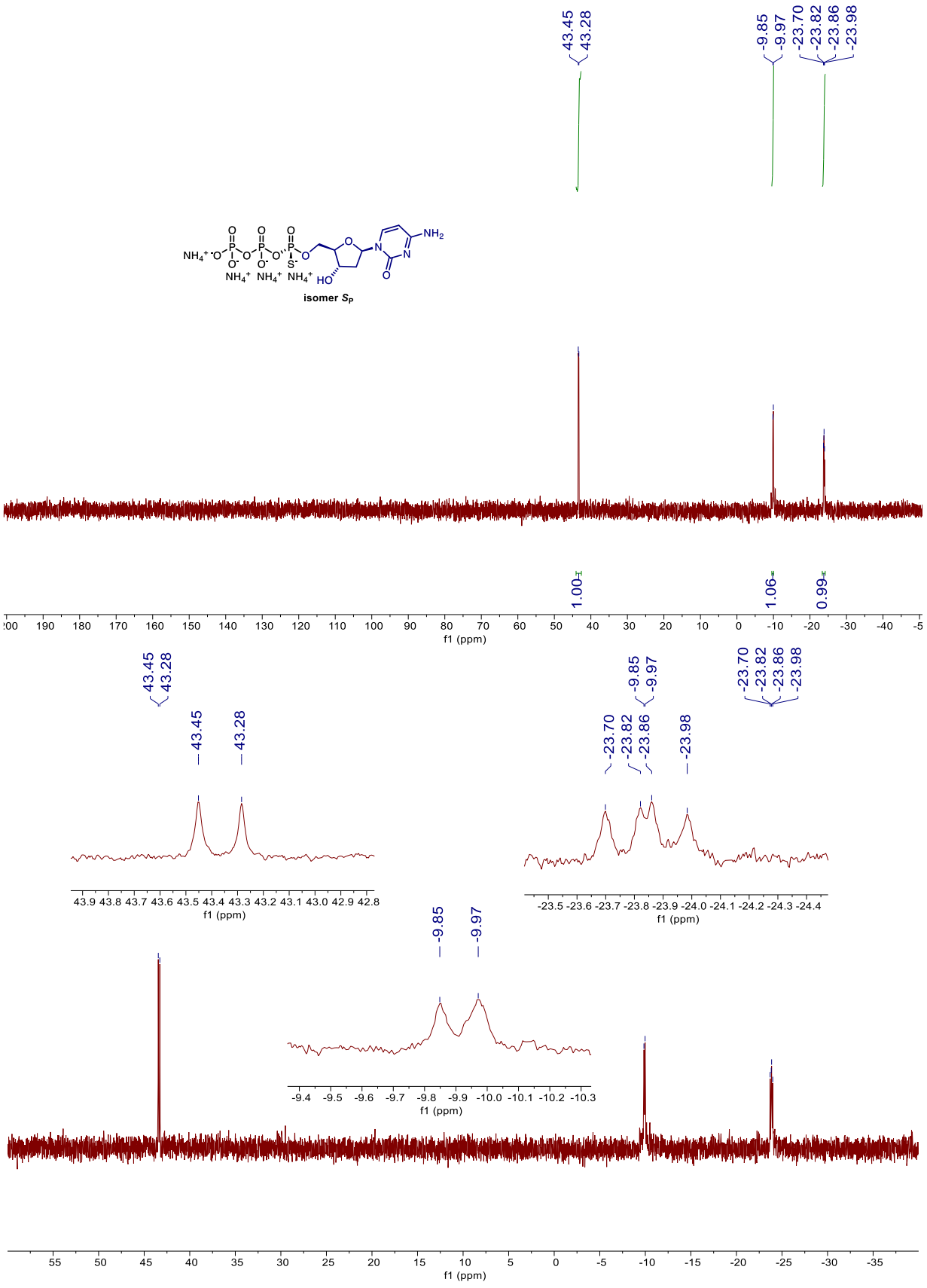
^{1}H NMR of compound (S_P)-27 (600 MHz, D_2O)



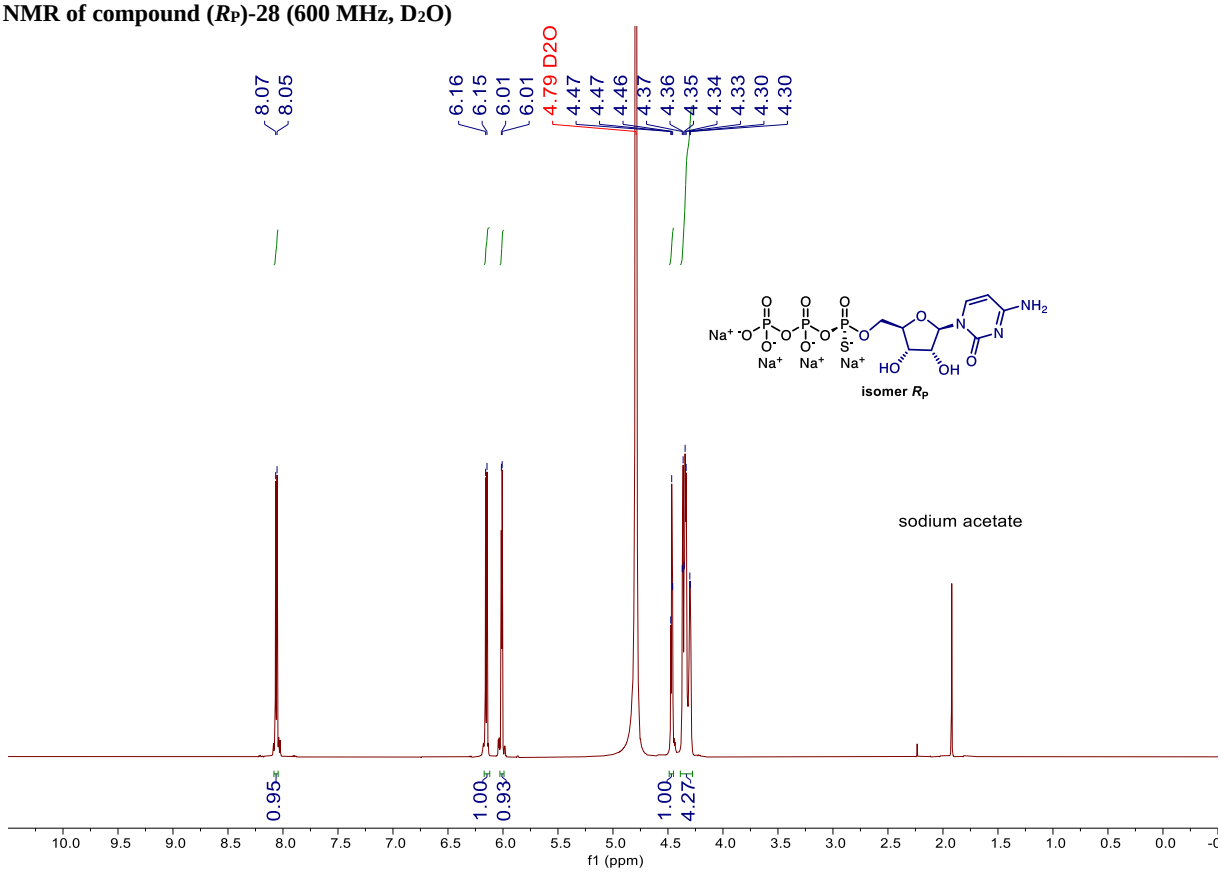
^{13}C NMR of compound (S_P)-27 (150 MHz, D_2O)



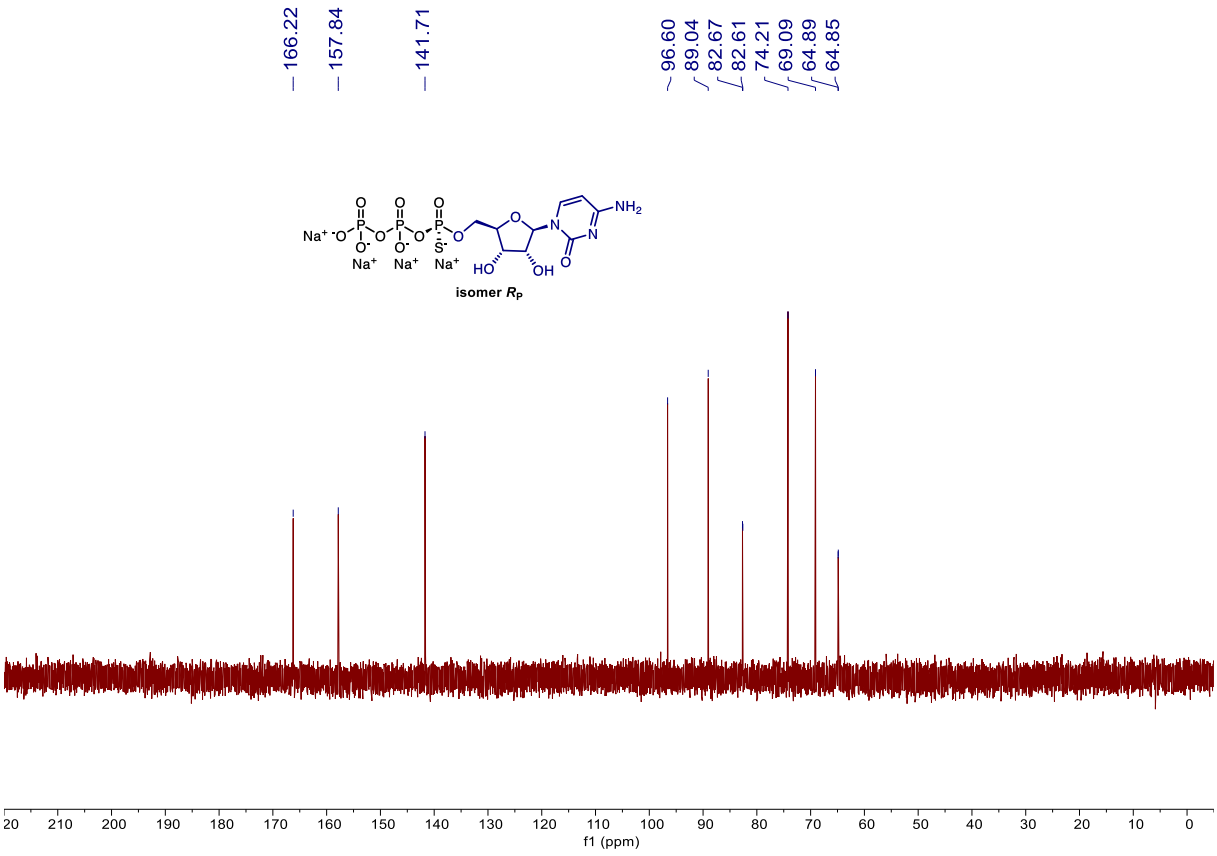
^{31}P NMR of compound (S_P)-27 (162 MHz, D_2O)



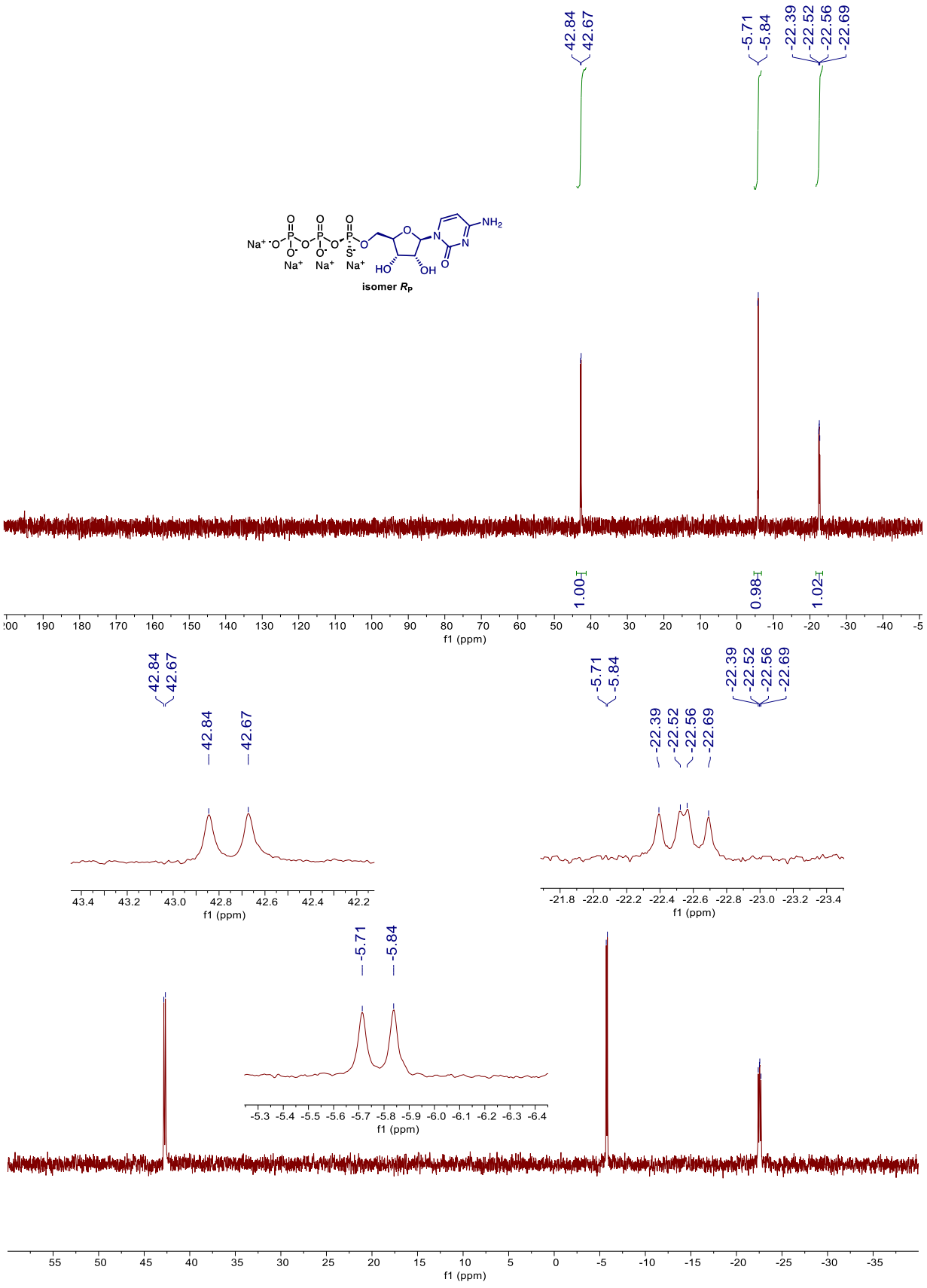
^{1}H NMR of compound (R_P)-28 (600 MHz, D_2O)



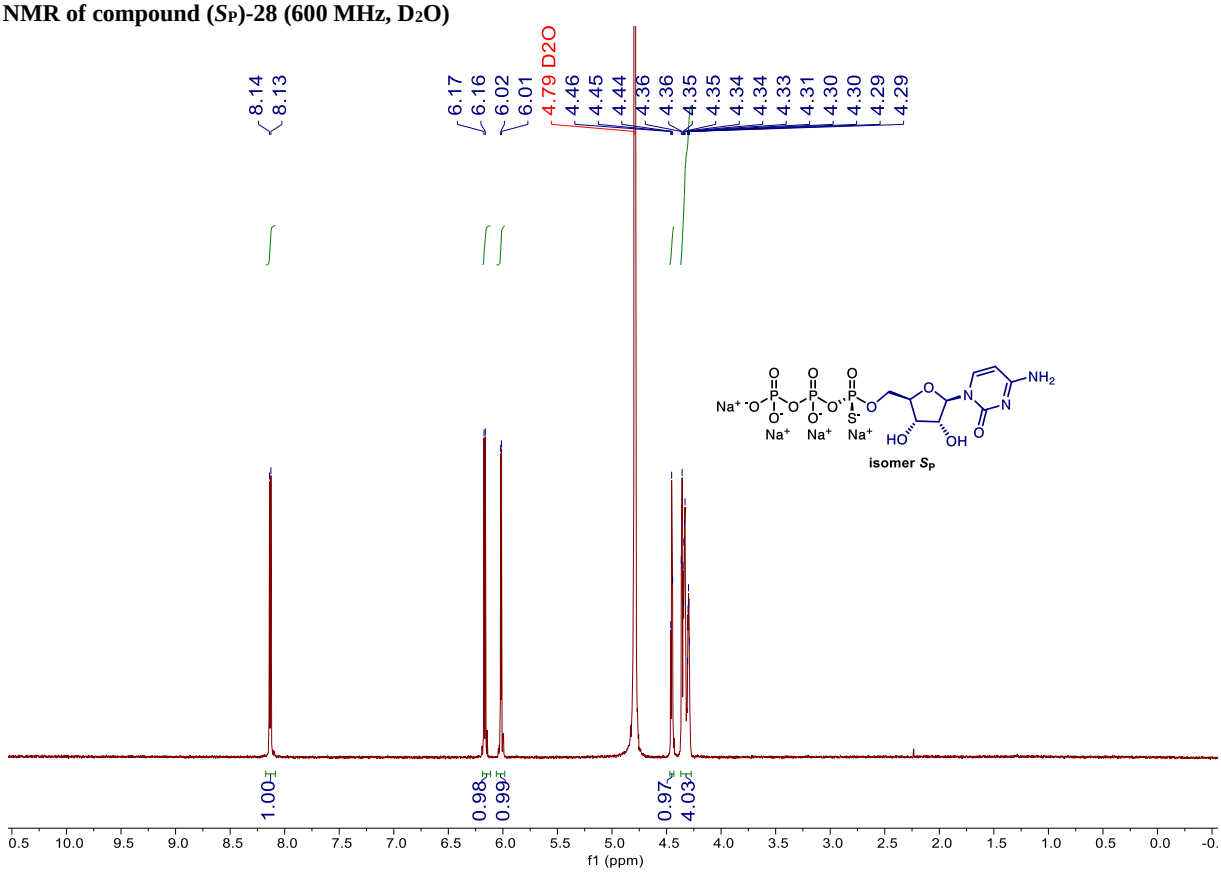
^{13}C NMR of compound (R_P)-28 (150 MHz, D_2O)



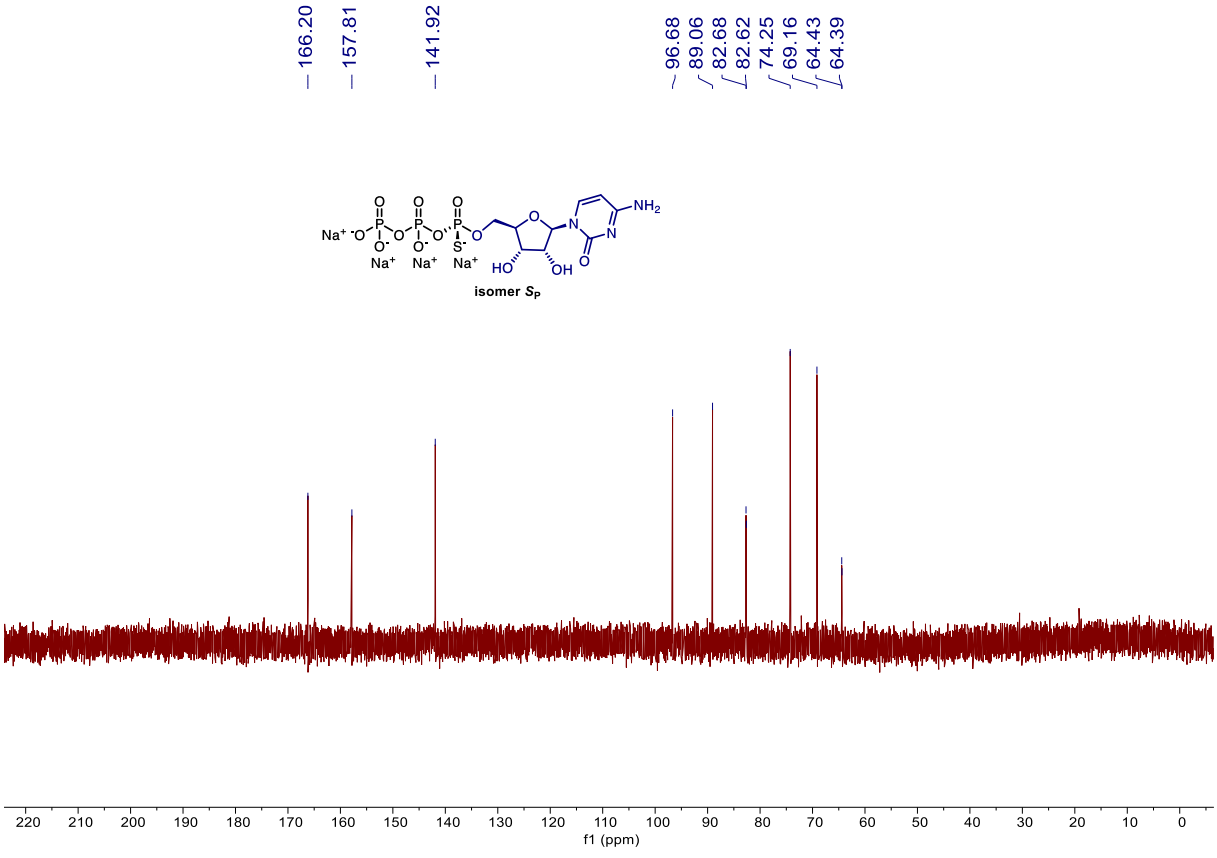
^{31}P NMR of compound (R_P)-28 (162 MHz, D_2O)



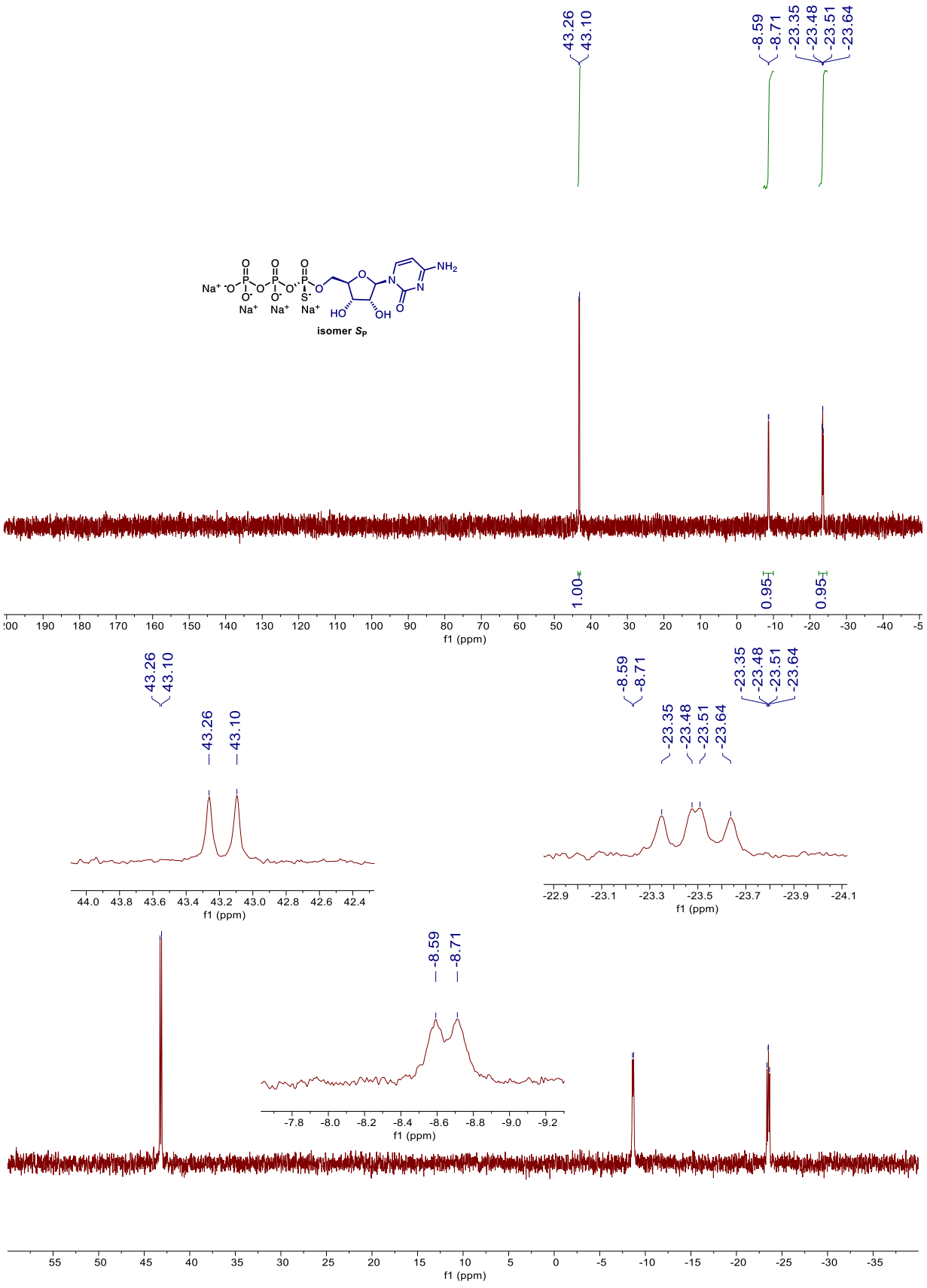
^{1}H NMR of compound (S_P)-28 (600 MHz, D_2O)



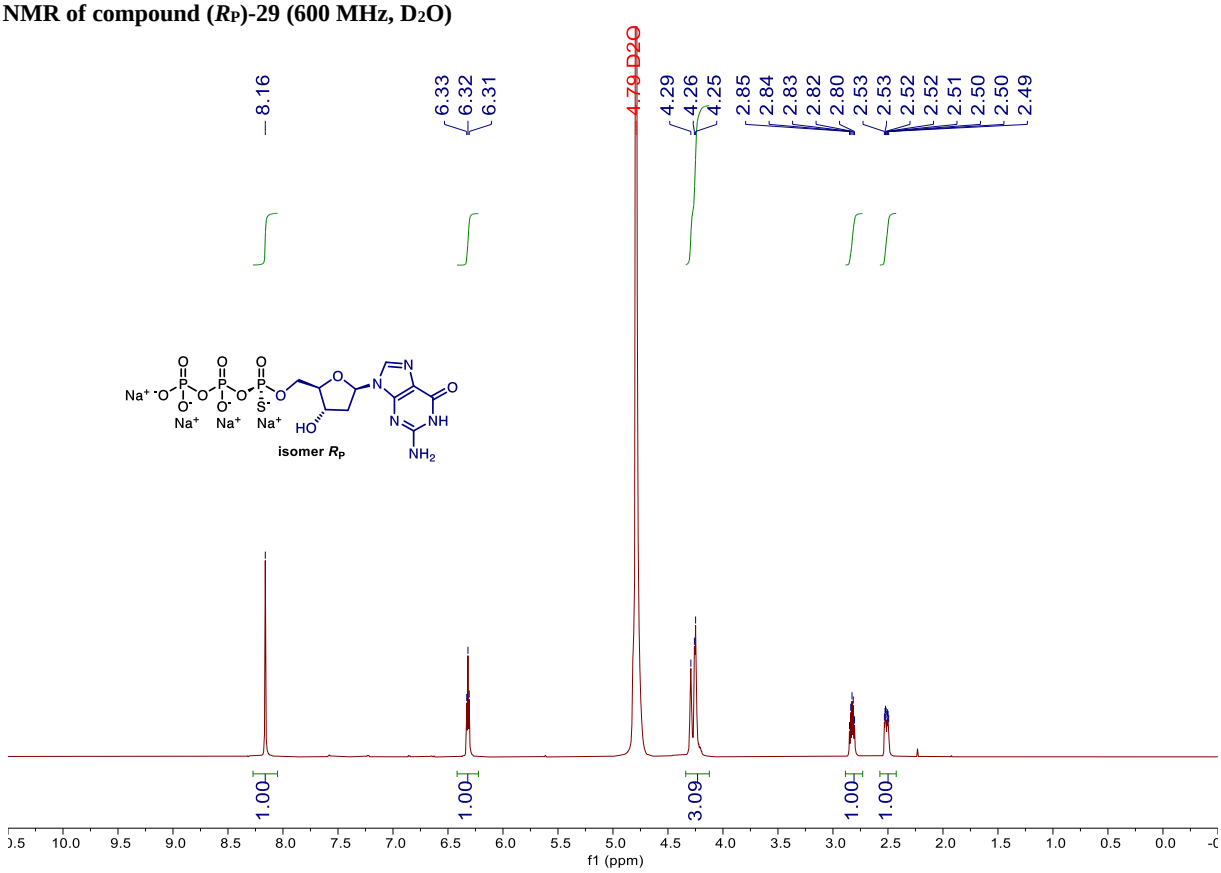
^{13}C NMR of compound (S_P)-28 (150 MHz, D_2O)



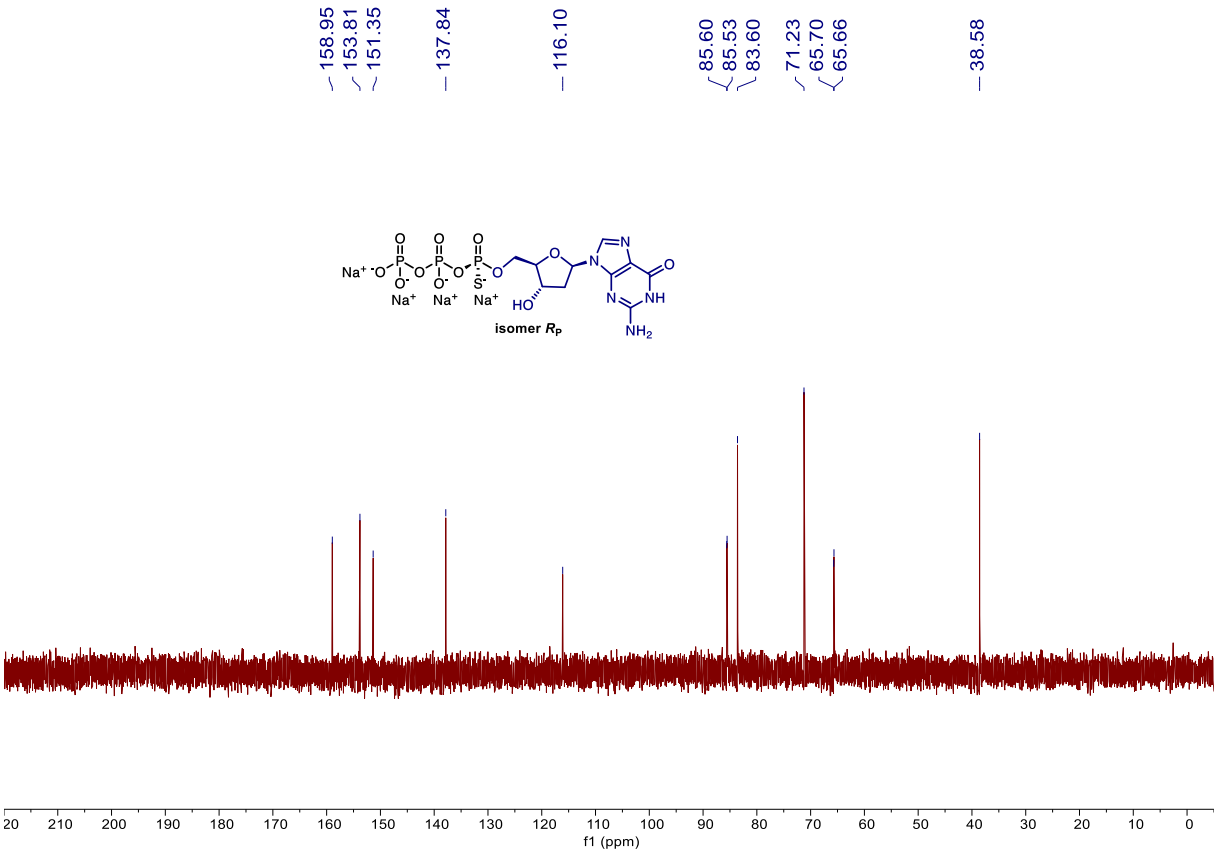
^{31}P NMR of compound (S_P)-28 (162 MHz, D_2O)



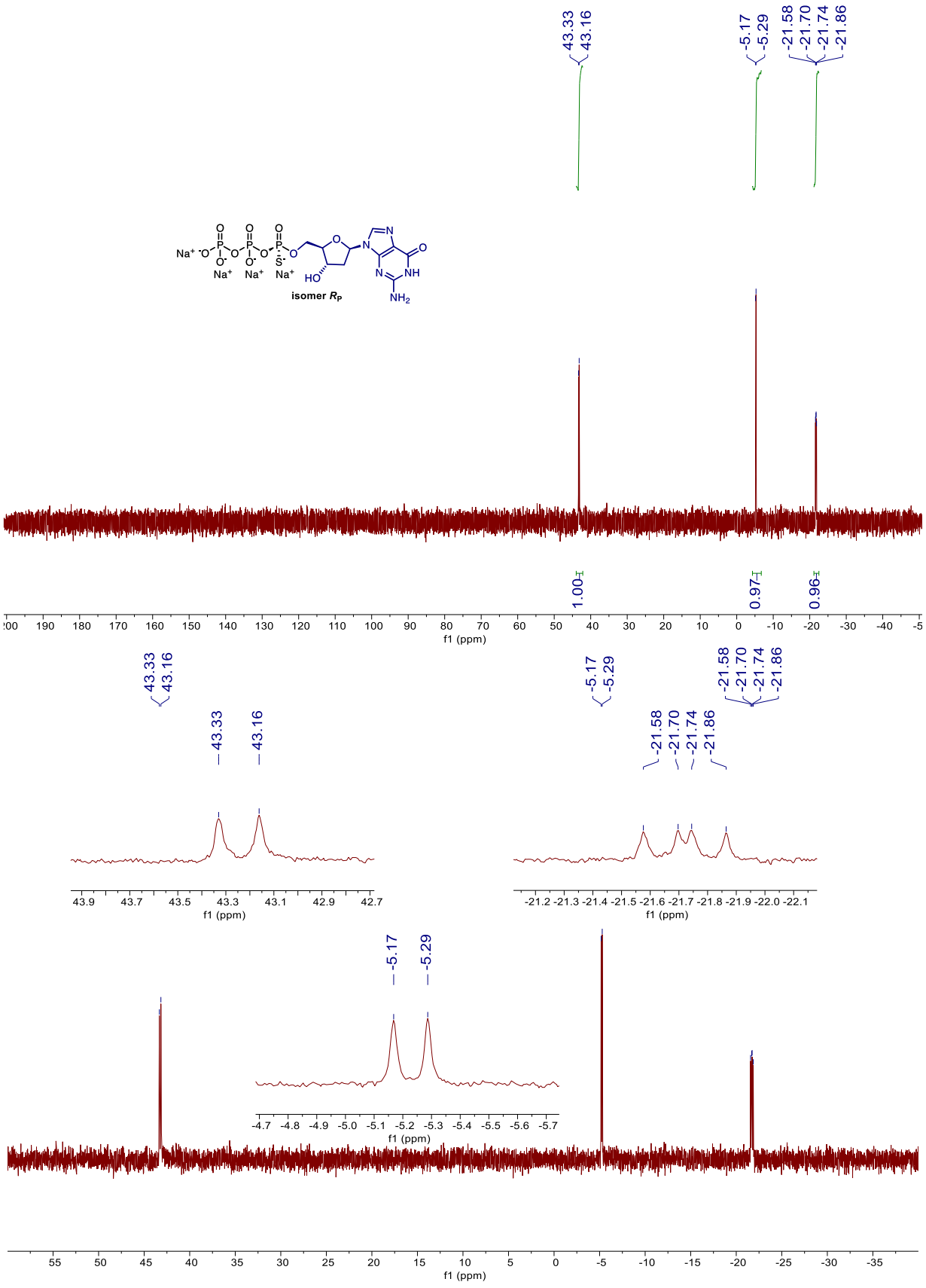
^{1}H NMR of compound (R_P)-29 (600 MHz, D_2O)



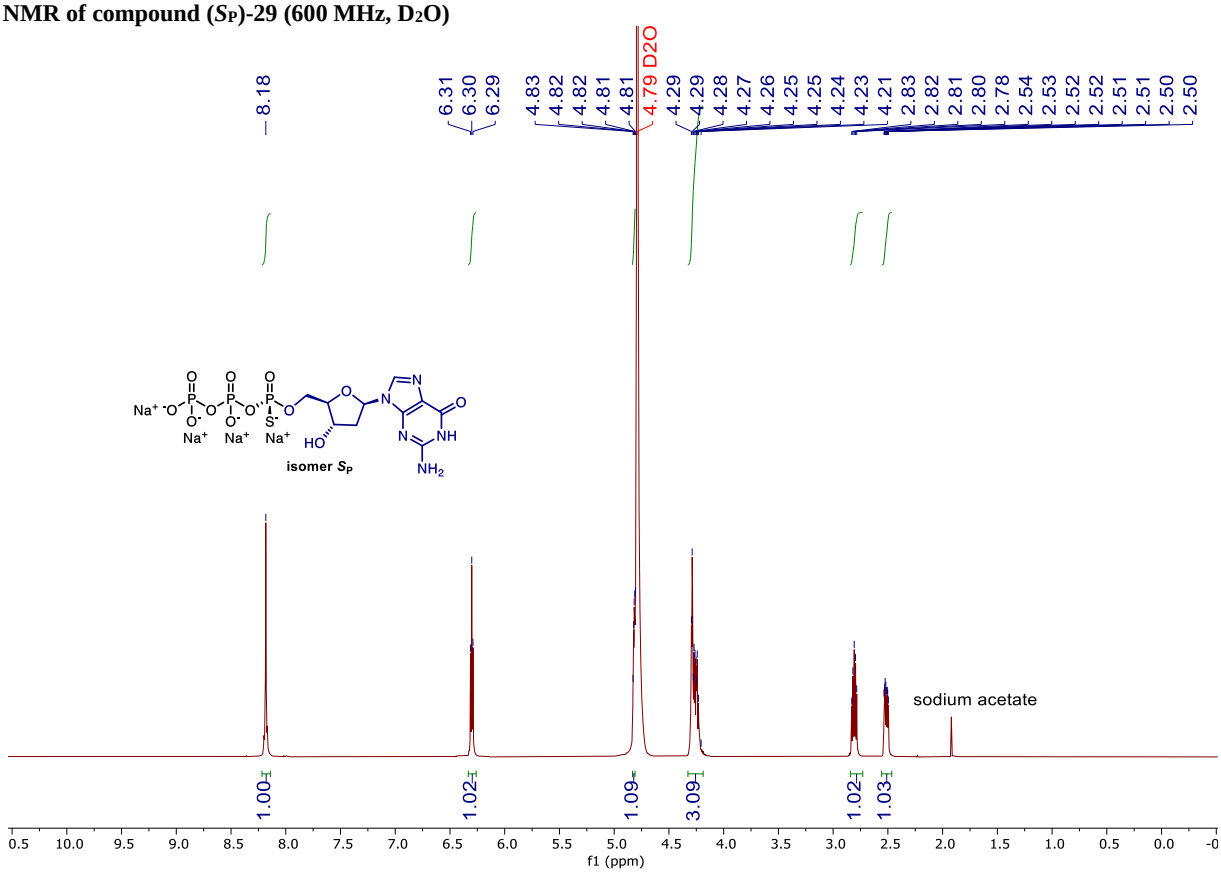
^{13}C NMR of compound (R_P)-29 (150 MHz, D_2O)



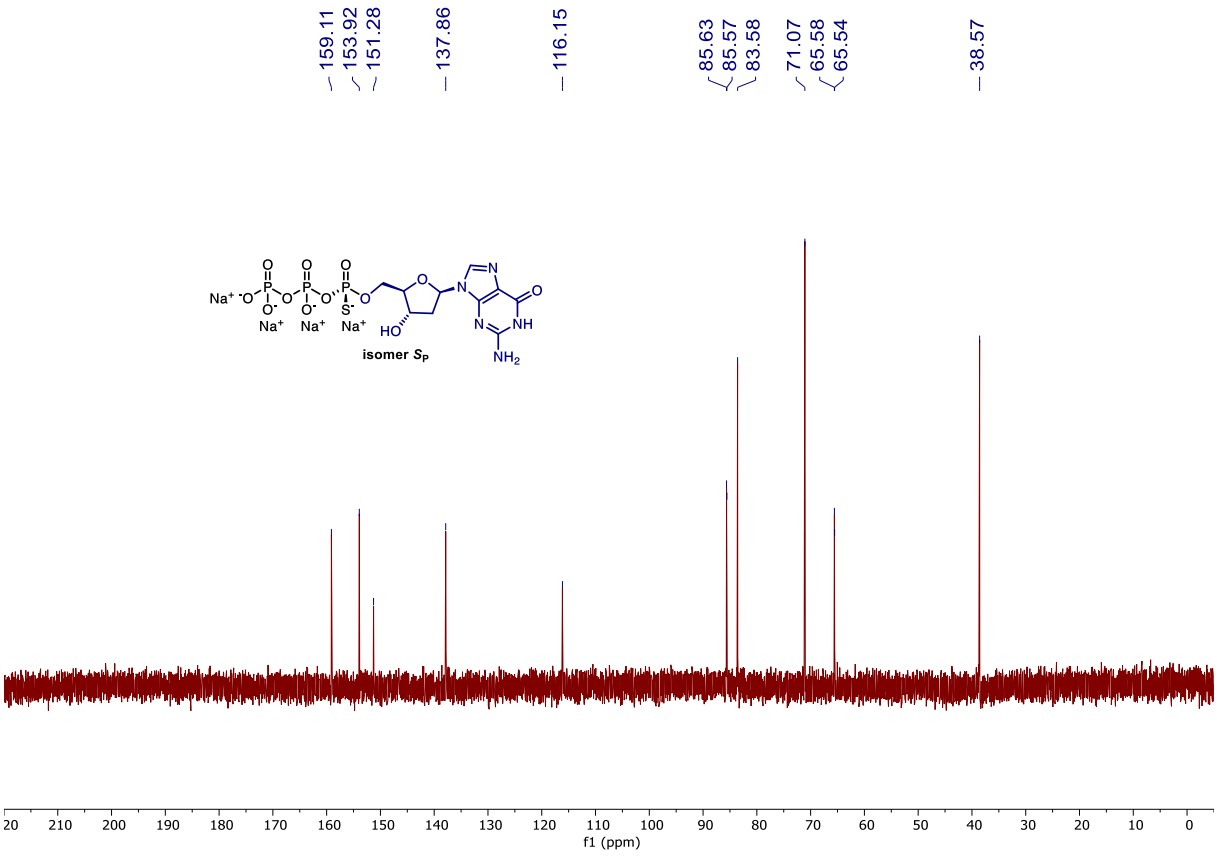
^{31}P NMR of compound (R_P)-29 (162 MHz, D_2O)



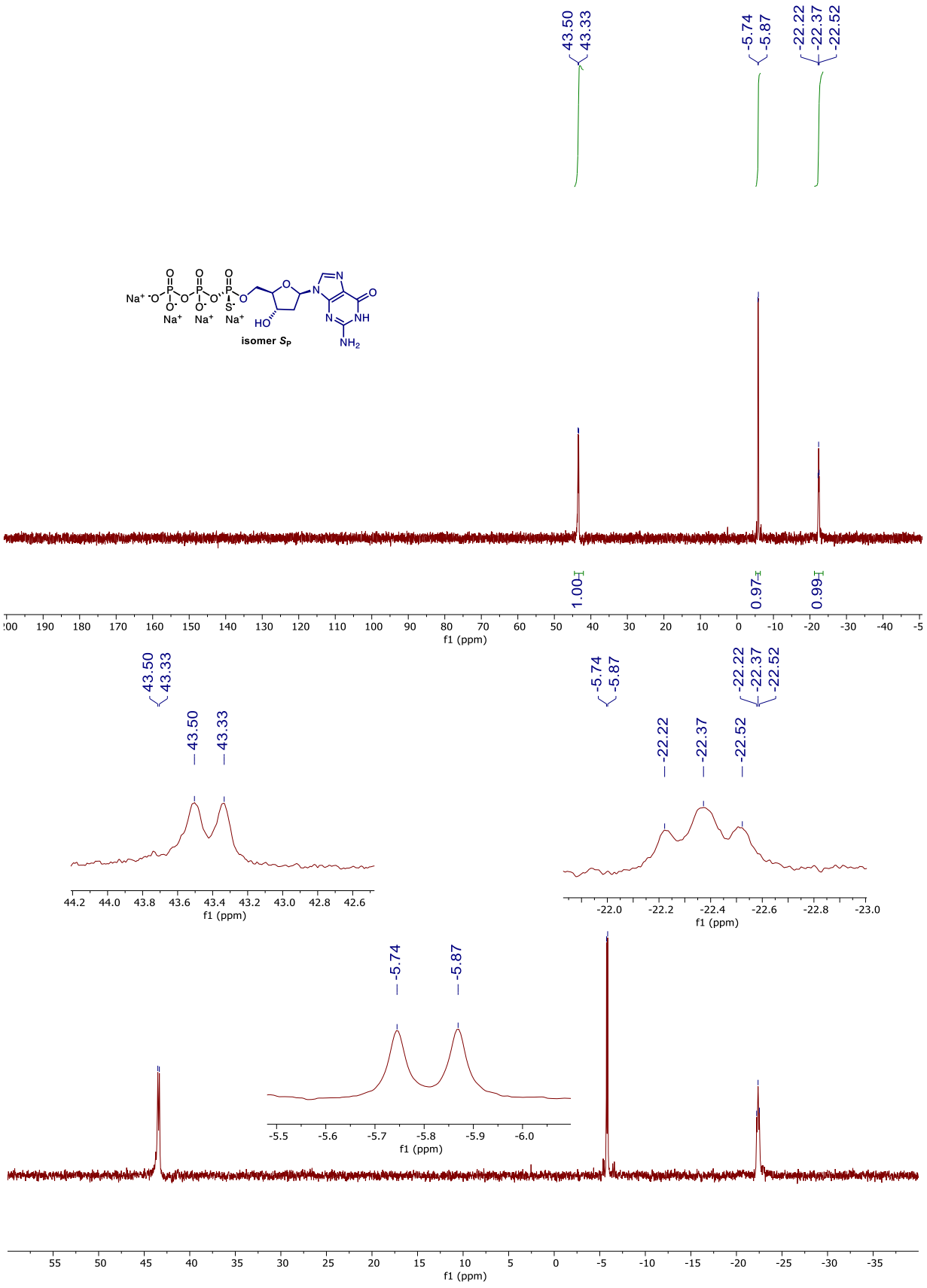
^{1}H NMR of compound (S_P)-29 (600 MHz, D_2O)



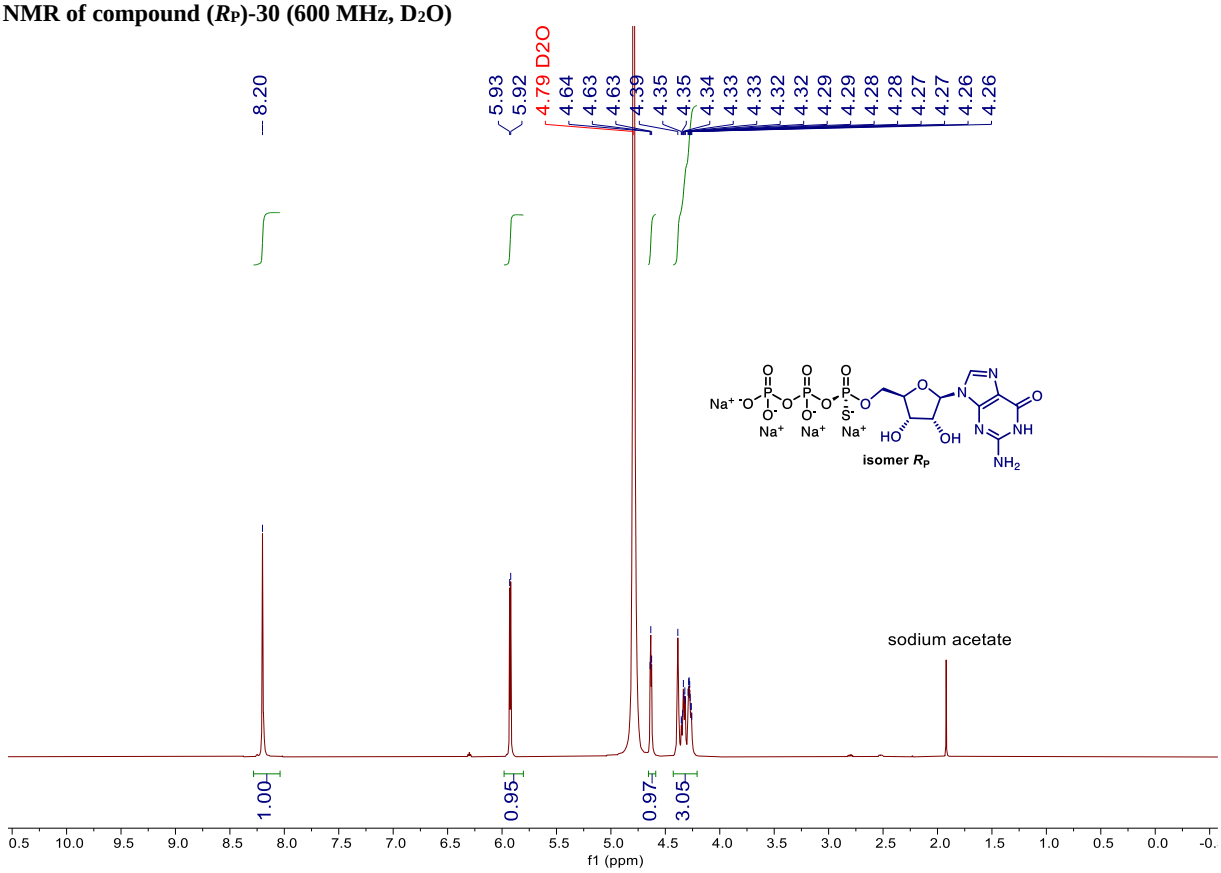
^{13}C NMR of compound (S_P)-29 (150 MHz, D_2O)



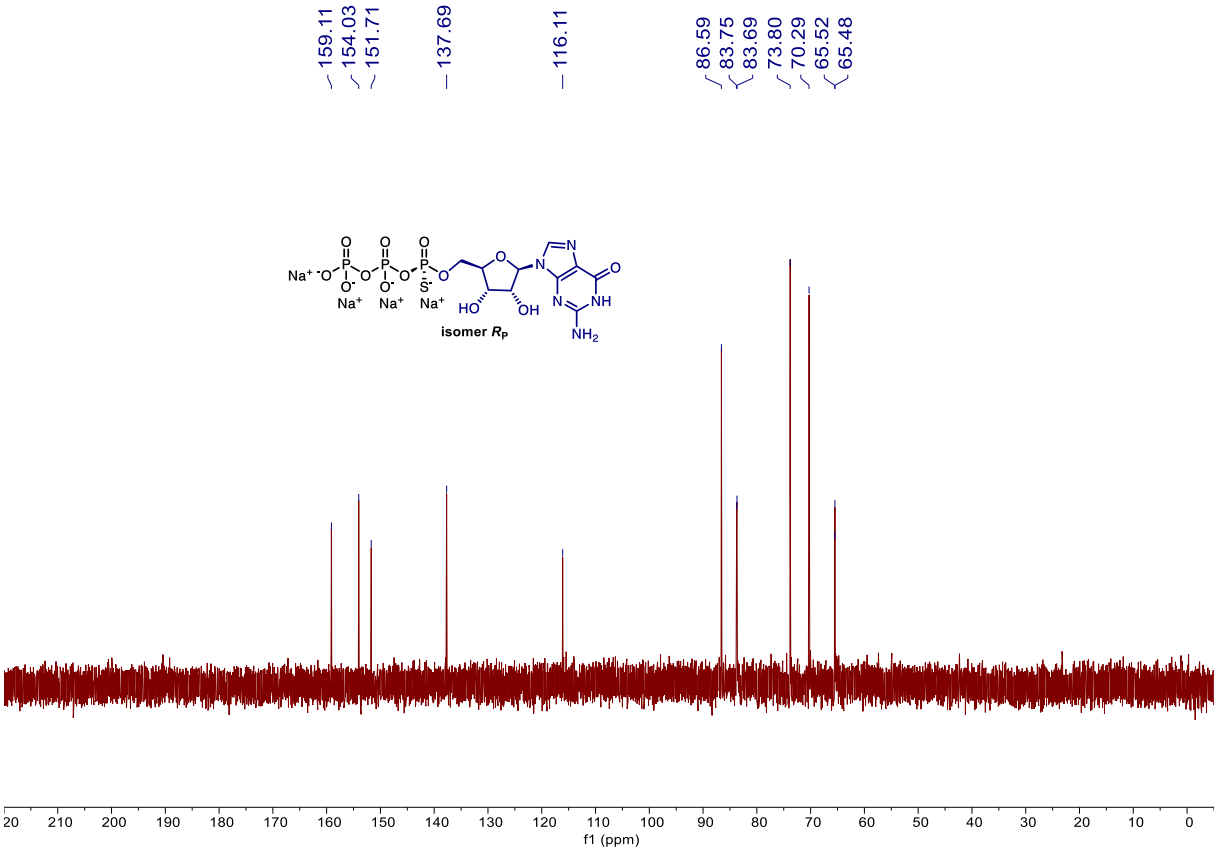
^{31}P NMR of compound (S_P)-29 (162 MHz, D_2O)



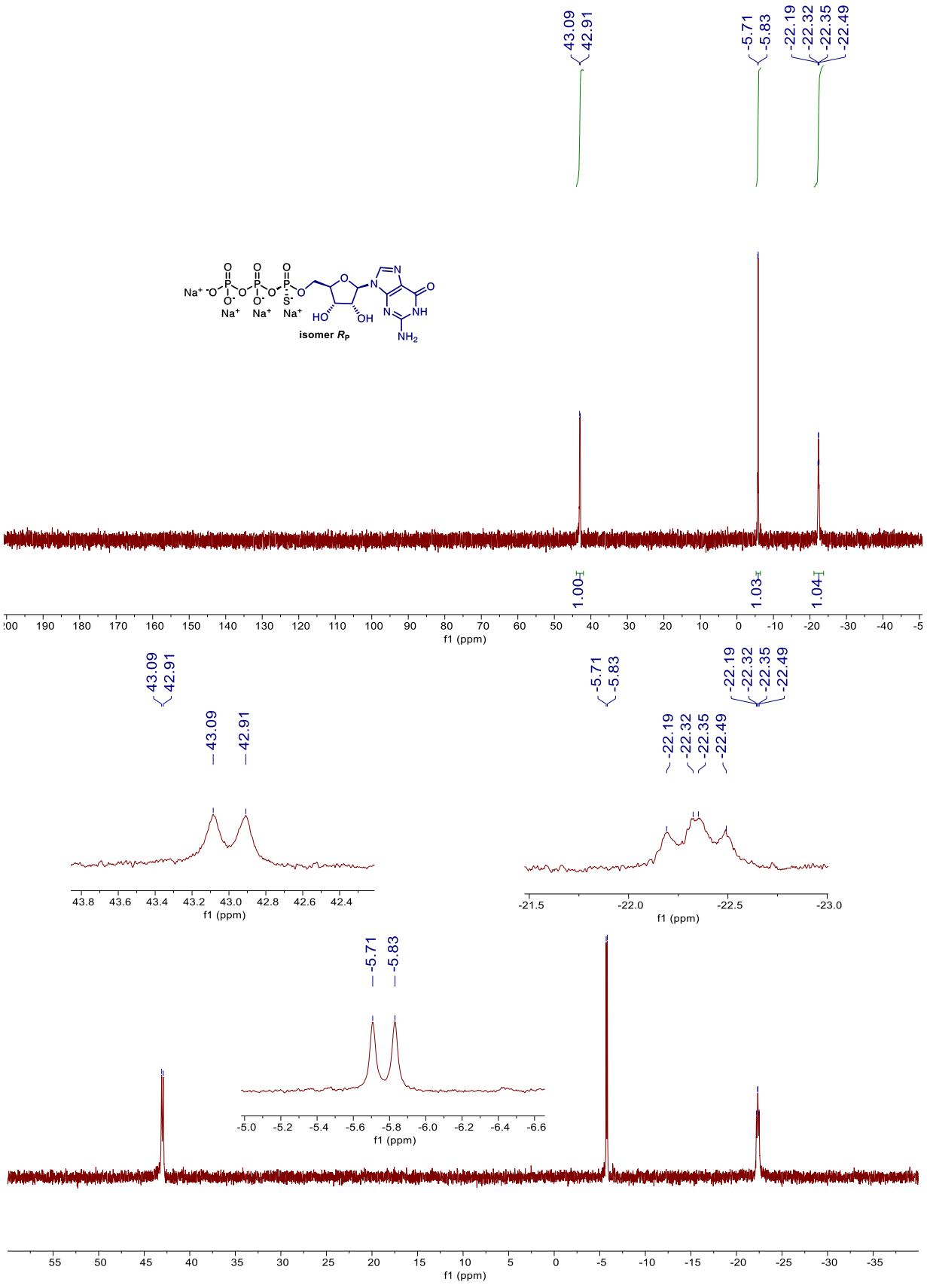
^{1}H NMR of compound (R_P)-30 (600 MHz, D_2O)



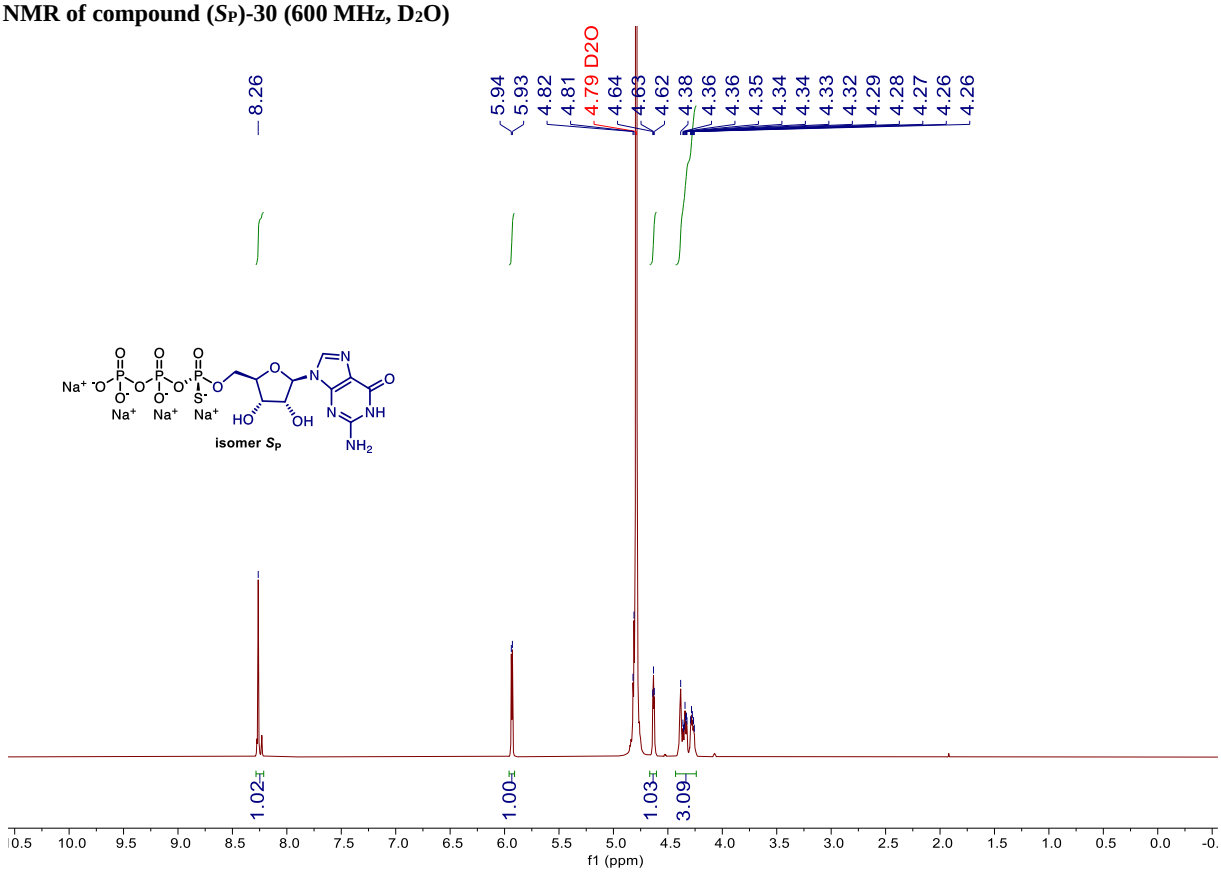
^{13}C NMR of compound (R_P)-30 (150 MHz, D_2O)



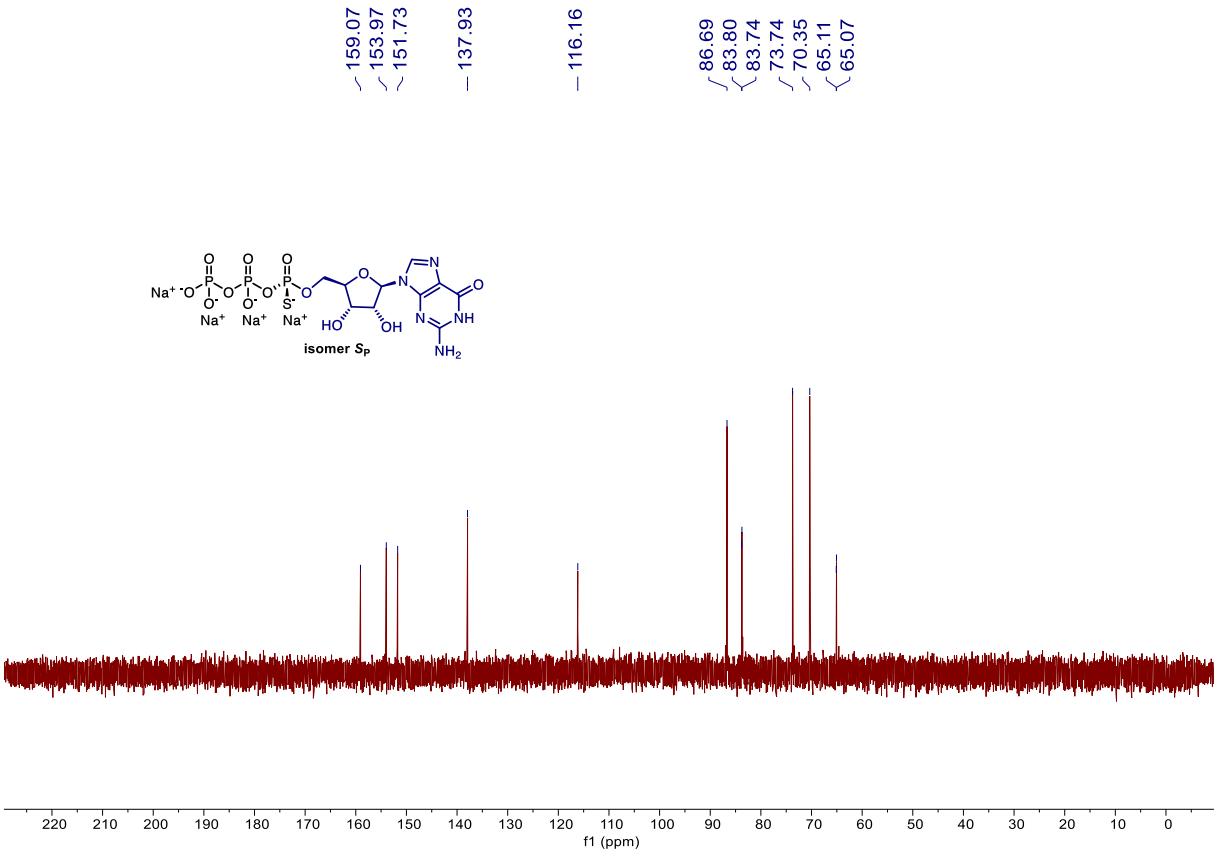
^{31}P NMR of compound (R_P)-30 (162 MHz, D_2O)



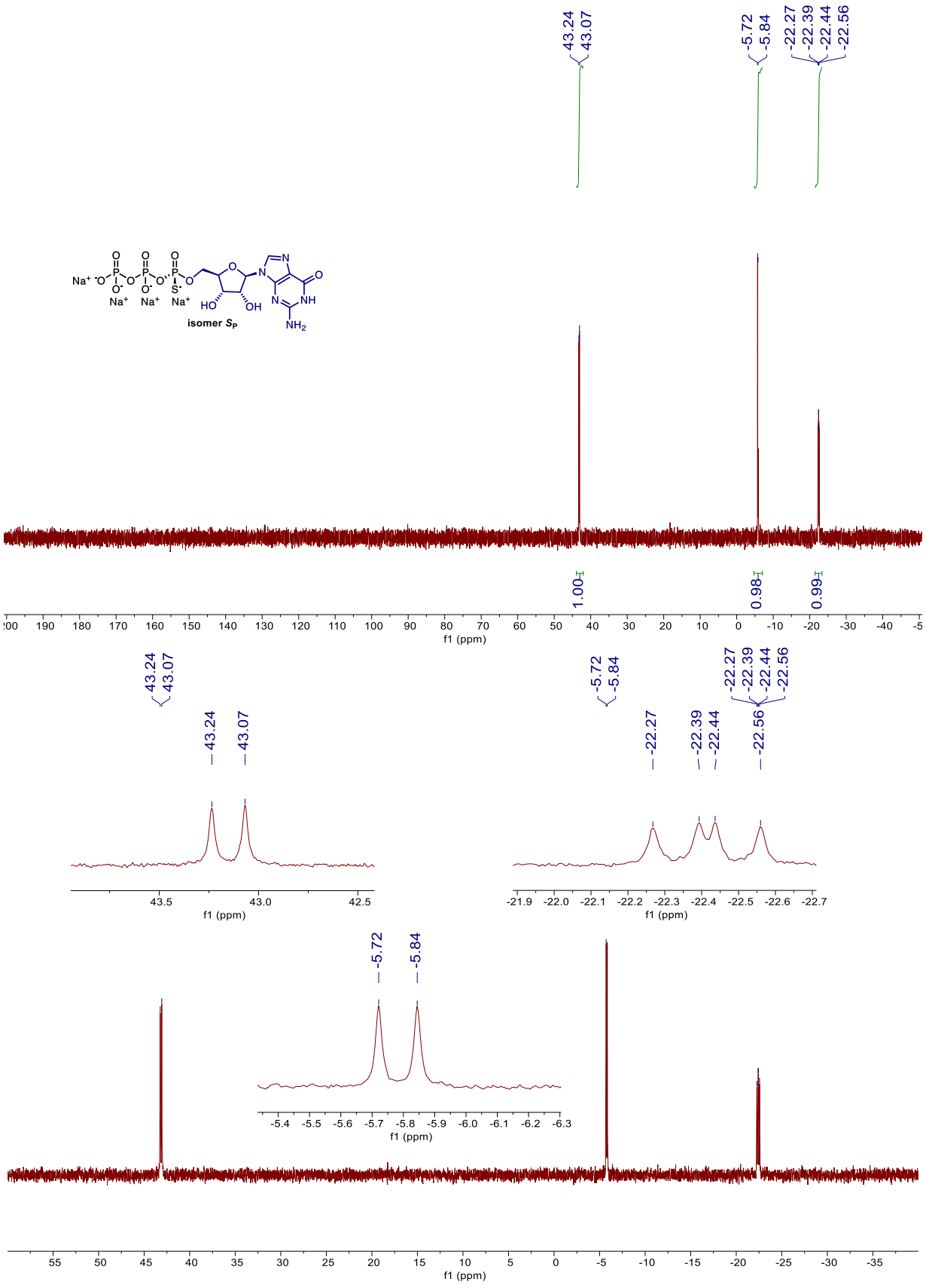
^{1}H NMR of compound (S_P)-30 (600 MHz, D_2O)



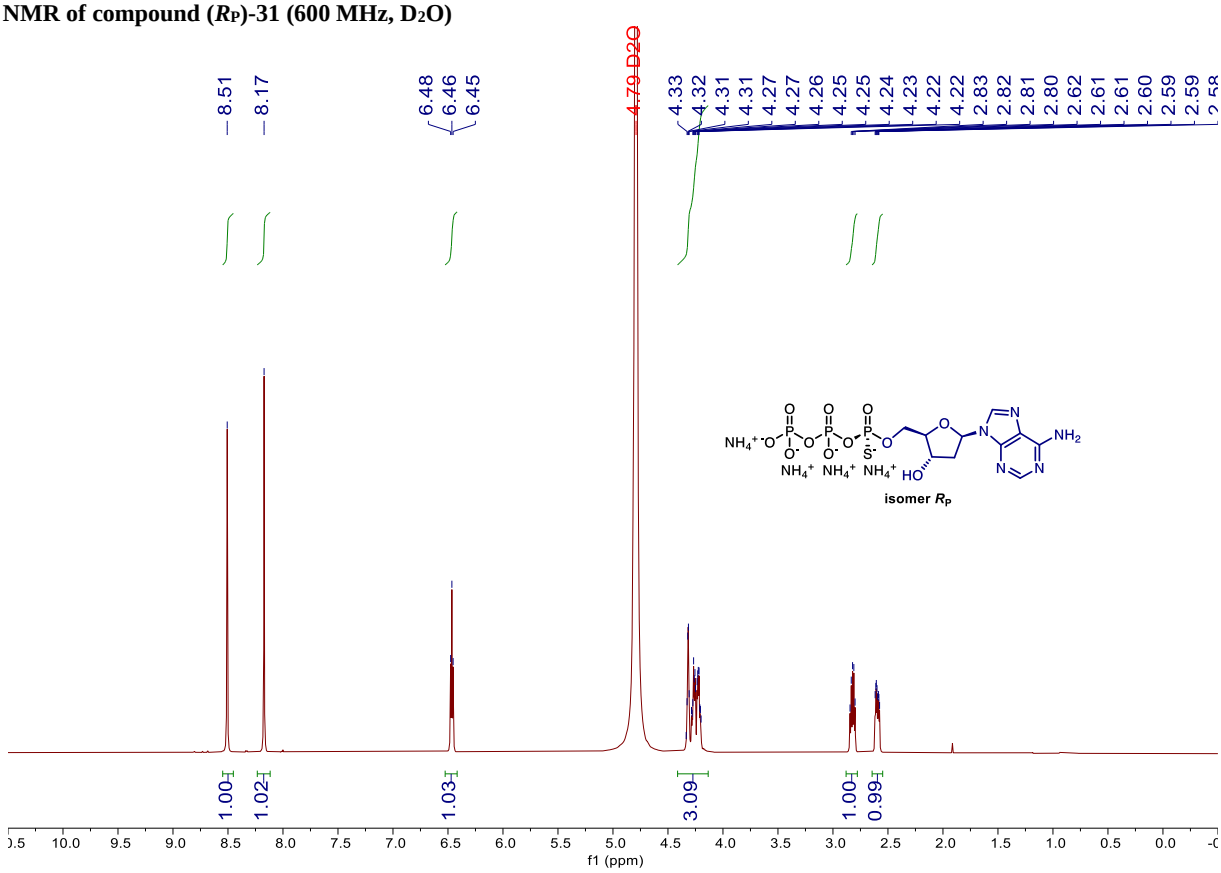
^{13}C NMR of compound (S_P)-30 (150 MHz, D_2O)



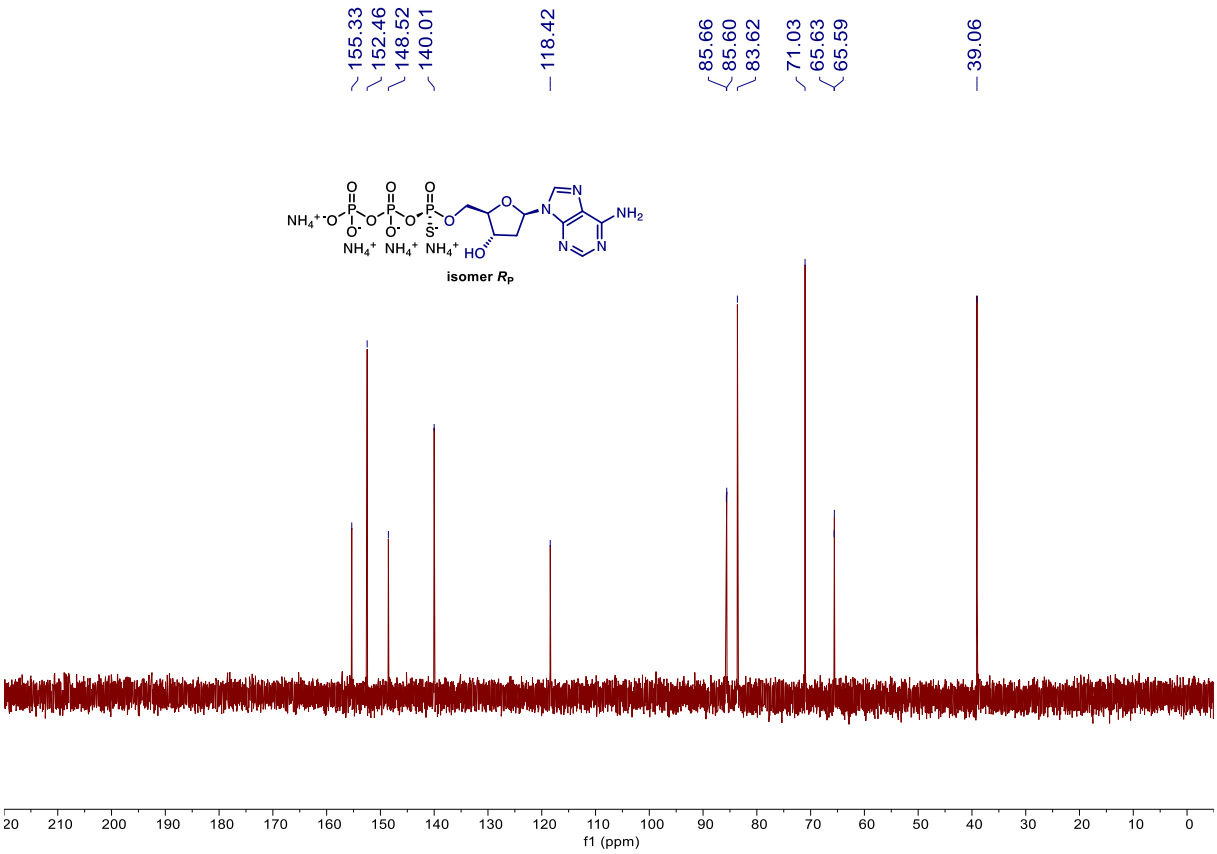
^{31}P NMR of compound (S_P)-30 (162 MHz, D_2O)



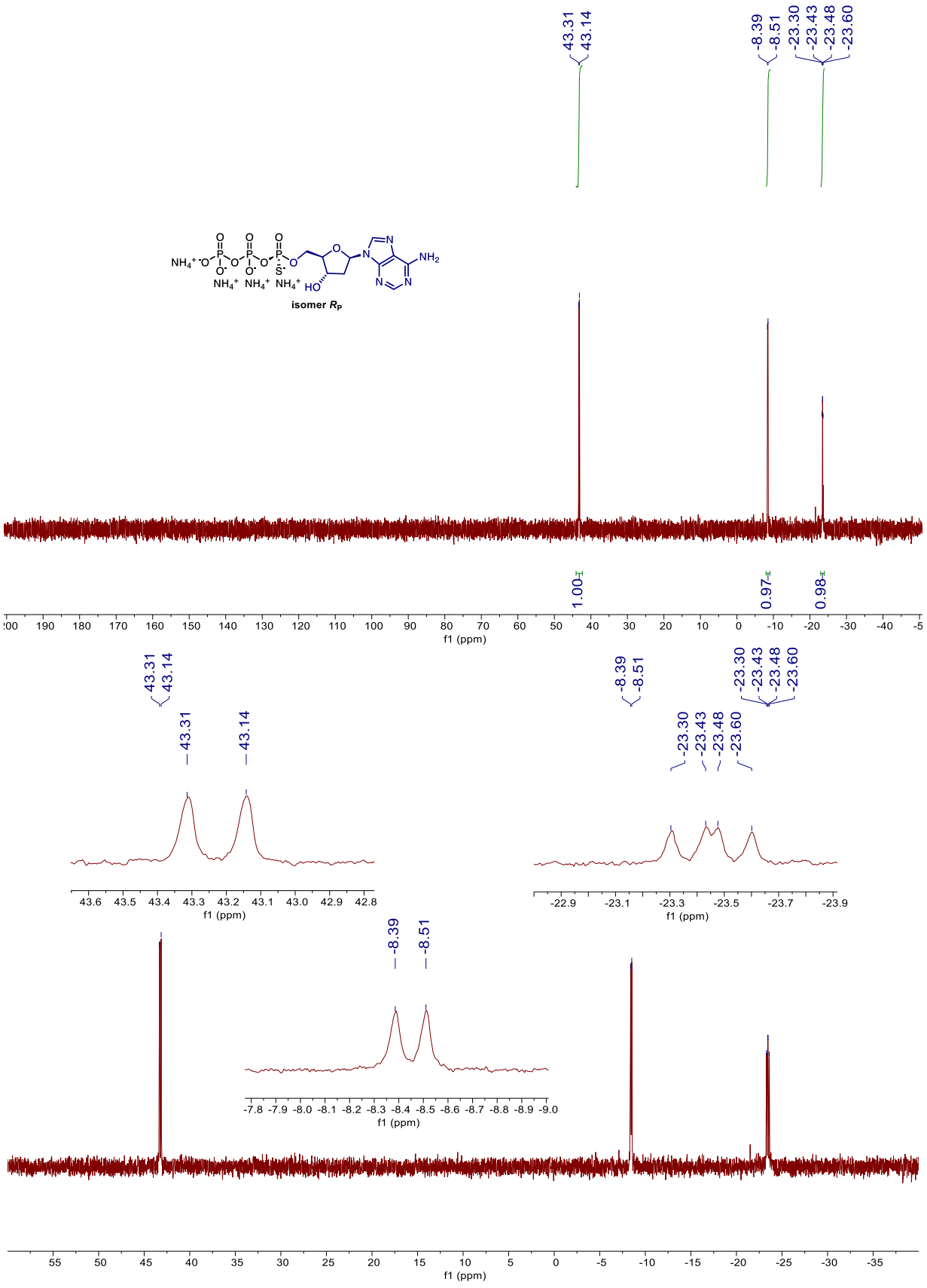
^{1}H NMR of compound (R_P)-31 (600 MHz, D_2O)



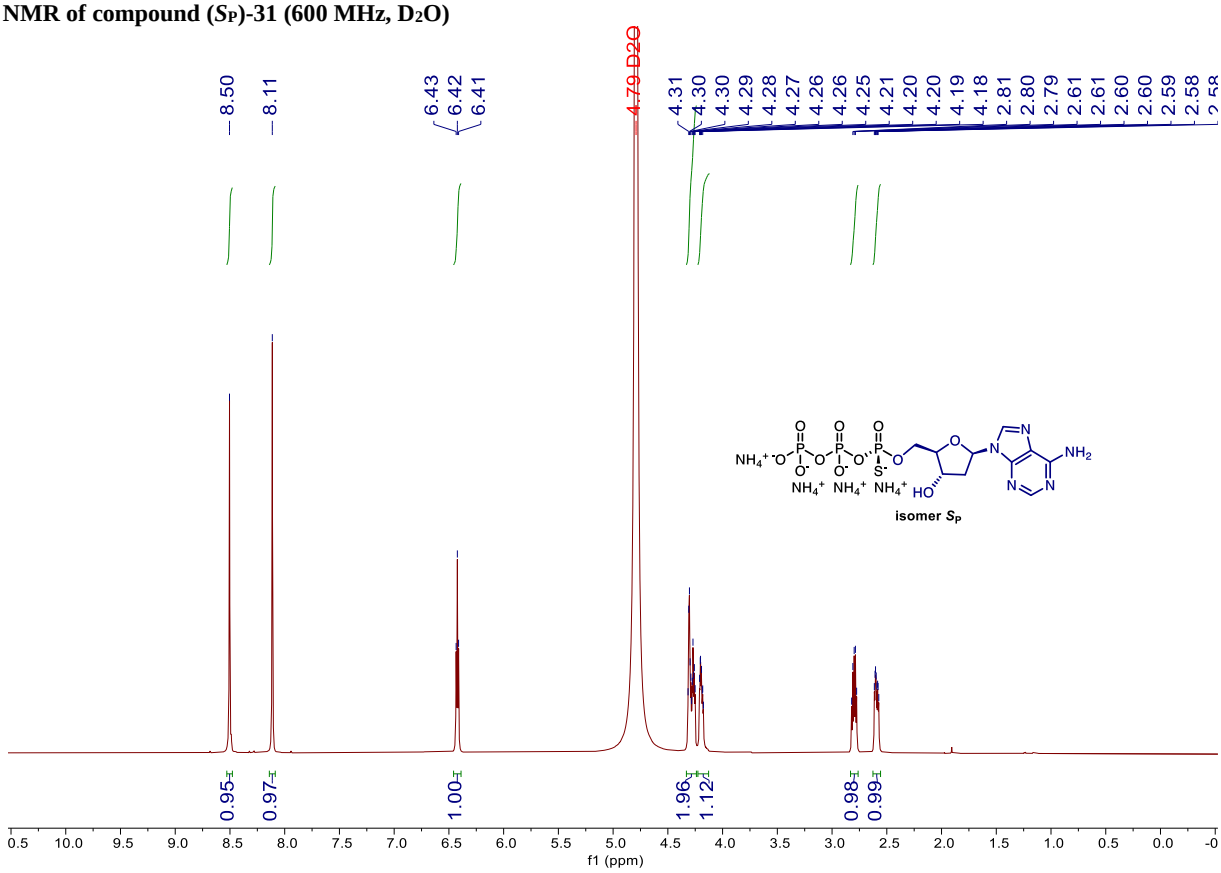
^{13}C NMR of compound (R_P)-31 (150 MHz, D_2O)



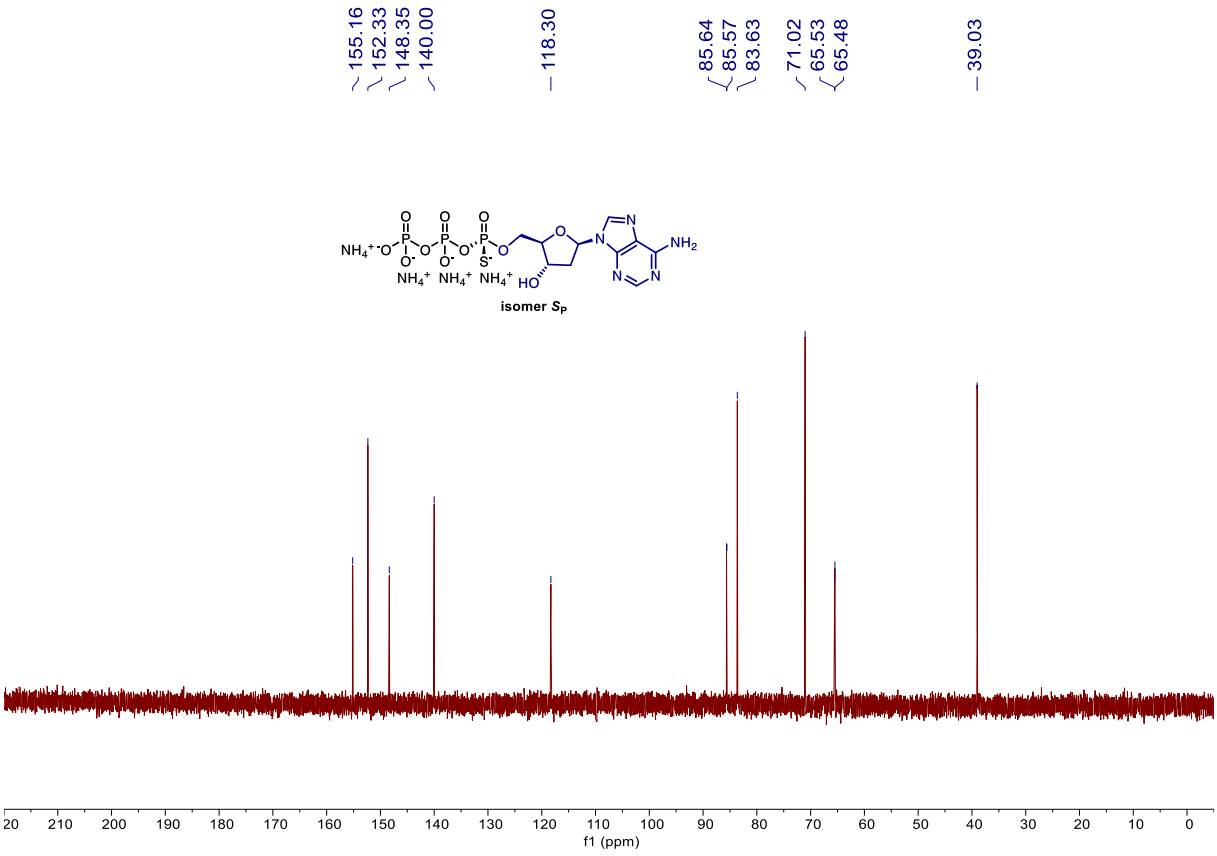
^{31}P NMR of compound (R_P)-31 (162 MHz, D_2O)



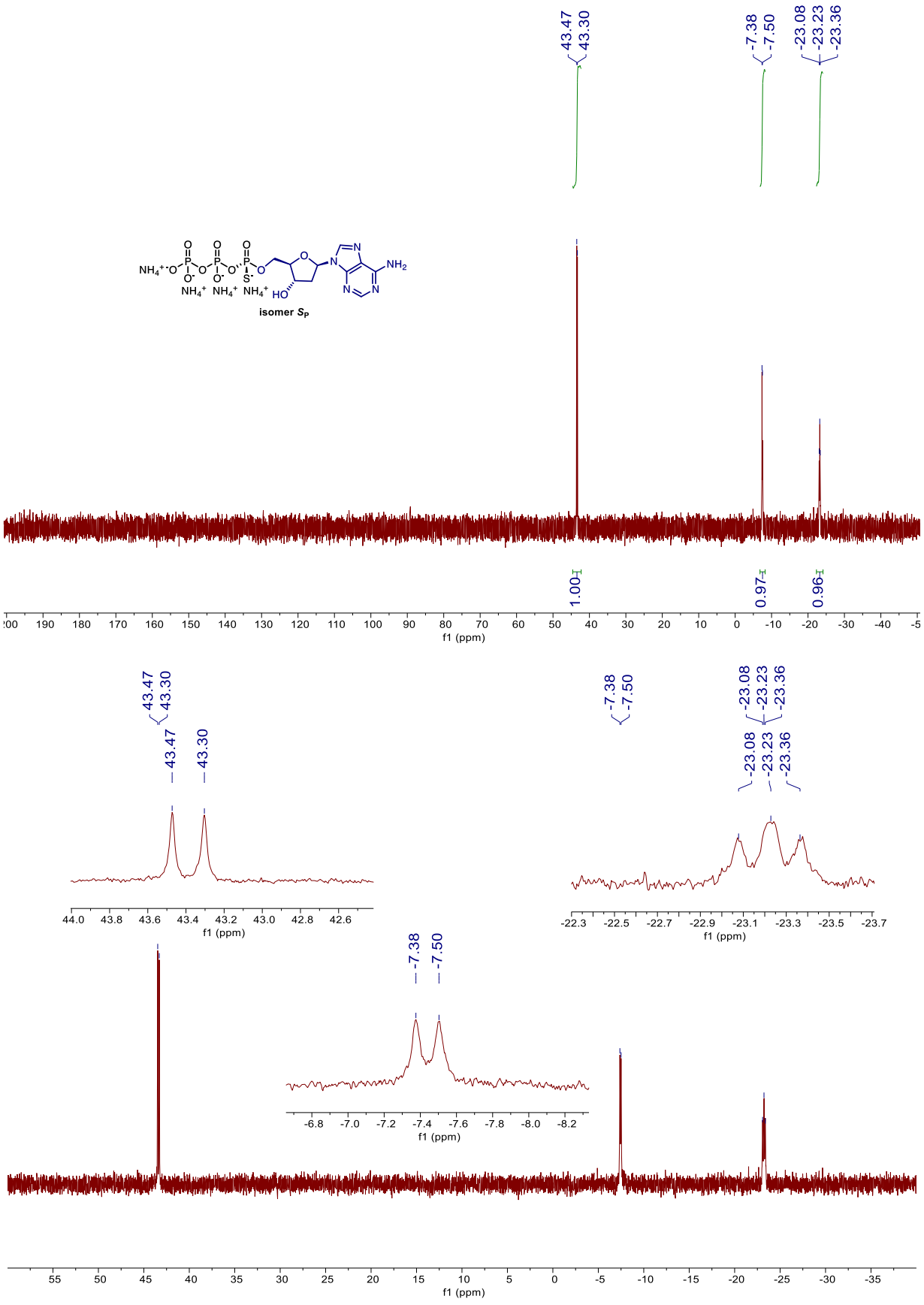
^{1}H NMR of compound (S_P)-31 (600 MHz, D_2O)



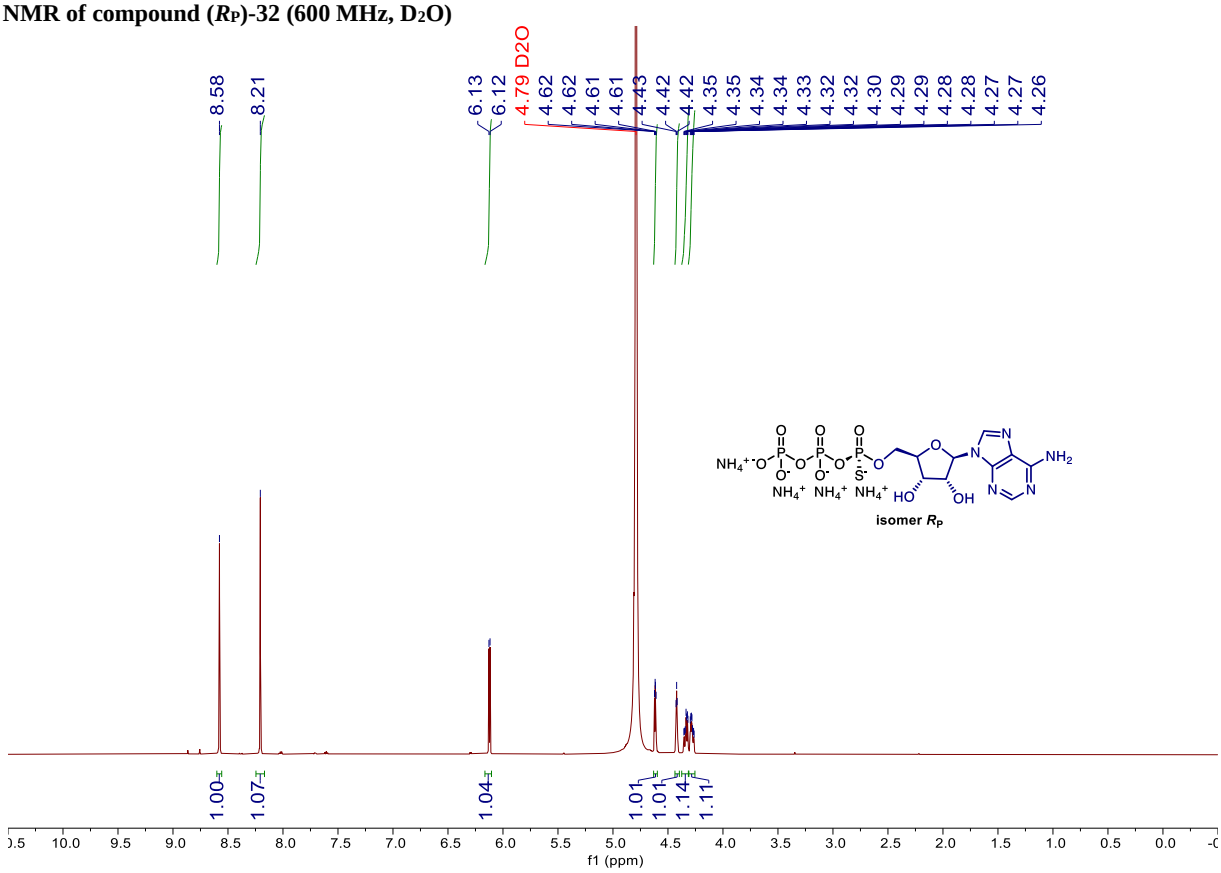
^{13}C NMR of compound (S_P)-31 (150 MHz, D_2O)



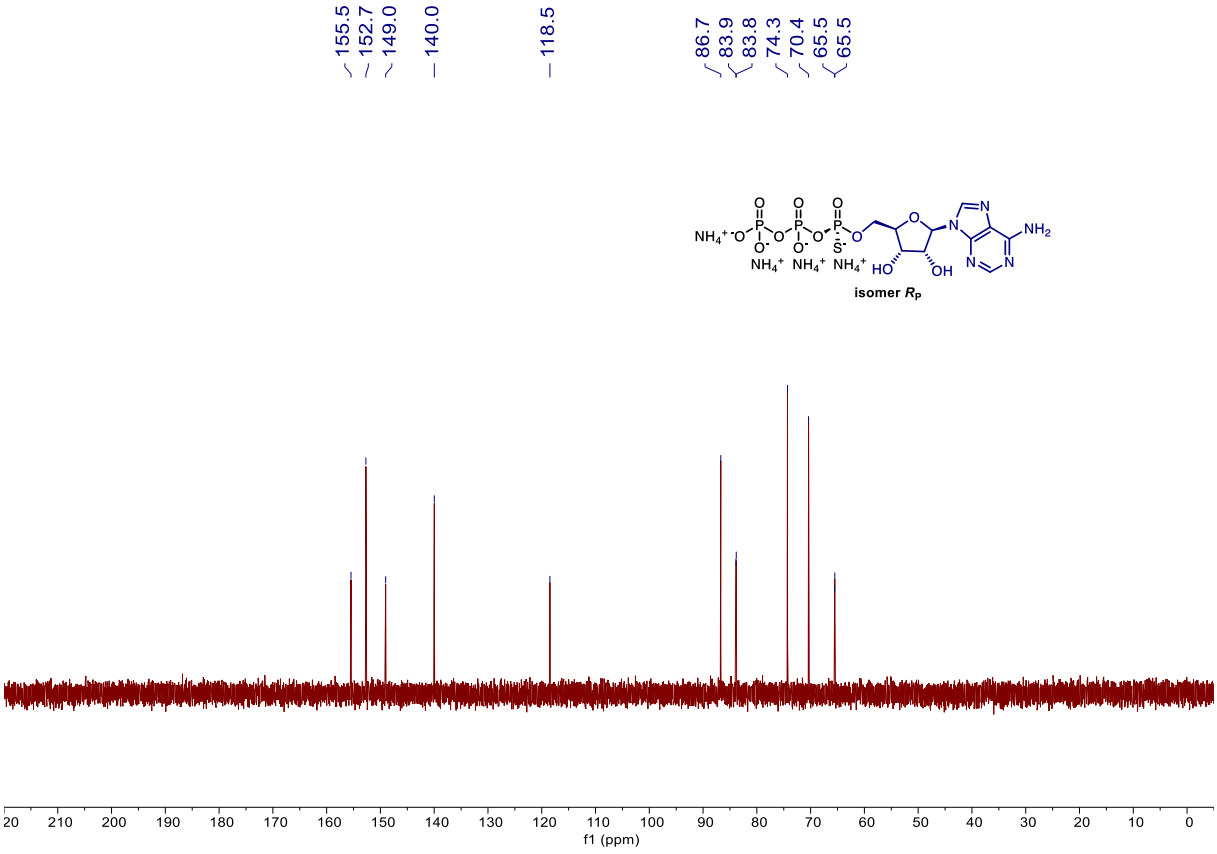
^{31}P NMR of compound (S_P)-31 (162 MHz, D_2O)



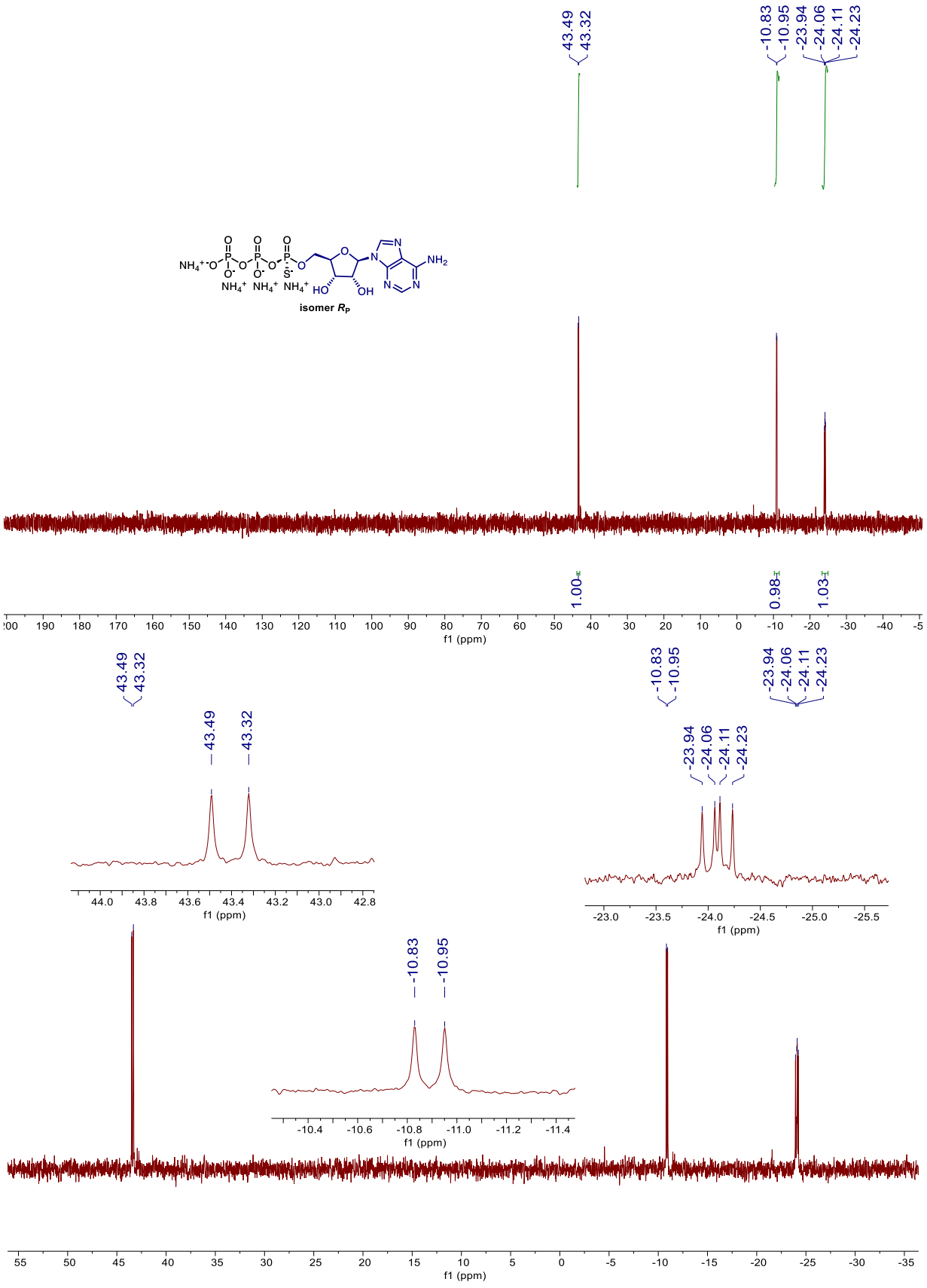
^{1}H NMR of compound (R_P)-32 (600 MHz, D_2O)



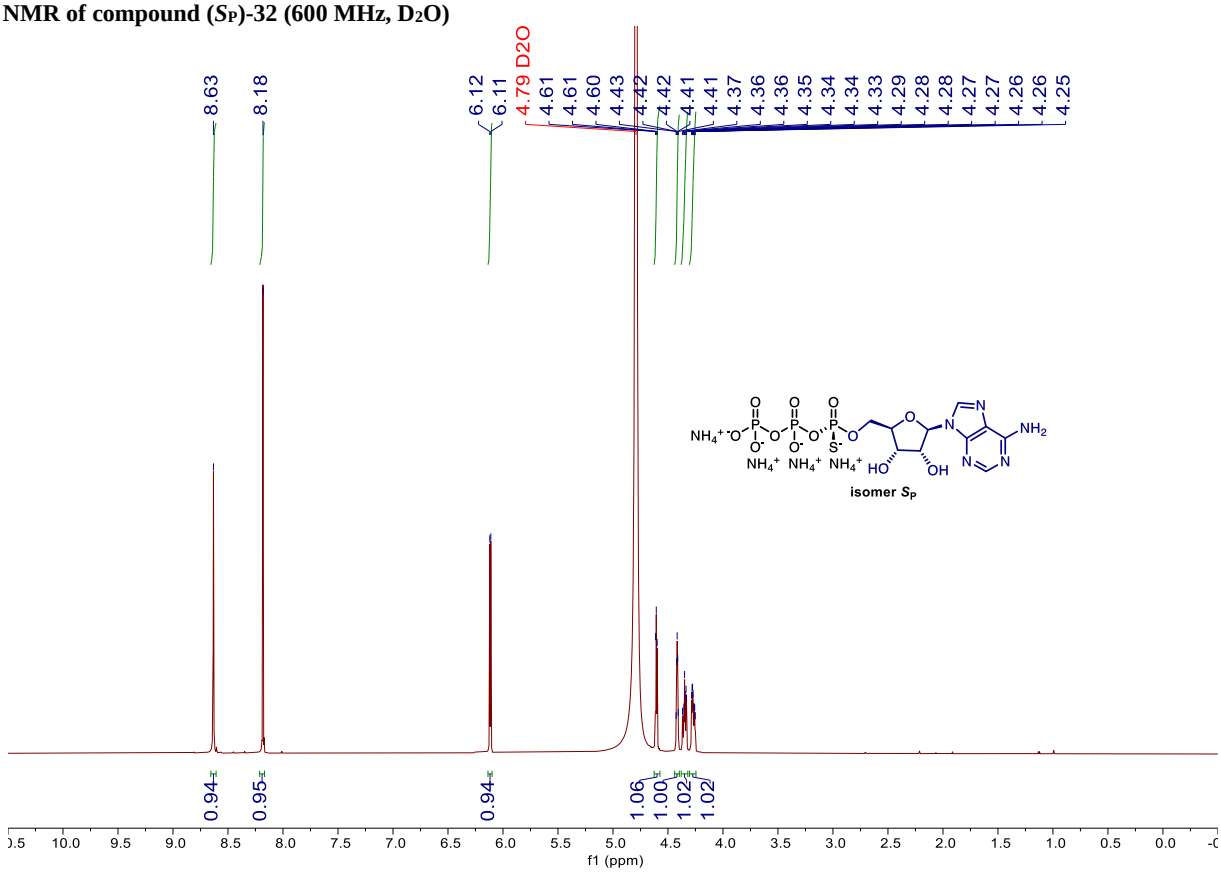
^{13}C NMR of compound (R_P)-32 (150 MHz, D_2O)



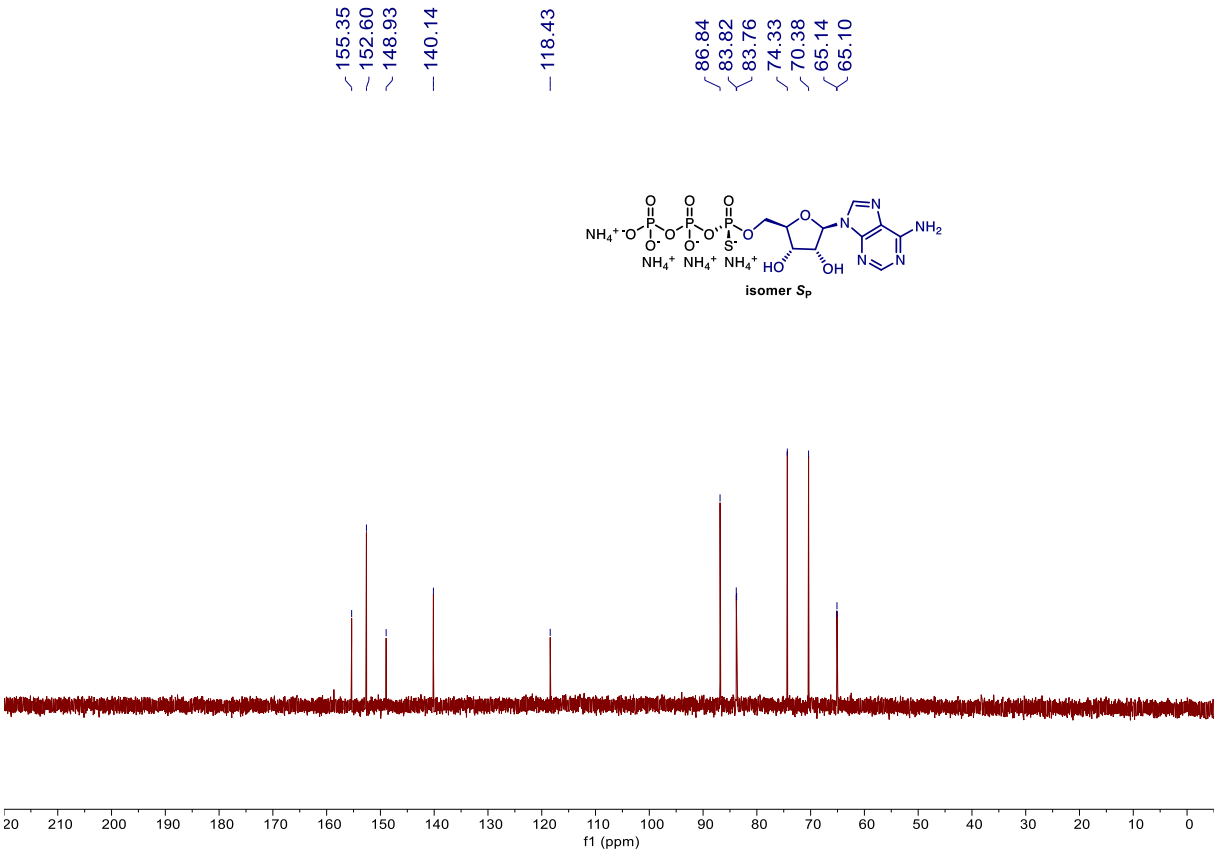
^{31}P NMR of compound (R_P)-32 (162 MHz, D_2O)



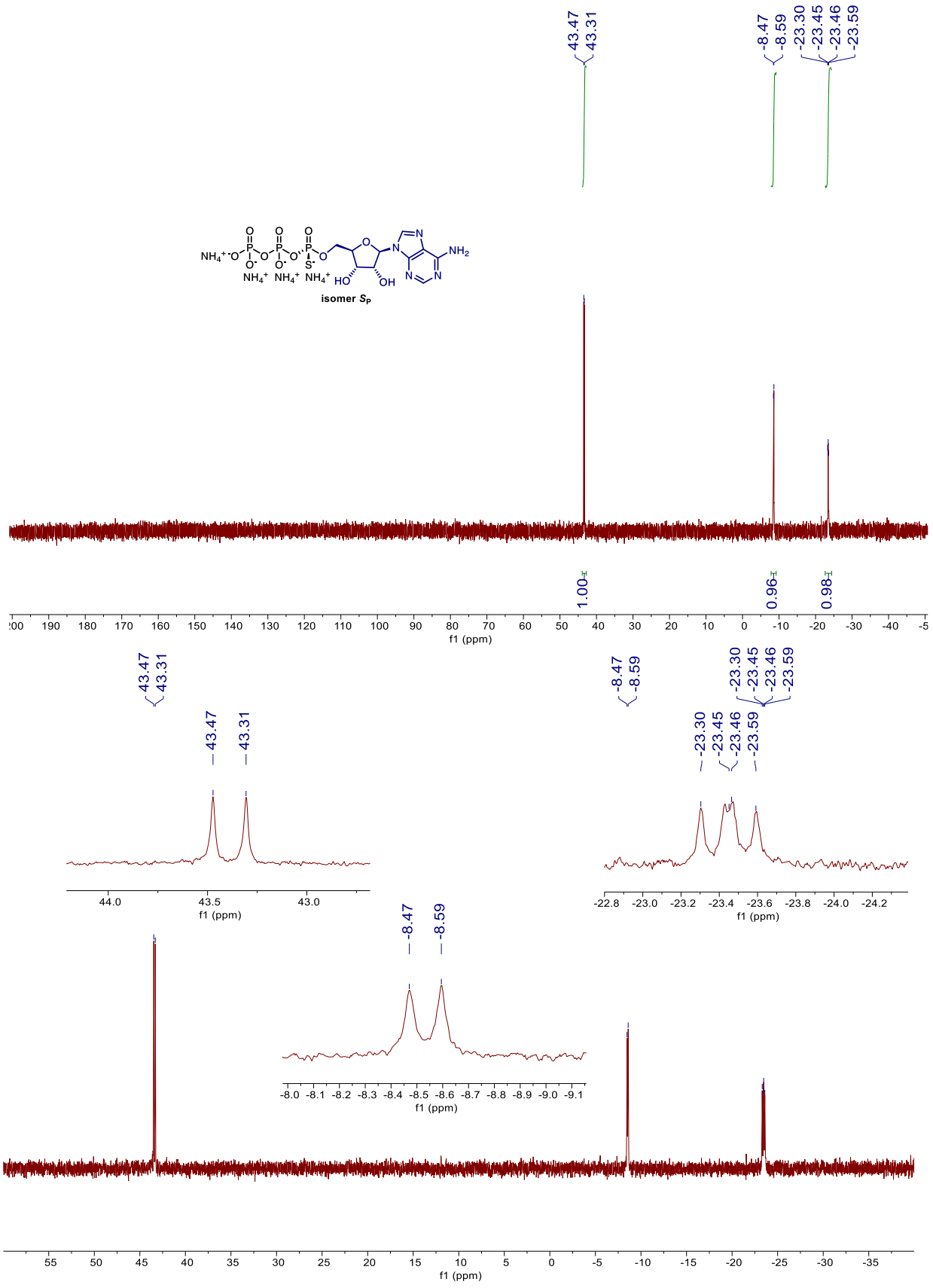
^{1}H NMR of compound (S_P)-32 (600 MHz, D_2O)



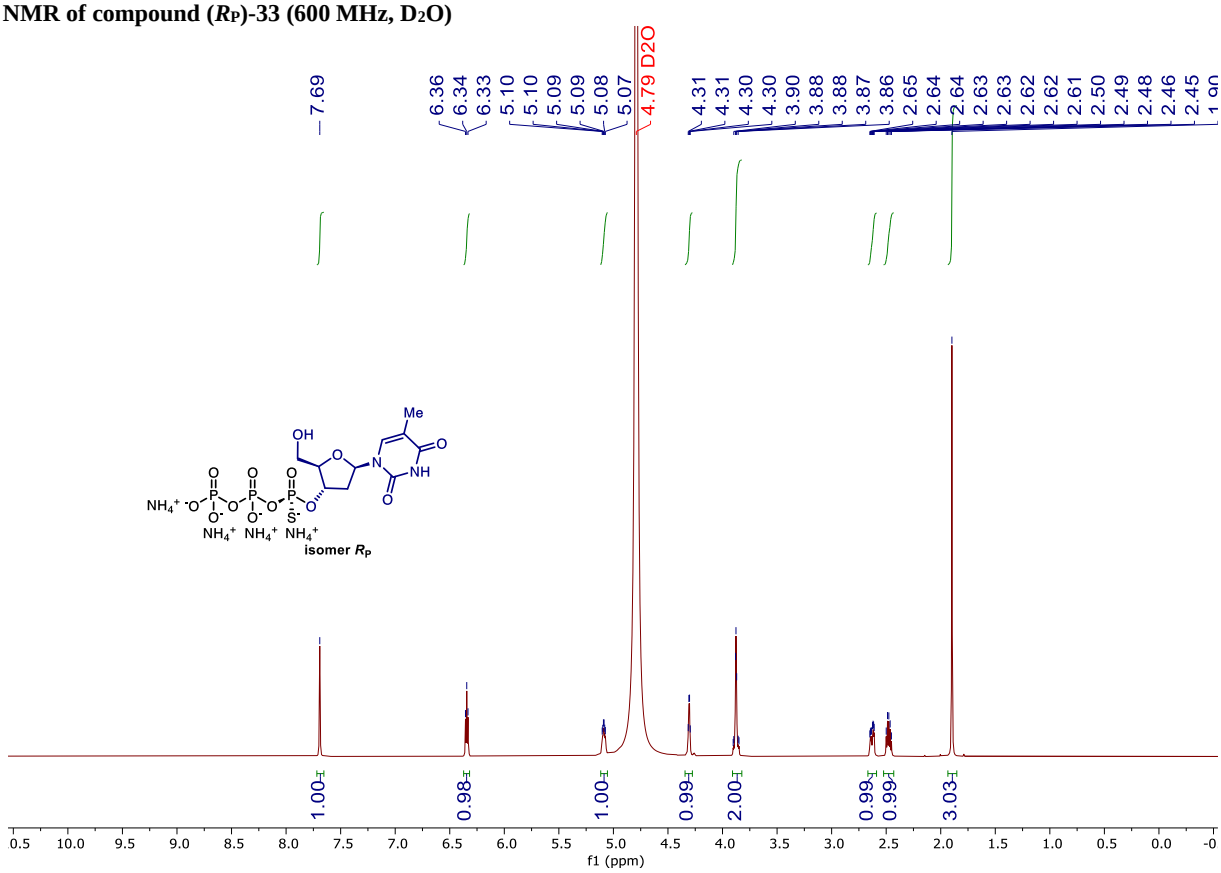
^{13}C NMR of compound (S_P)-32 (150 MHz, D_2O)



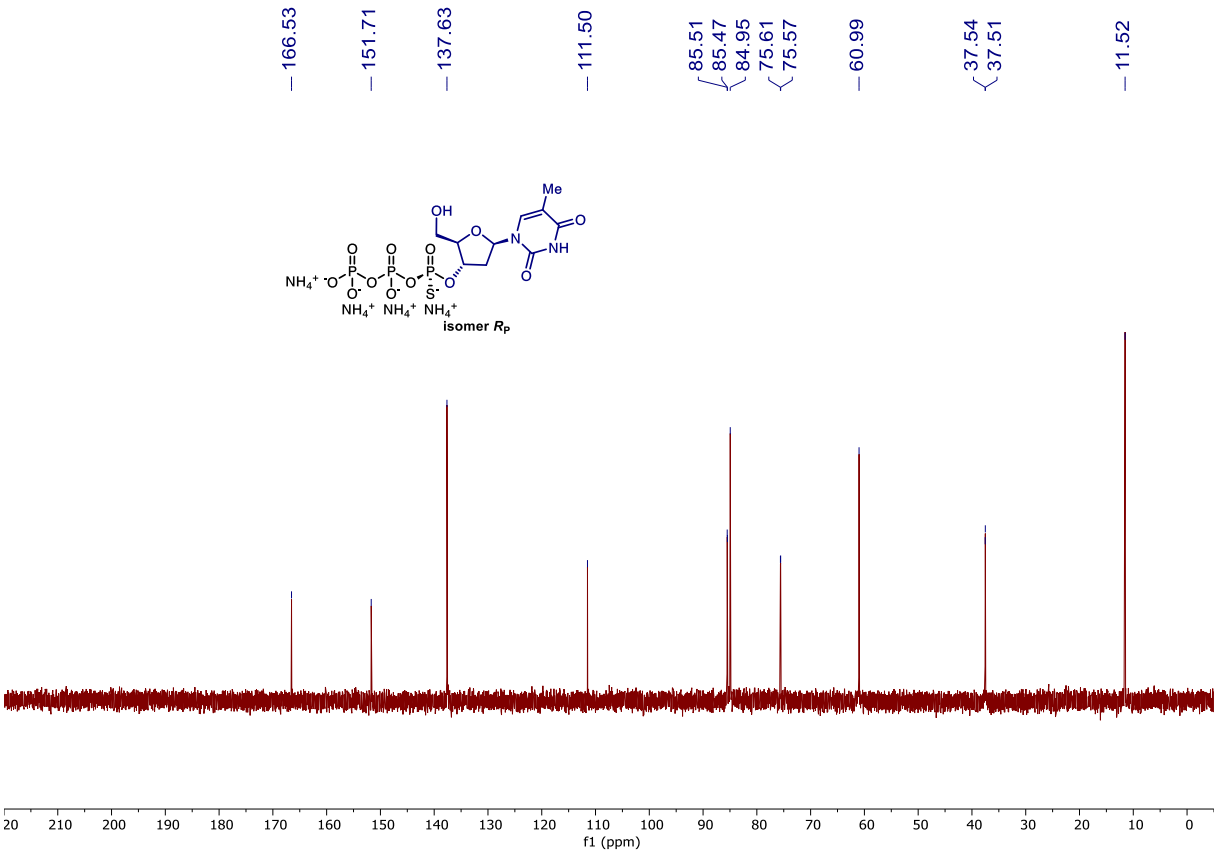
^{31}P NMR of compound (S_P)-32 (162 MHz, D_2O)



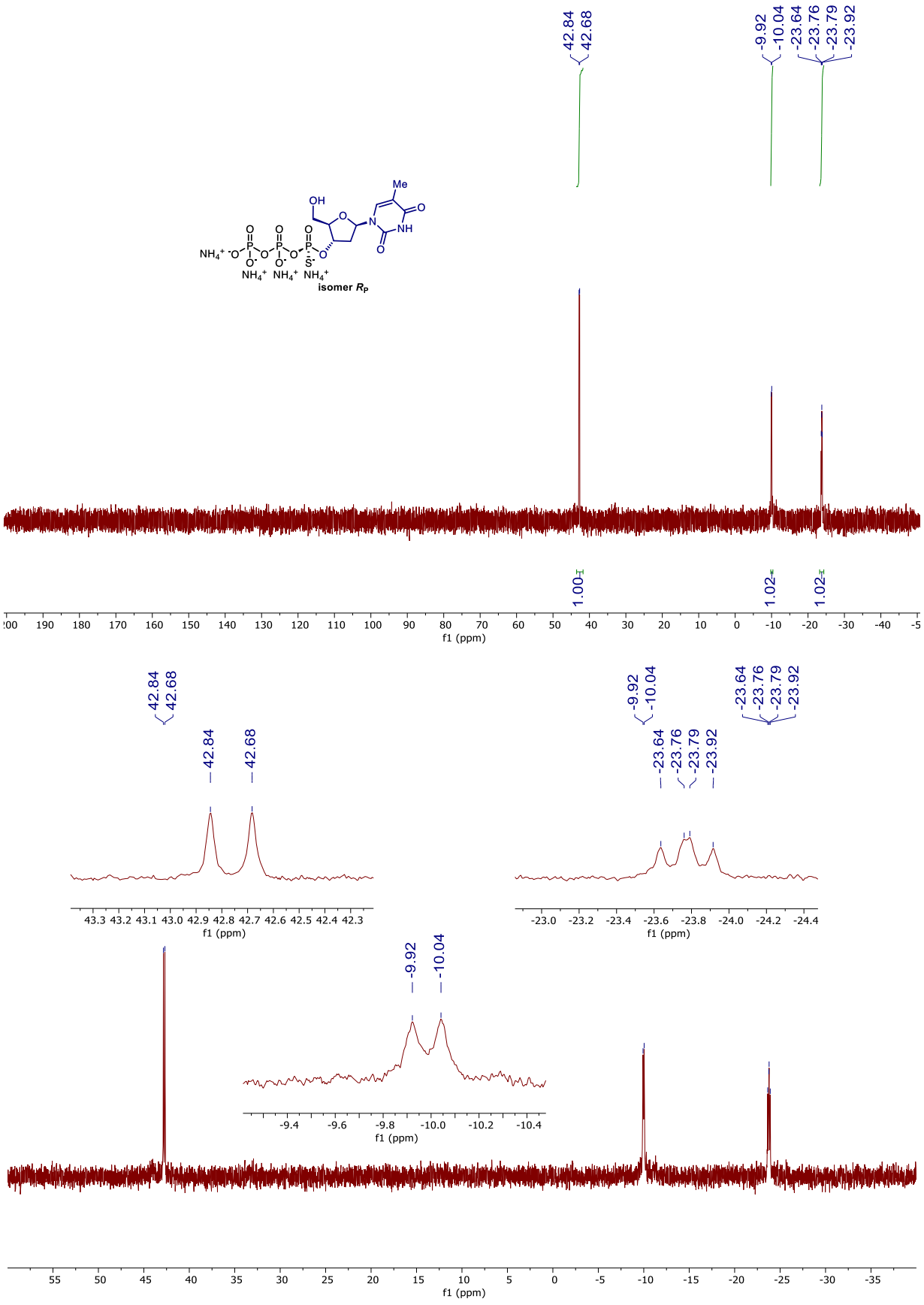
^{1}H NMR of compound (R_P)-33 (600 MHz, D_2O)



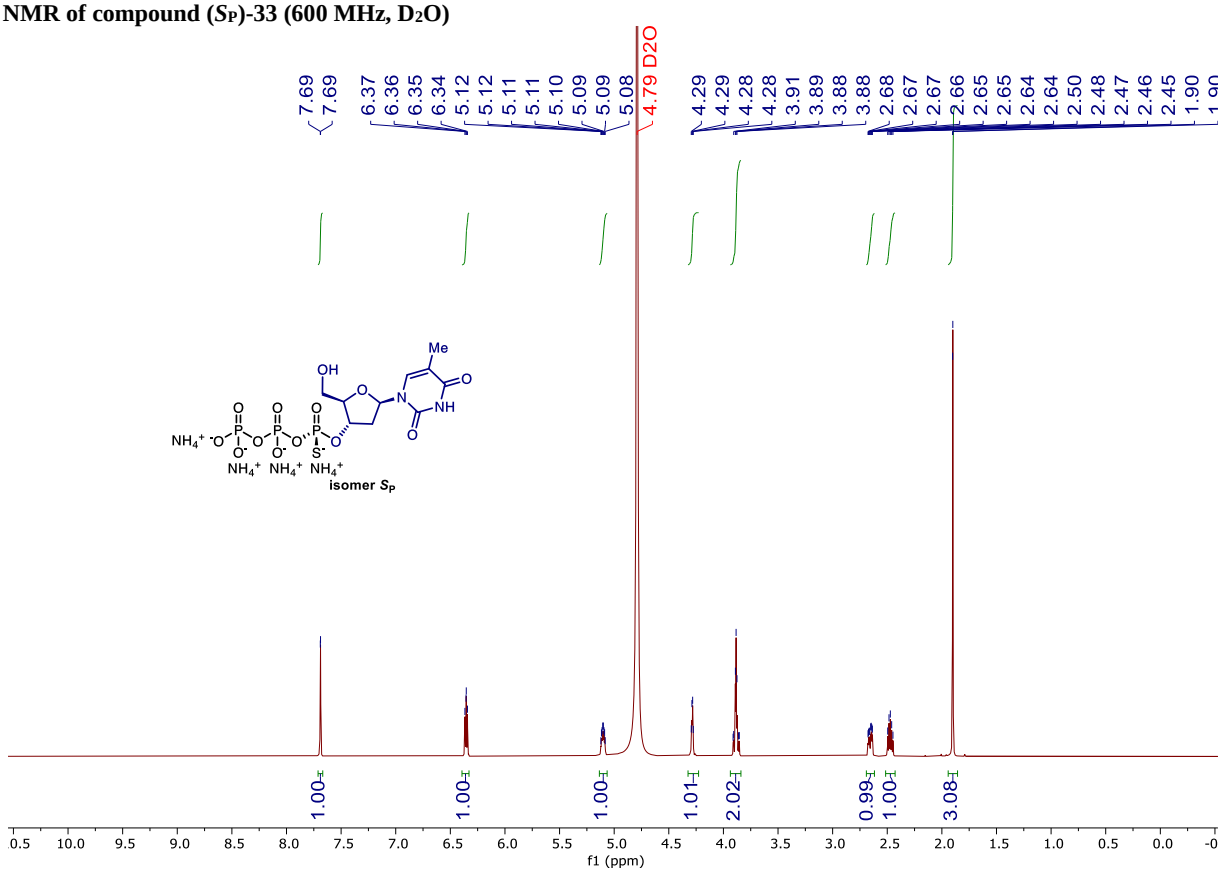
^{13}C NMR of compound (R_P)-33 (150 MHz, D_2O)



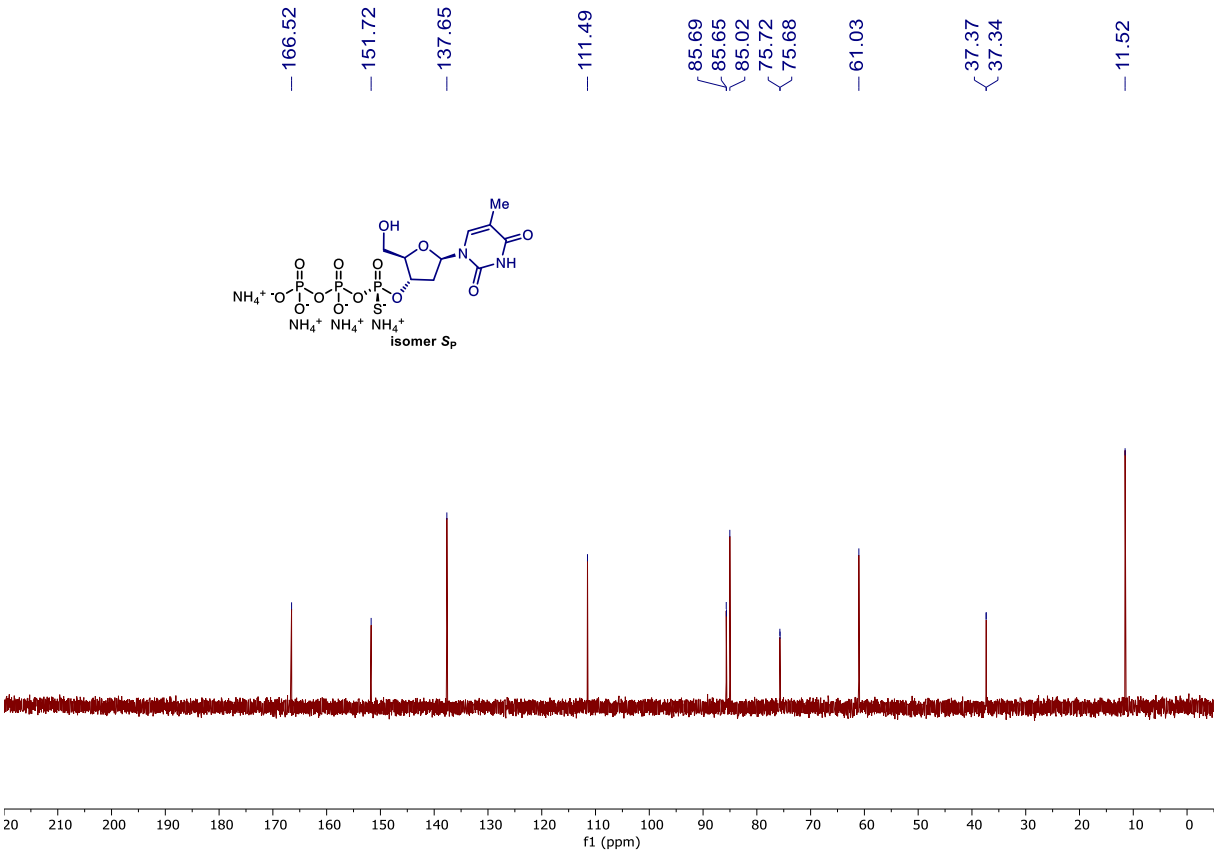
^{31}P NMR of compound (R_P)-33 (162 MHz, D_2O)



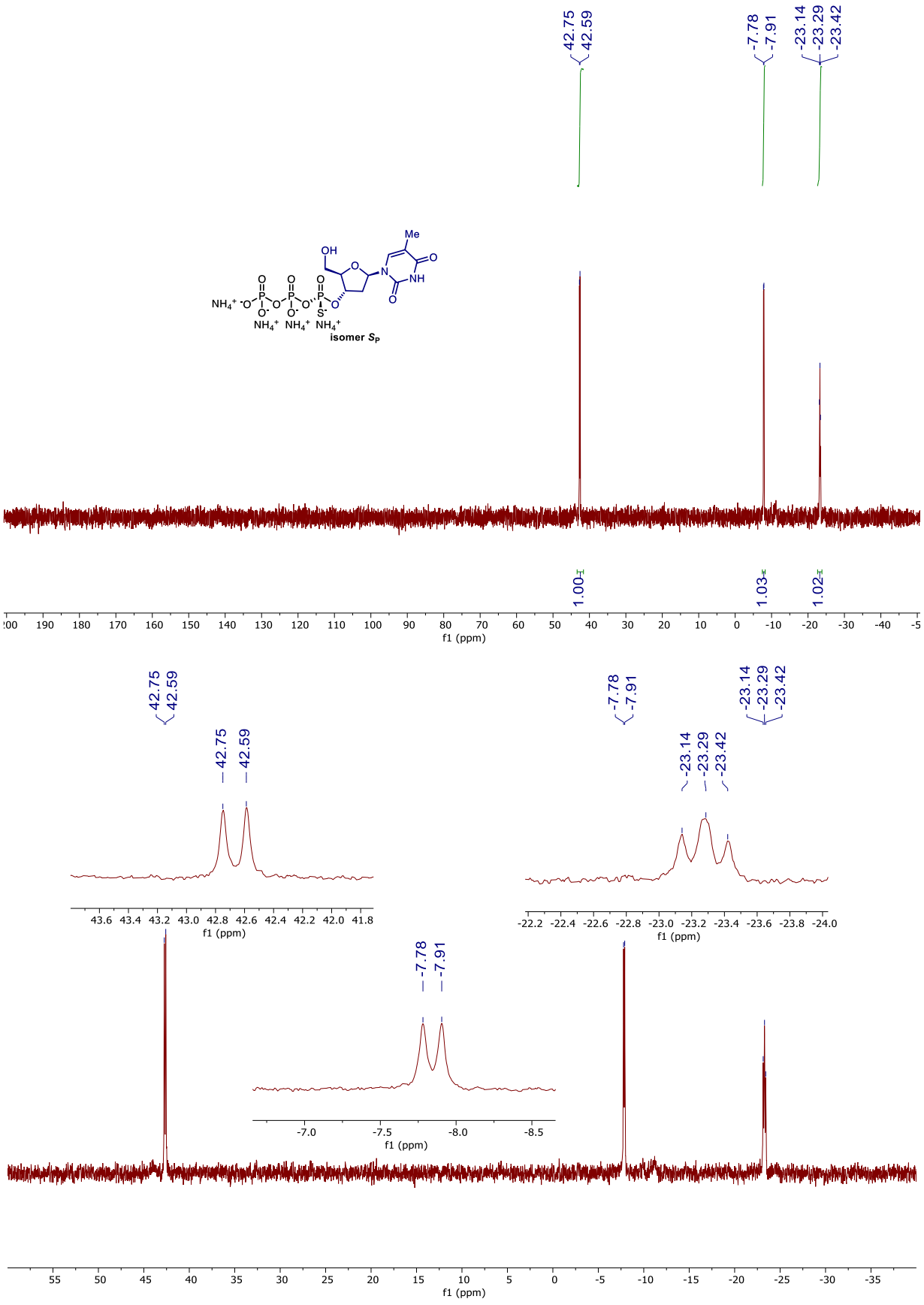
^{1}H NMR of compound (S_P)-33 (600 MHz, D_2O)



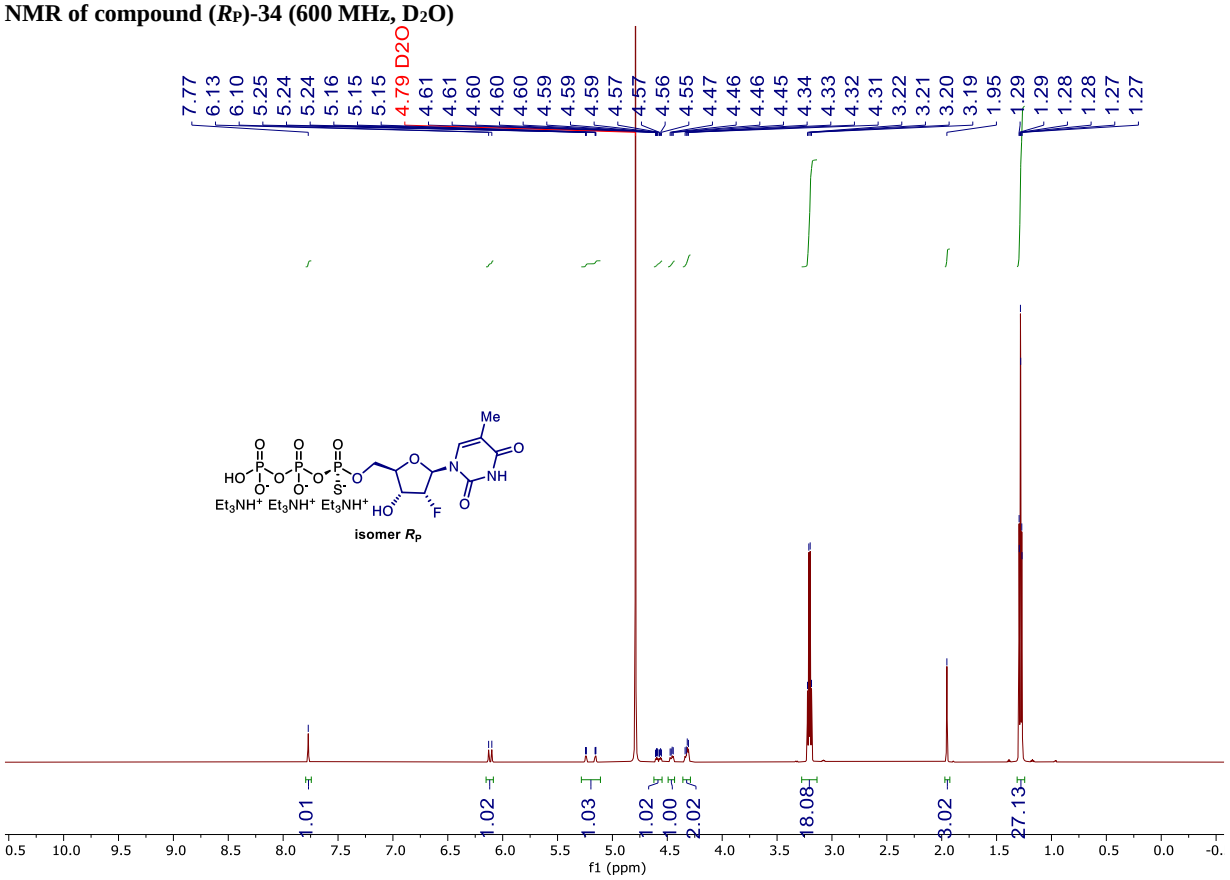
^{13}C NMR of compound (S_P)-33 (150 MHz, D_2O)

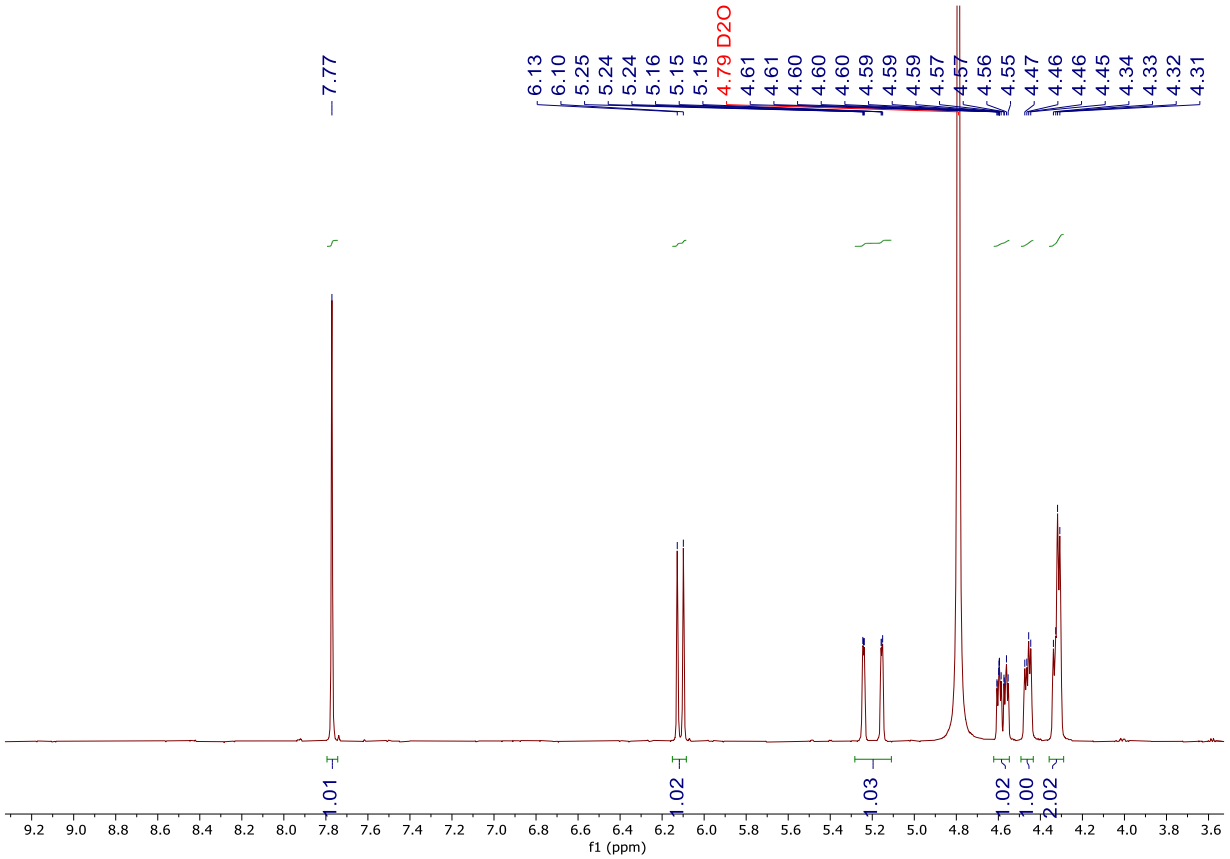


^{31}P NMR of compound (S_P)-33 (162 MHz, D_2O)

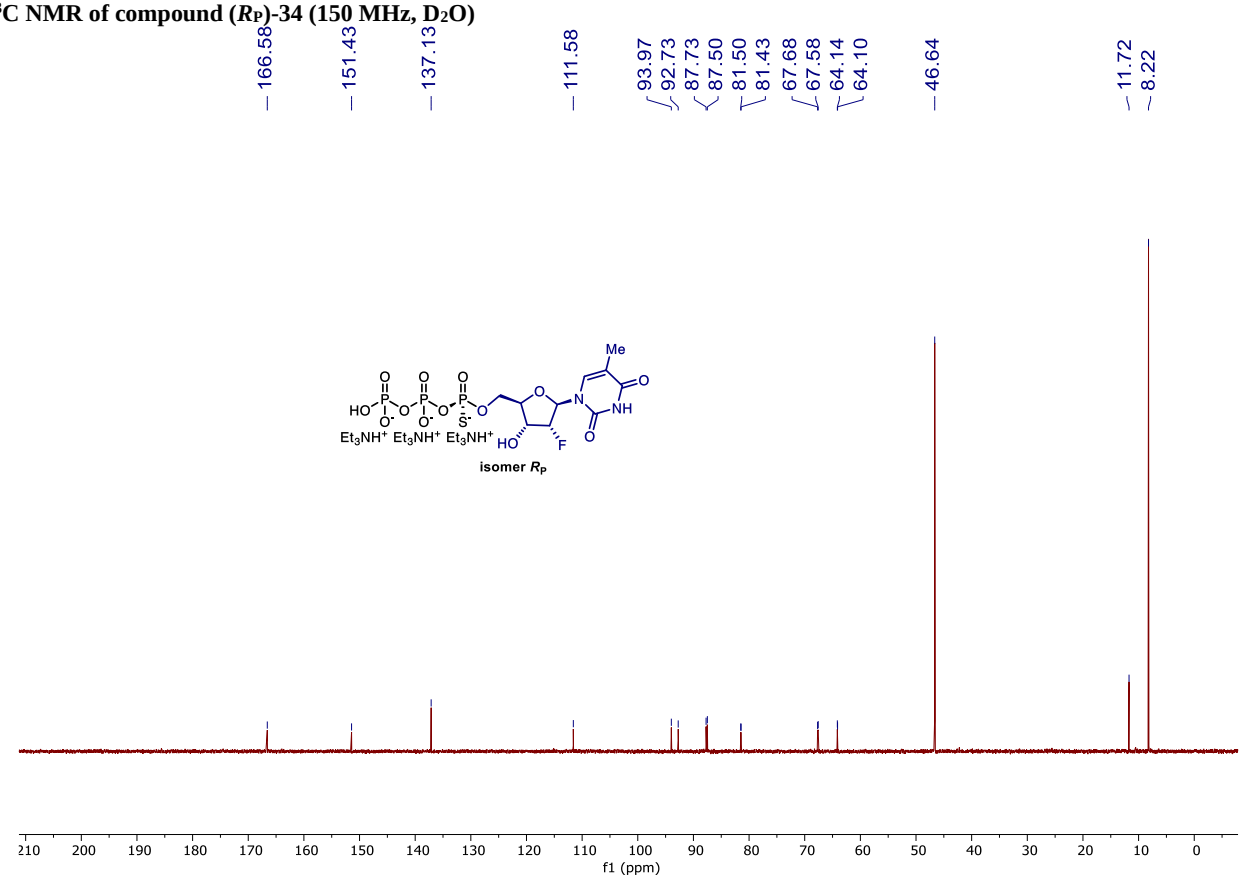


^{1}H NMR of compound (R_P)-34 (600 MHz, D_2O)

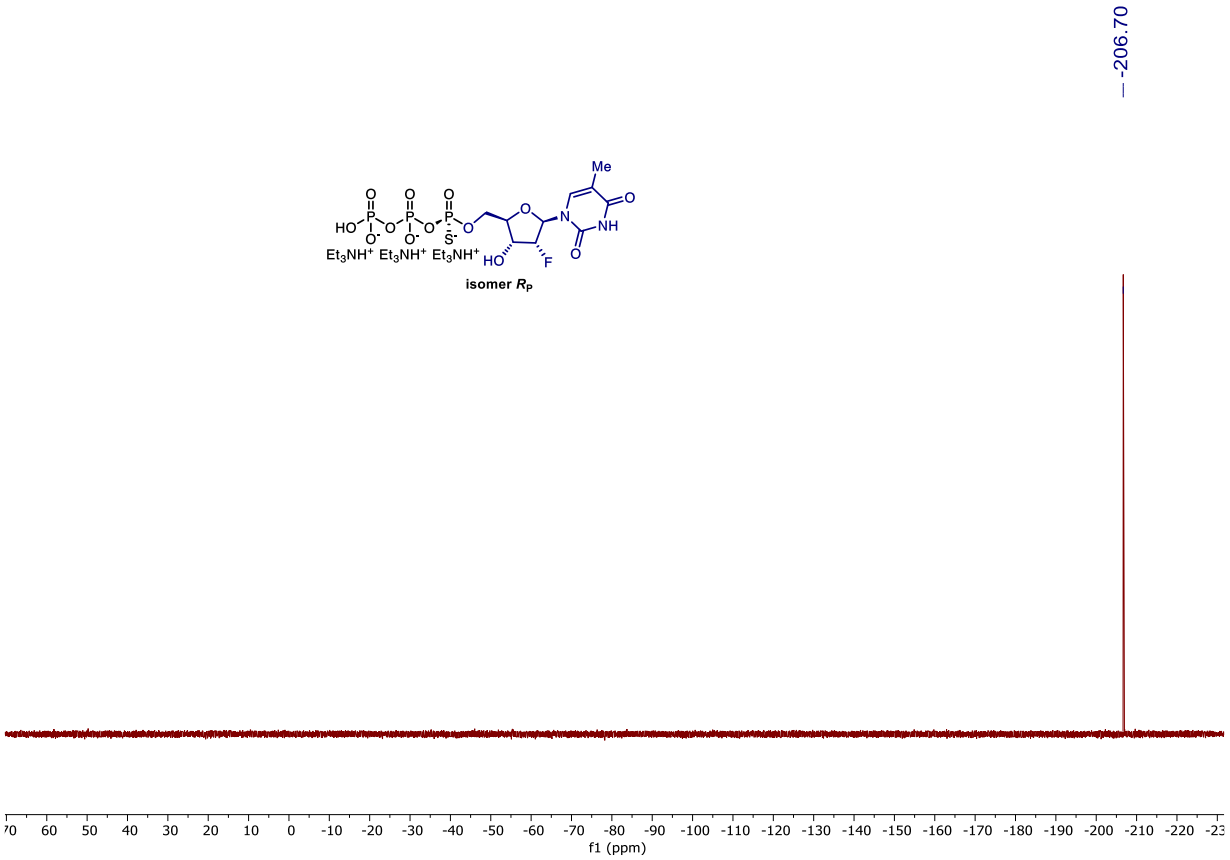




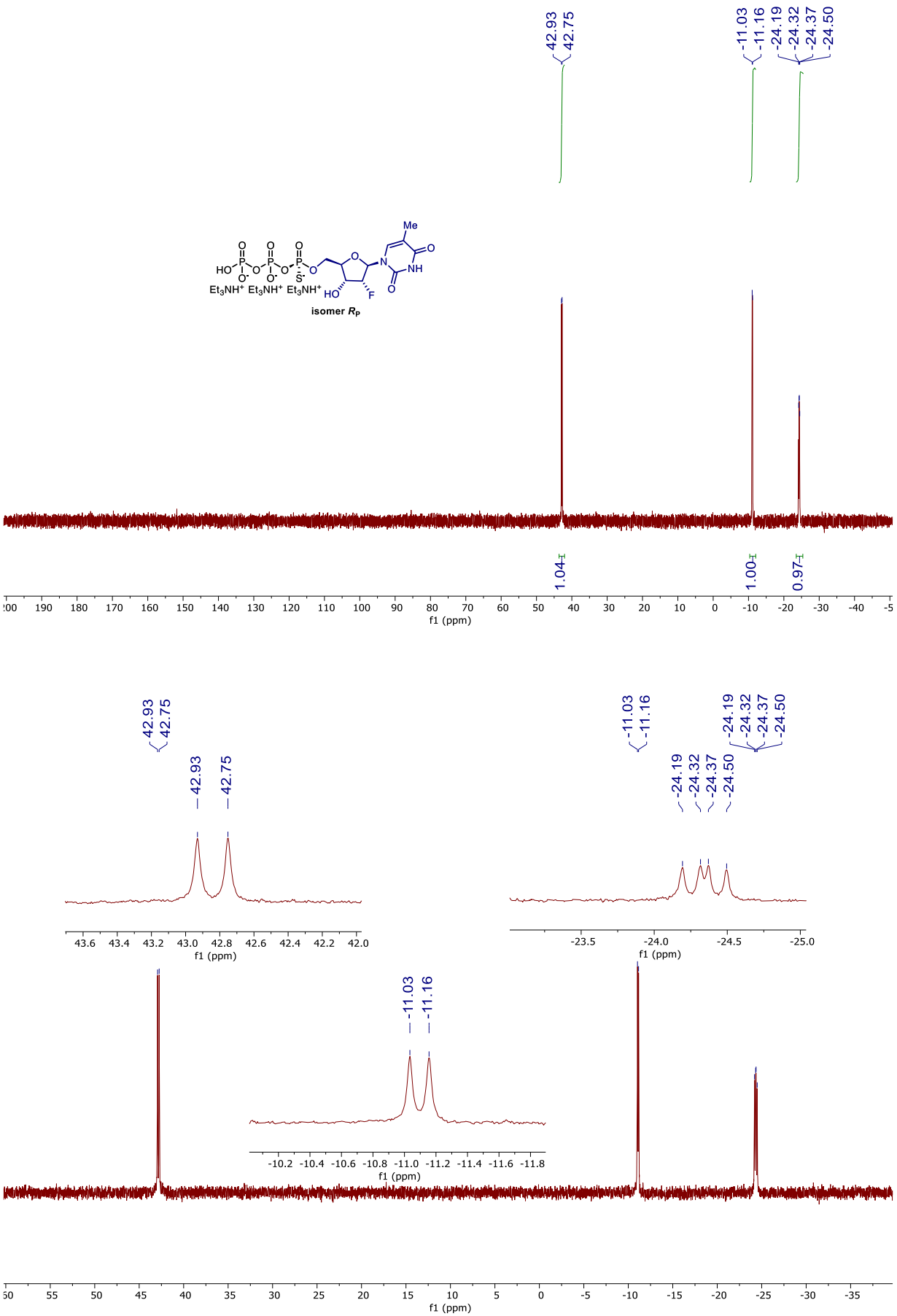
^{13}C NMR of compound (R_P)-34 (150 MHz, D_2O)



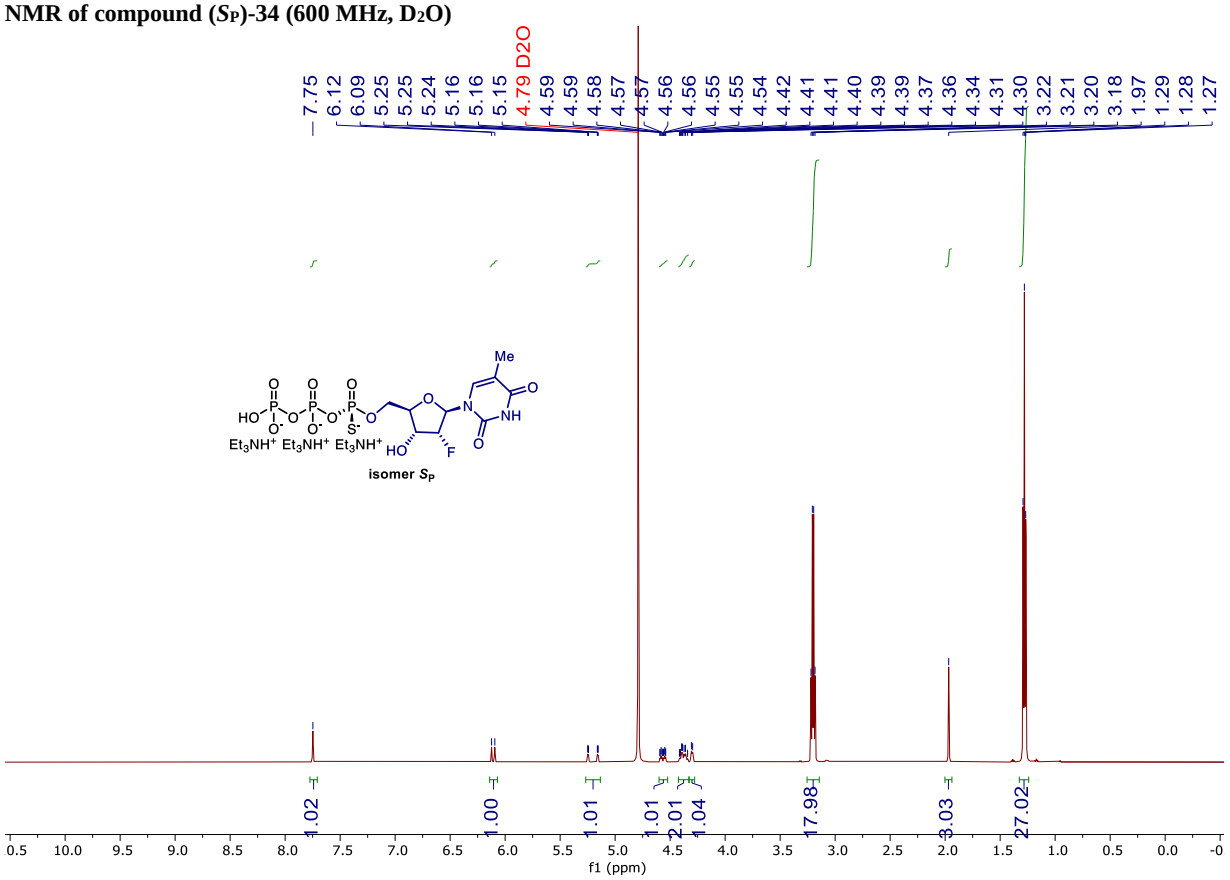
^{19}F NMR of compound (R_P)-34 (376 MHz, D_2O)

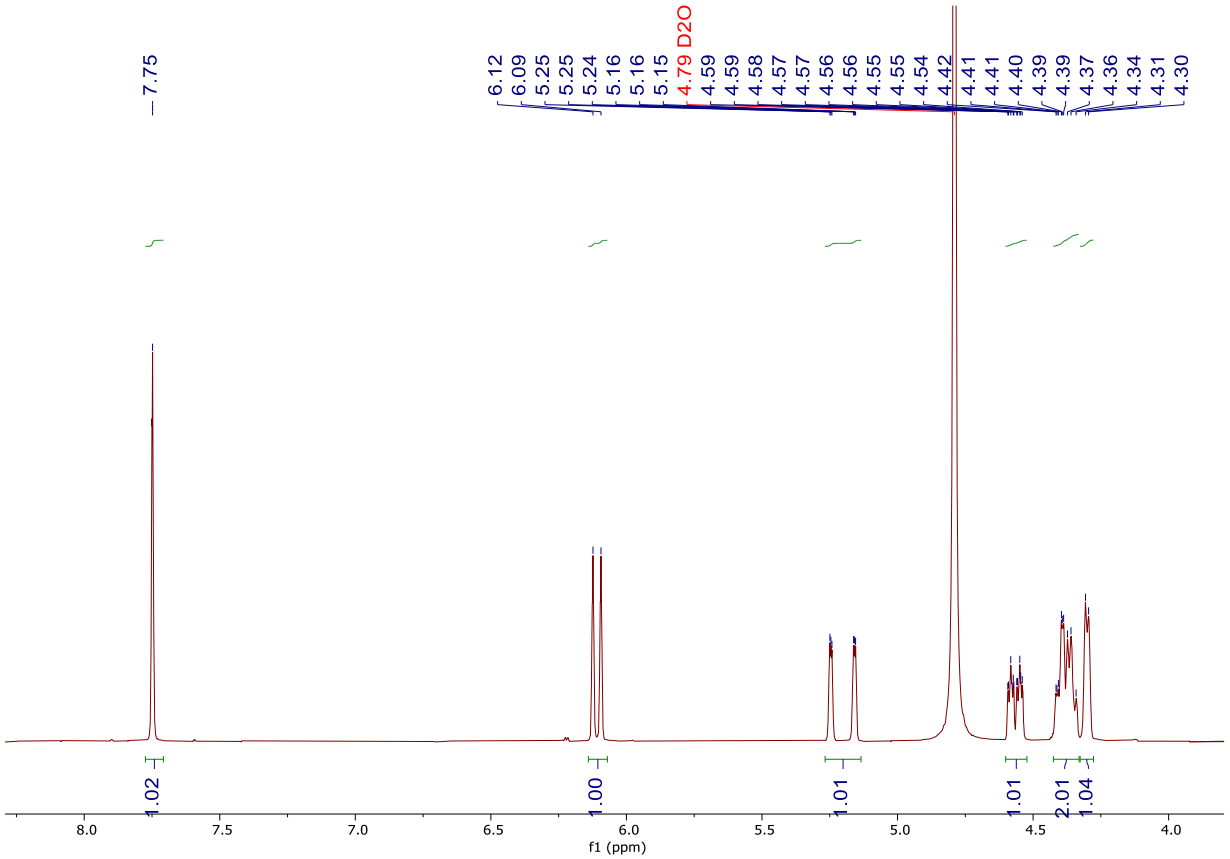


^{31}P NMR of compound (R_P)-34 (162 MHz, D_2O)

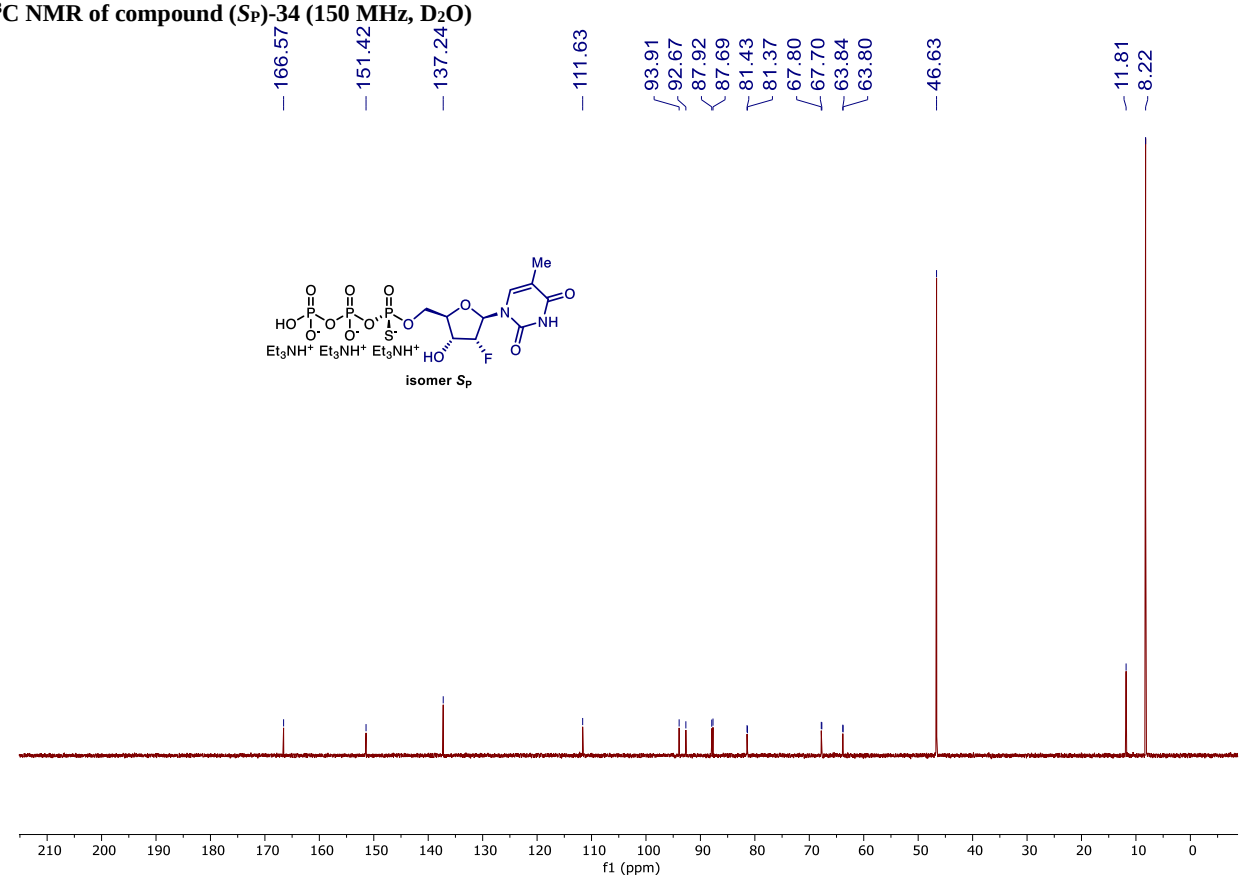


^{1}H NMR of compound (S_P)-34 (600 MHz, D_2O)

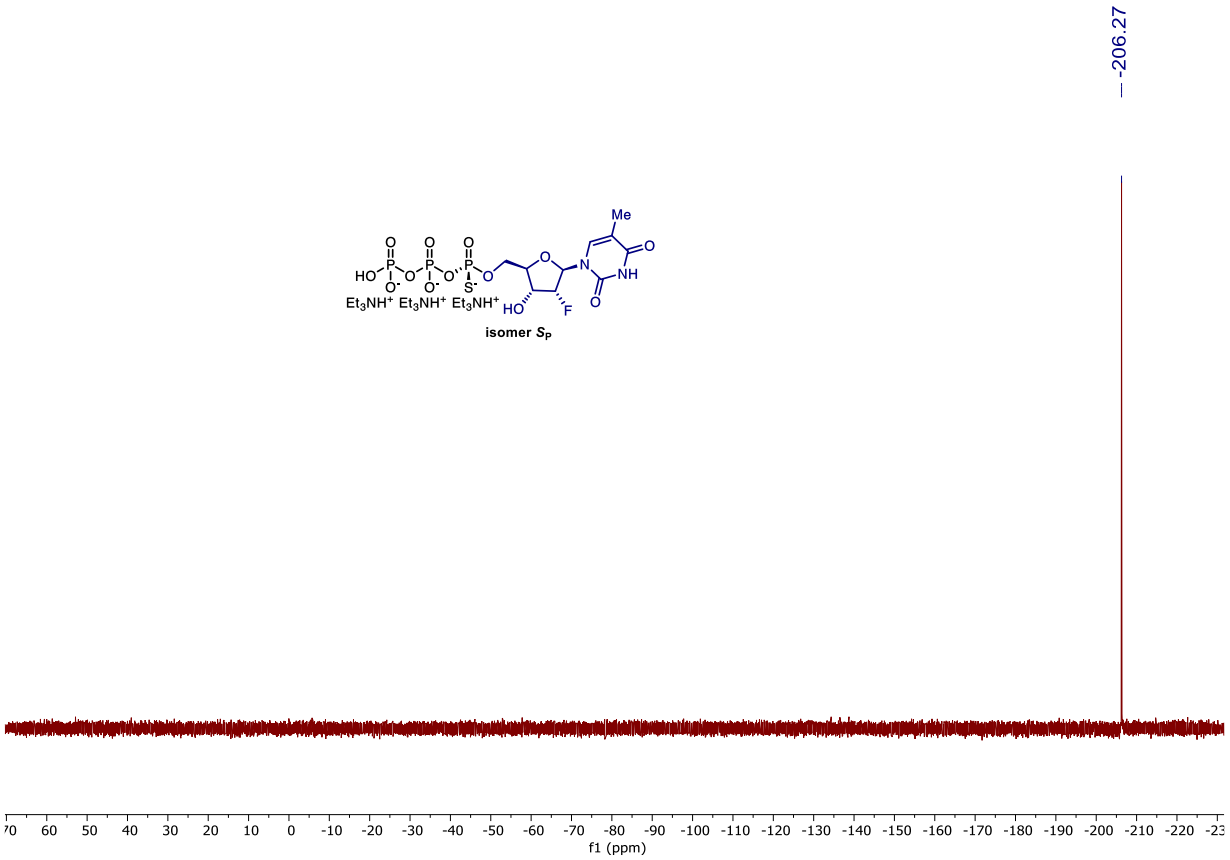




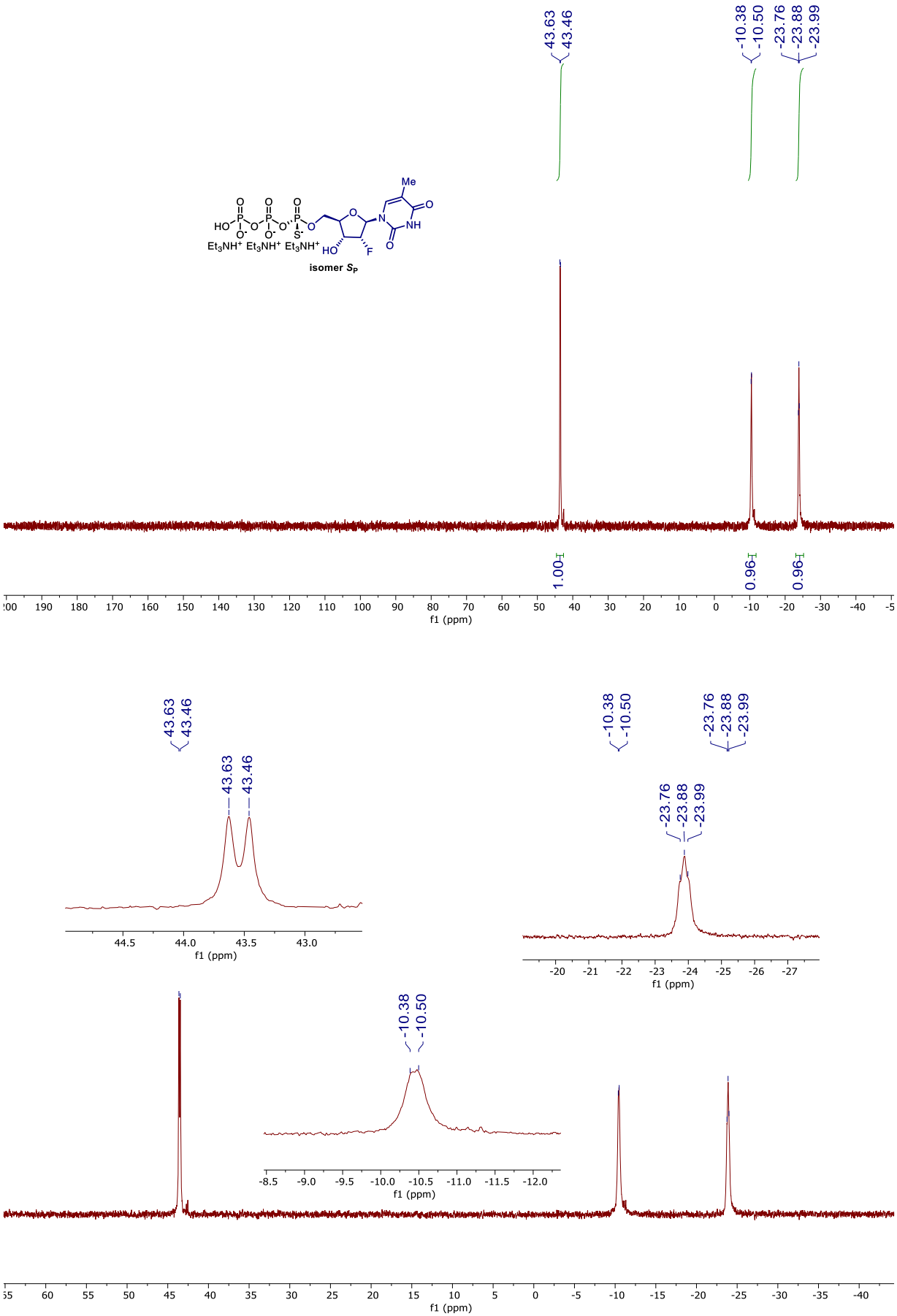
^{13}C NMR of compound (S_P)-34 (150 MHz, D_2O)



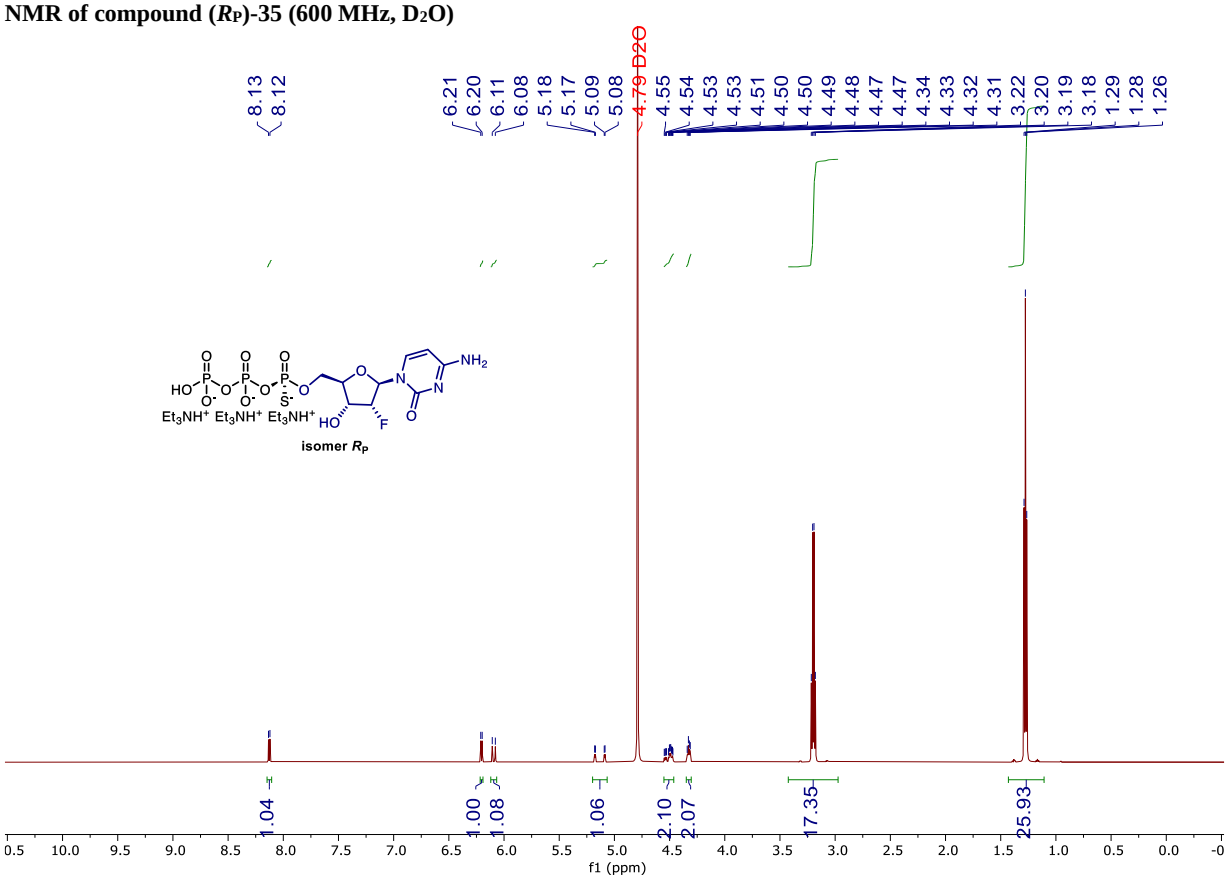
^{19}F NMR of compound (S_P)-34 (376 MHz, D_2O)

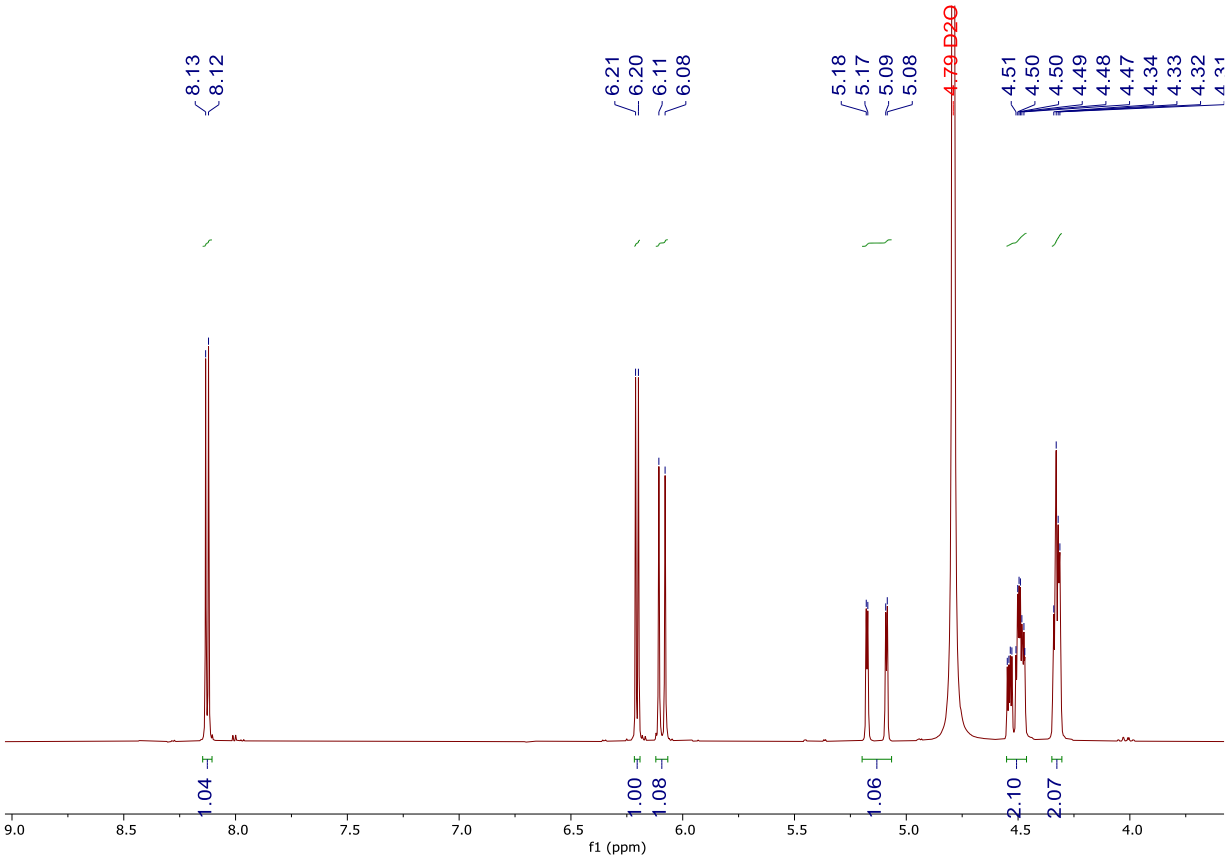


^{31}P NMR of compound (S_P)-34 (162 MHz, D_2O)

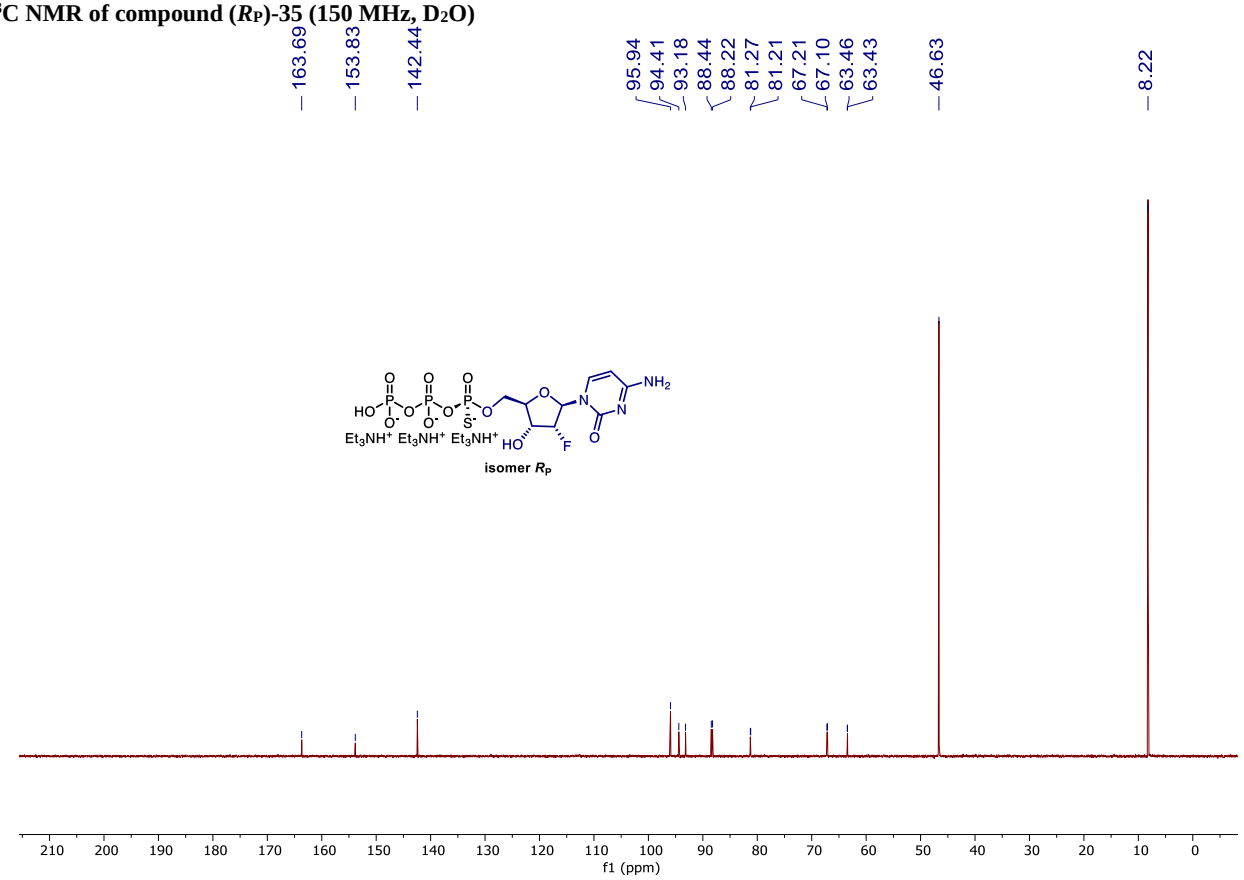


^{1}H NMR of compound (R_P)-35 (600 MHz, D_2O)

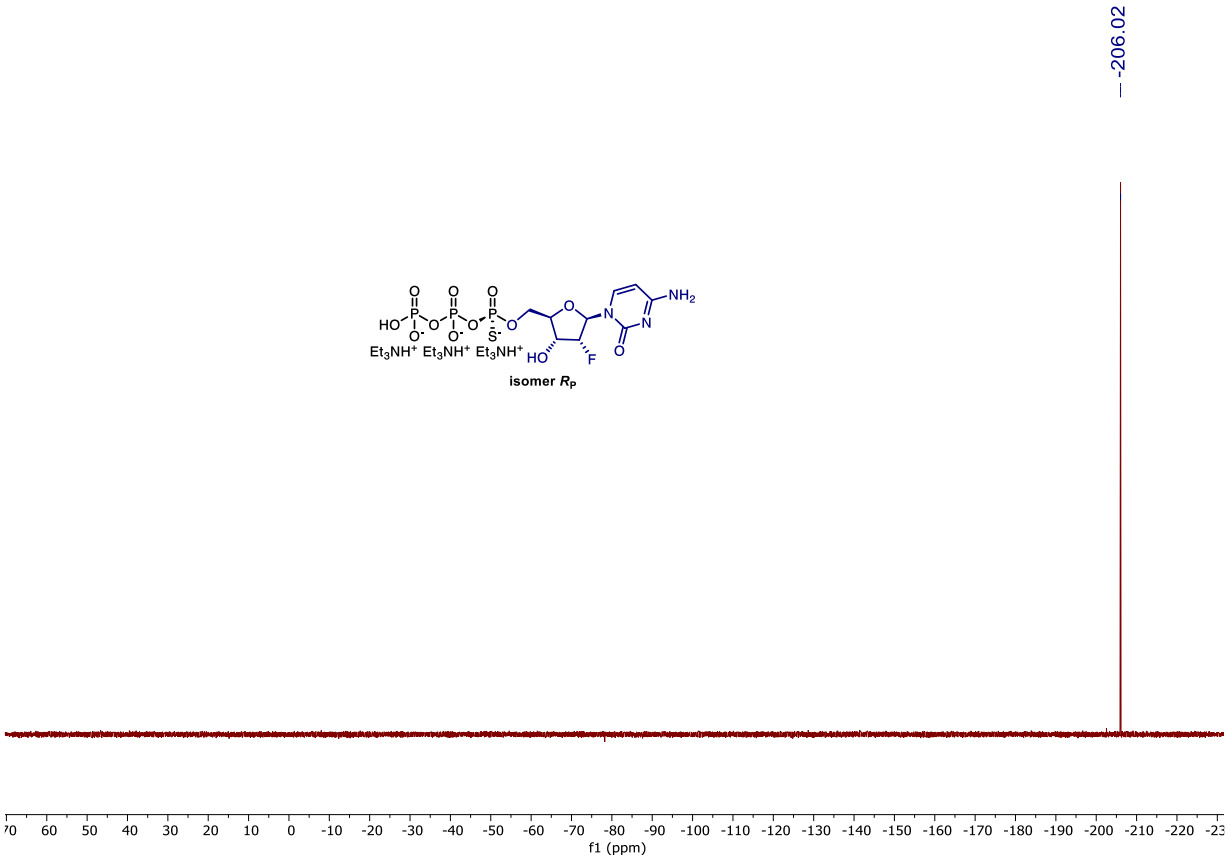




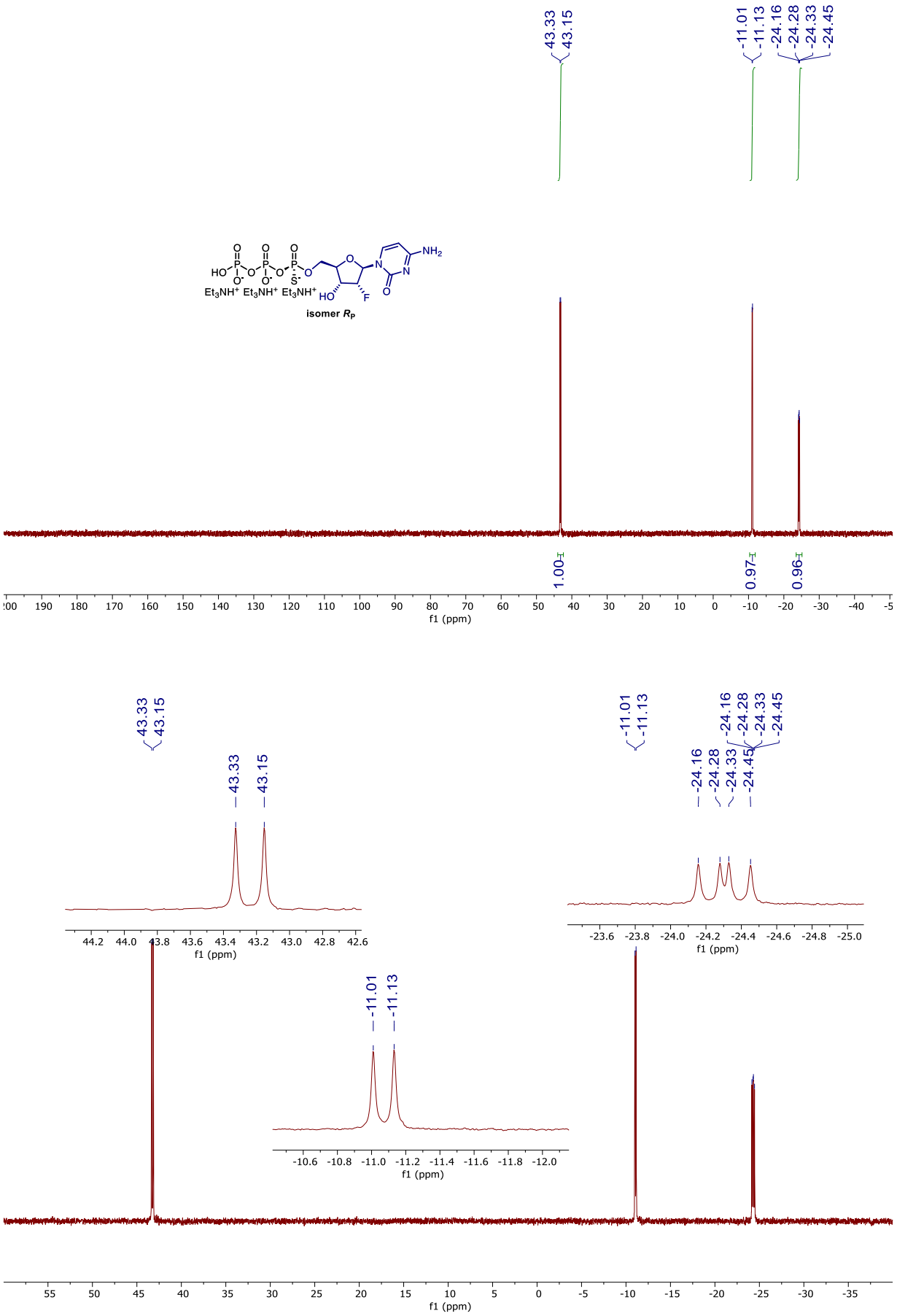
^{13}C NMR of compound (R_P)-35 (150 MHz, D_2O)



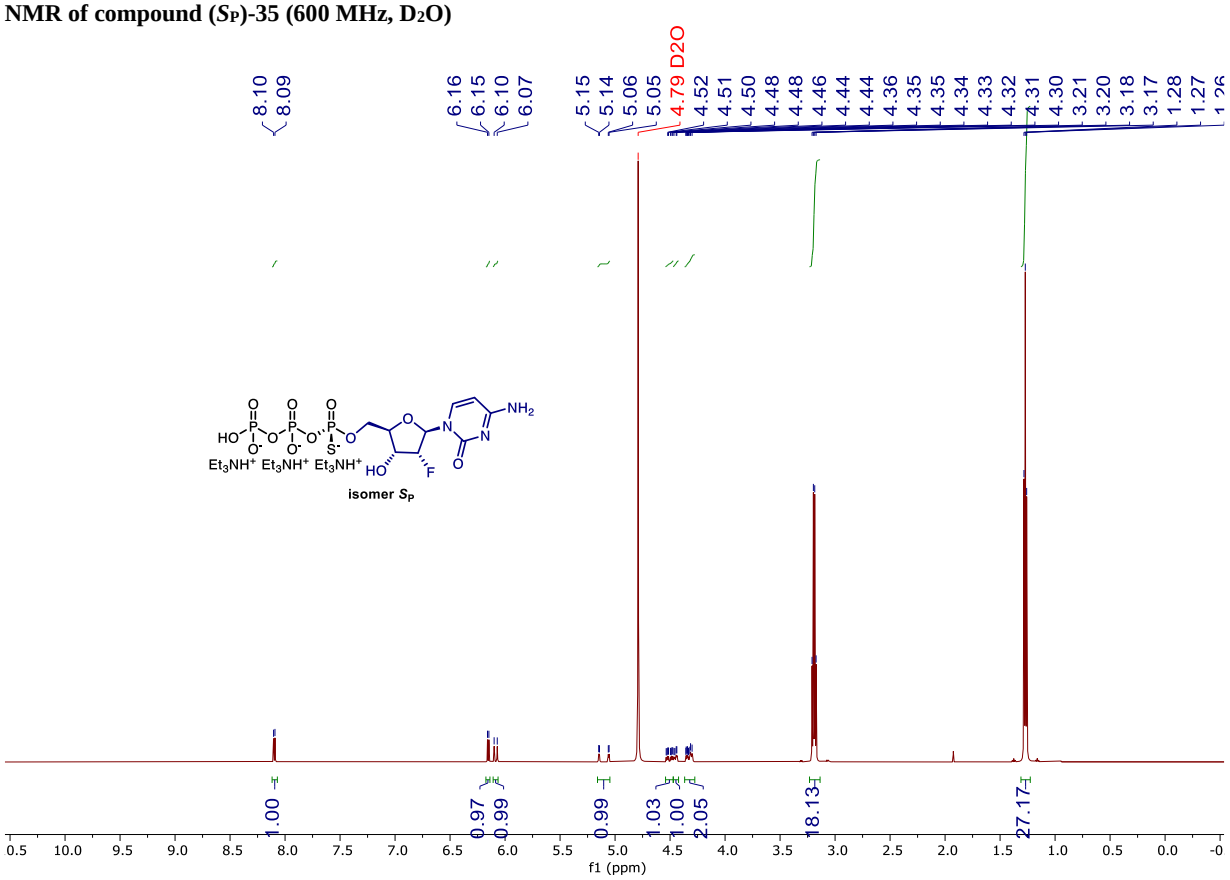
^{19}F NMR of compound (R_P)-35 (376 MHz, D_2O)

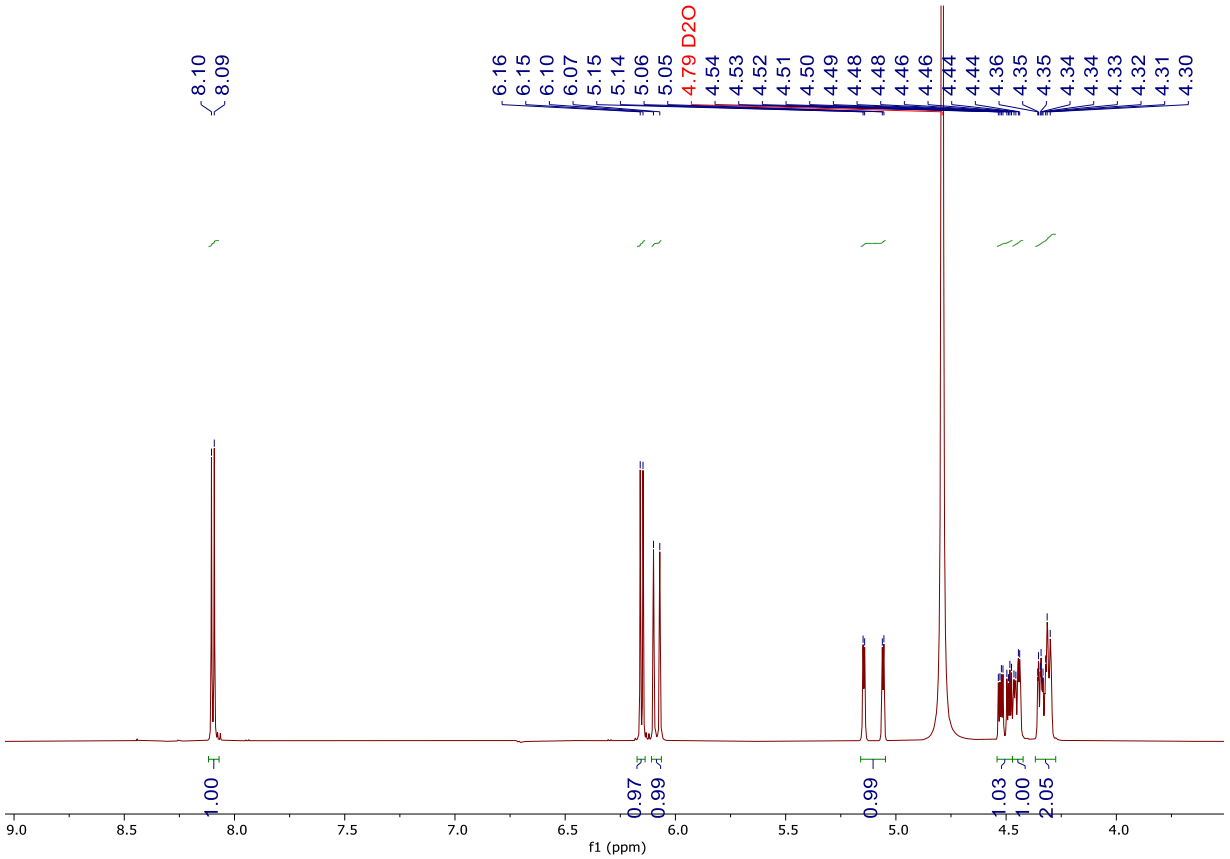


^{31}P NMR of compound (R_P)-35 (162 MHz, D_2O)

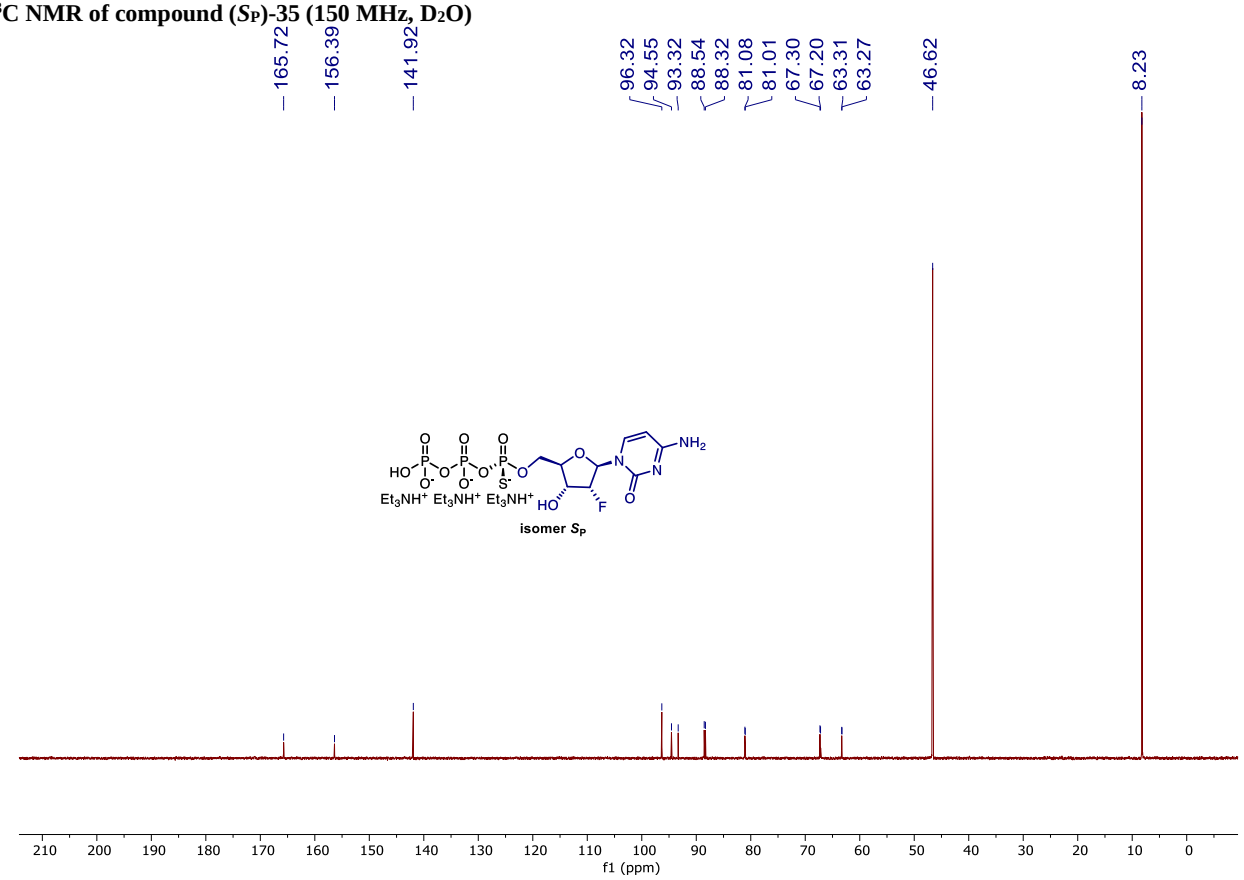


^{1}H NMR of compound (S_P)-35 (600 MHz, D_2O)

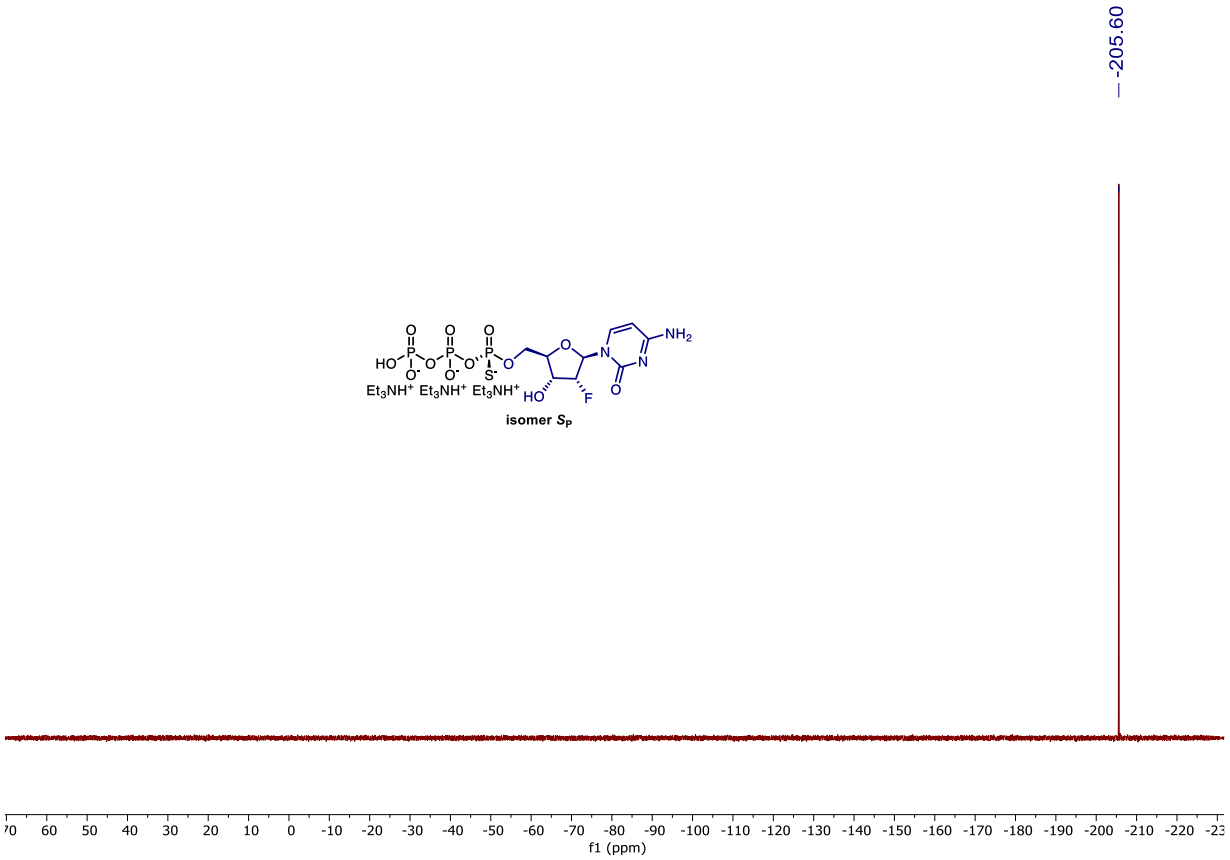




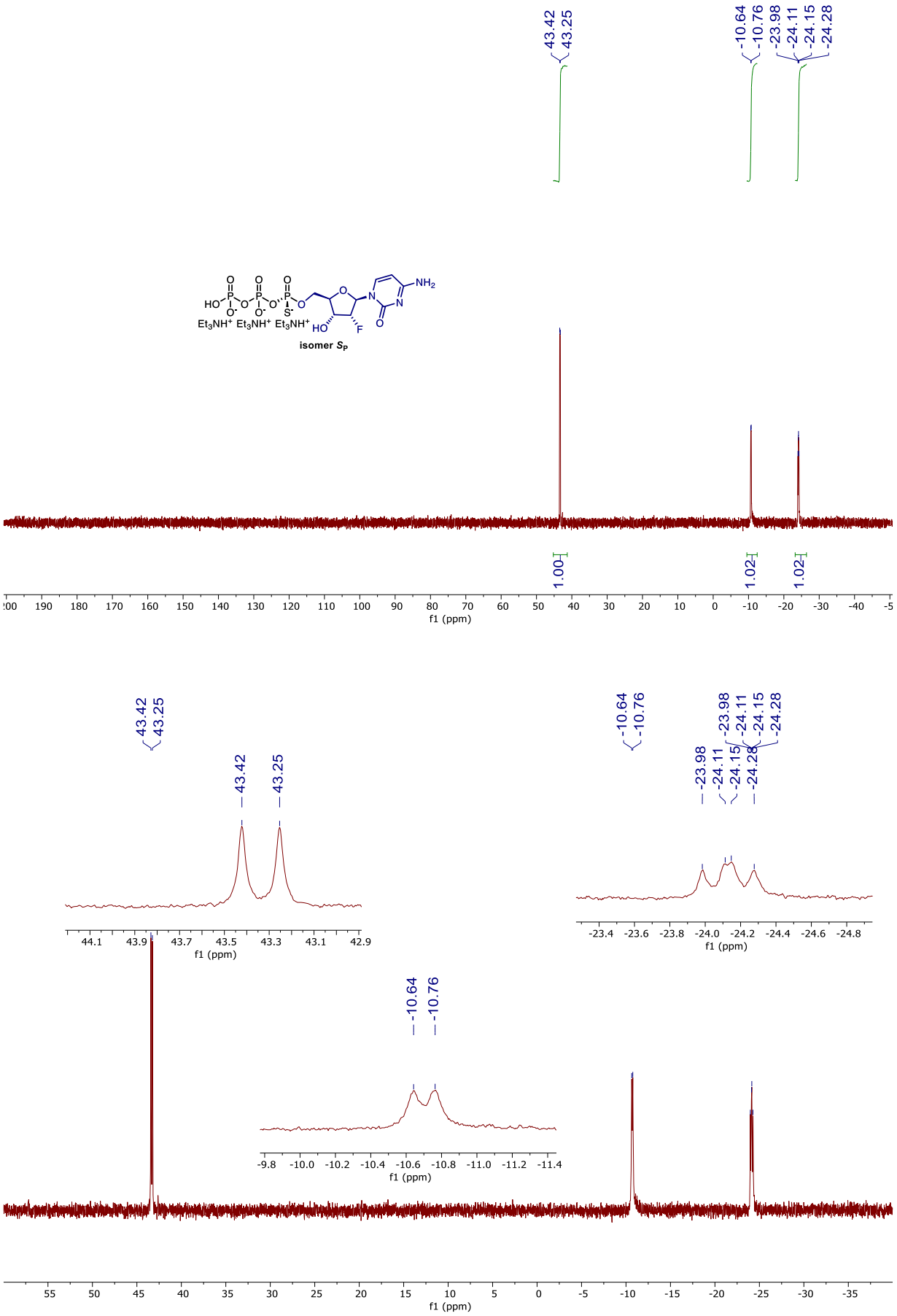
^{13}C NMR of compound (S_P)-35 (150 MHz, D_2O)



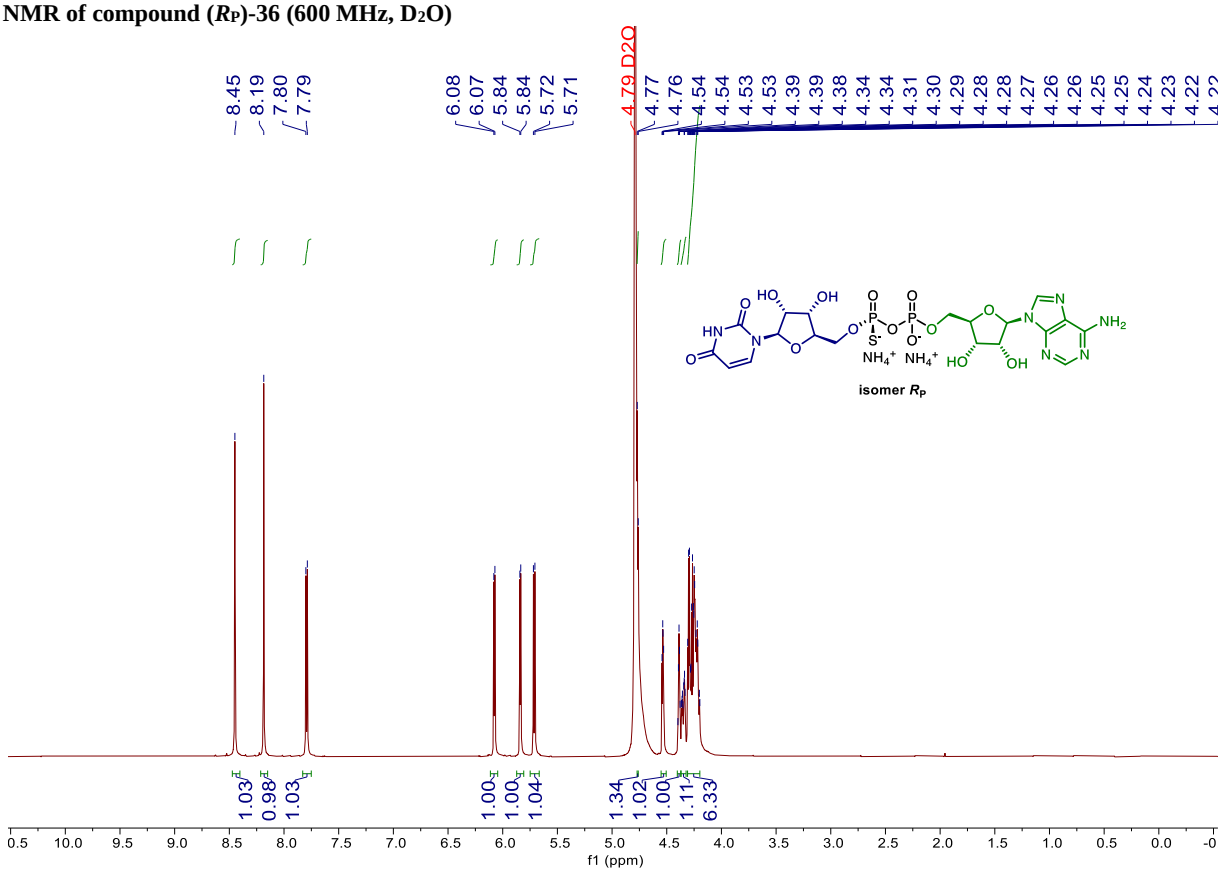
^{19}F NMR of compound (S_P)-35 (376 MHz, D_2O)



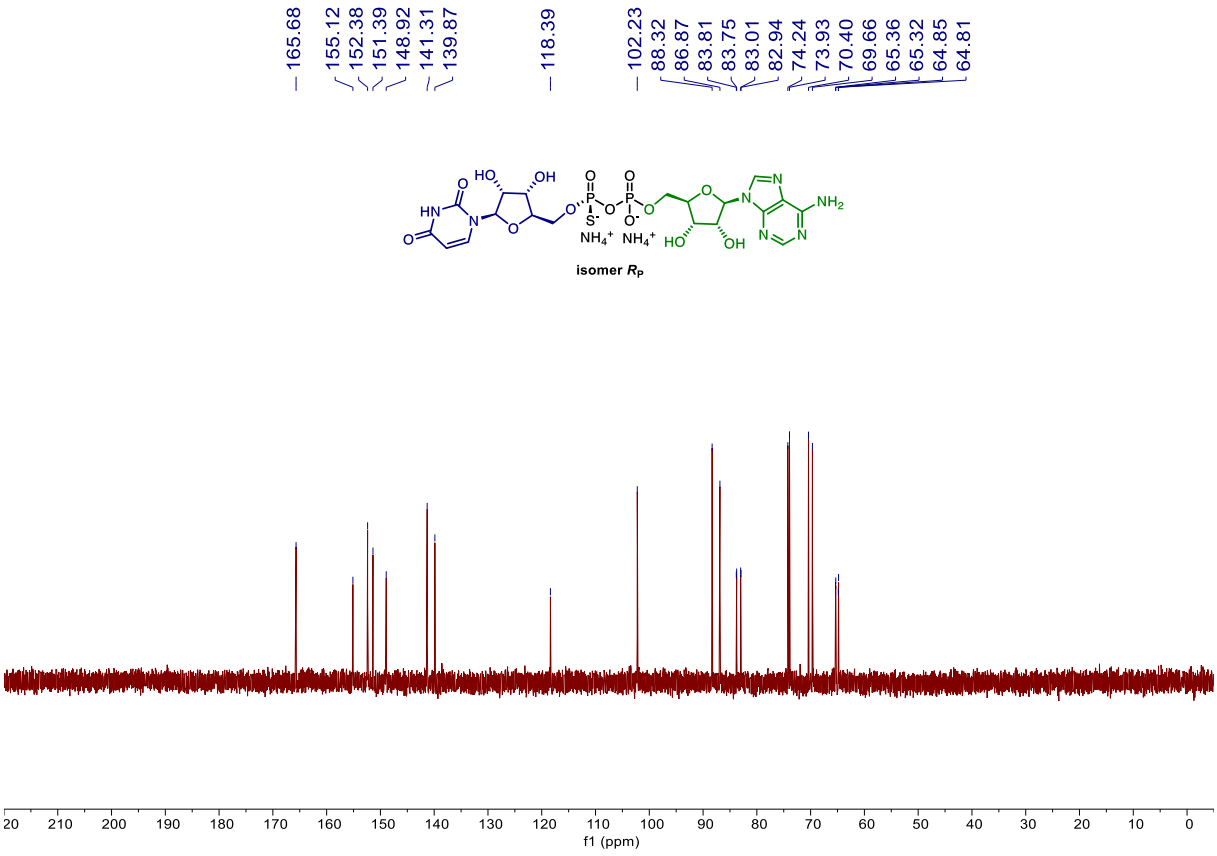
^{31}P NMR of compound (S_P)-35 (162 MHz, D_2O)



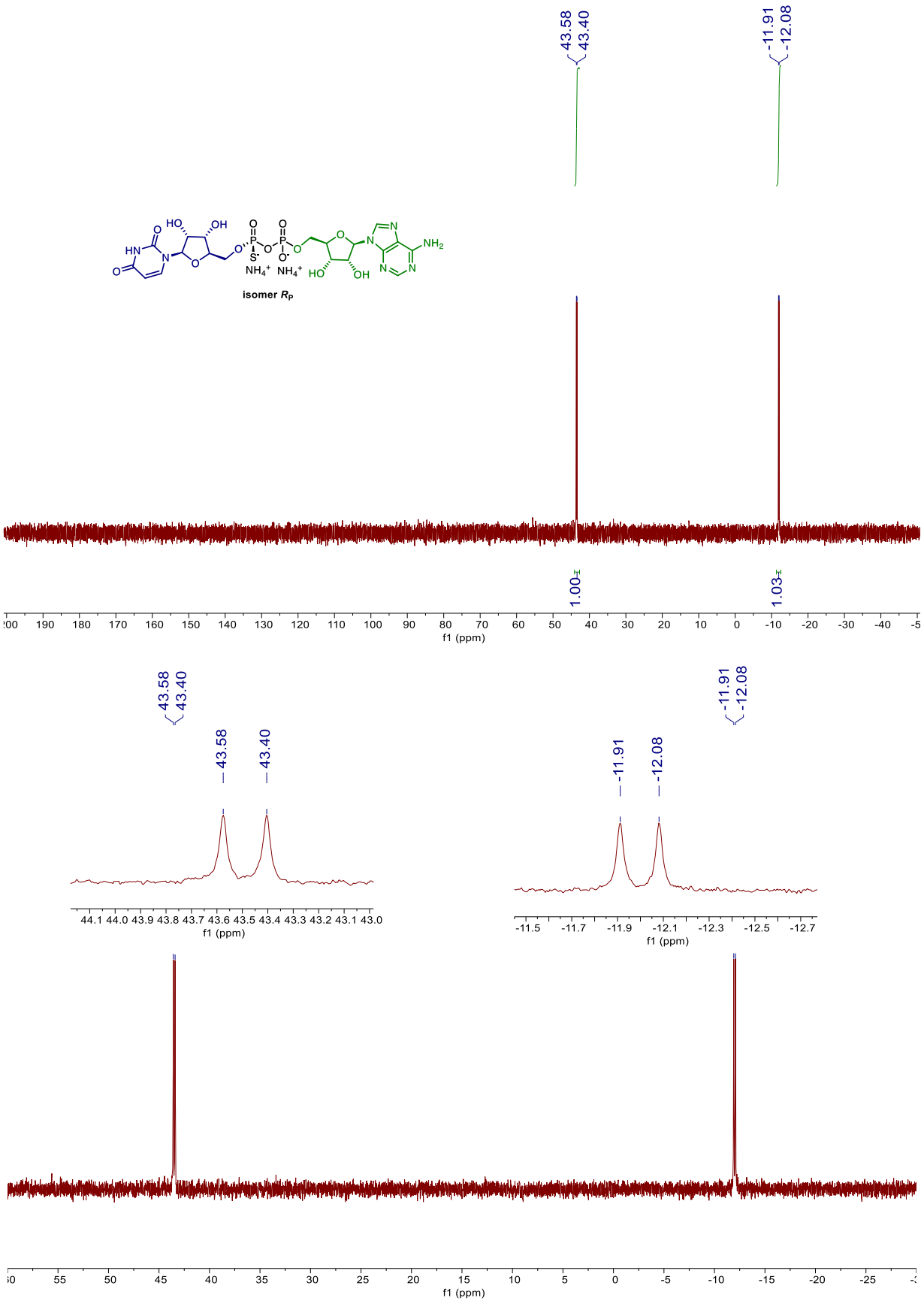
^{1}H NMR of compound (R_P)-36 (600 MHz, D_2O)



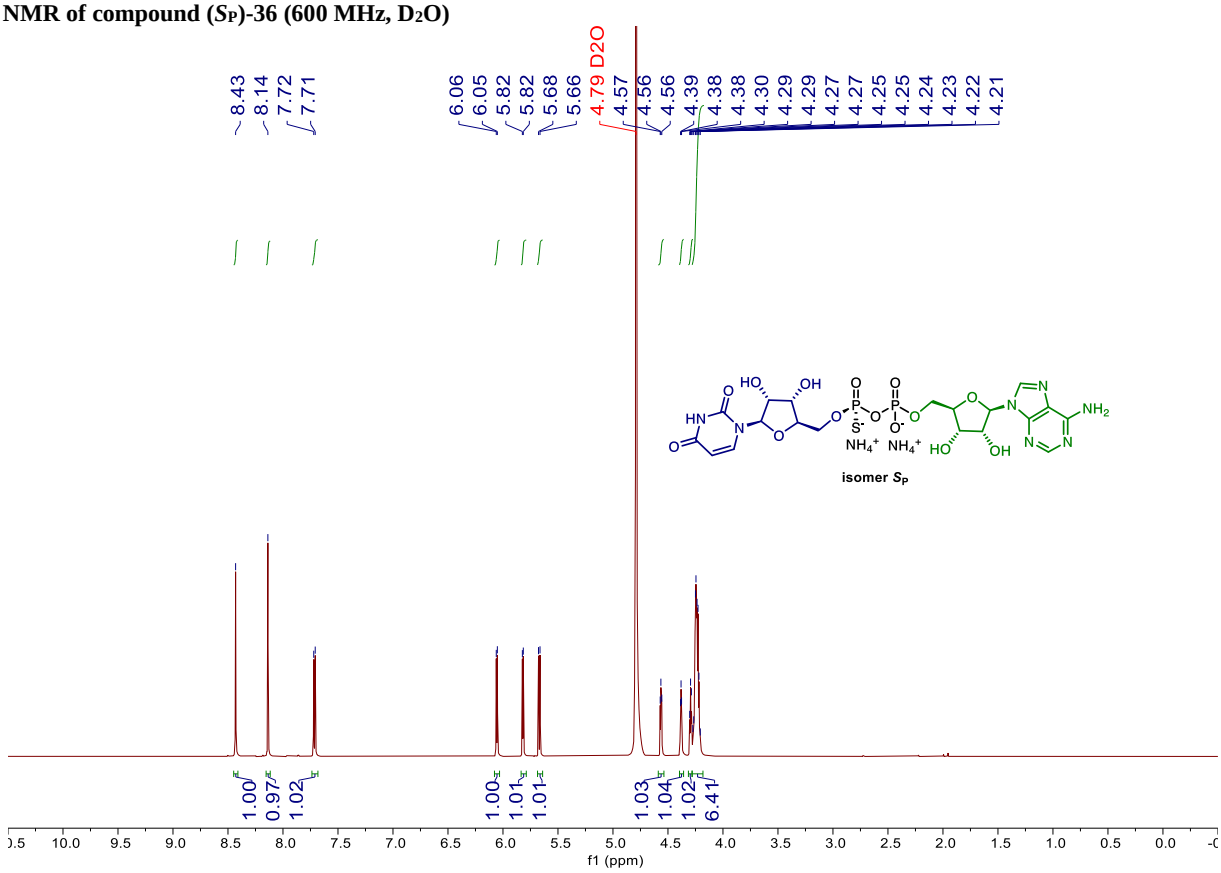
^{13}C NMR of compound (R_P)-36 (150 MHz, D_2O)



^{31}P NMR of compound (R_P)-36 (162 MHz, D_2O)

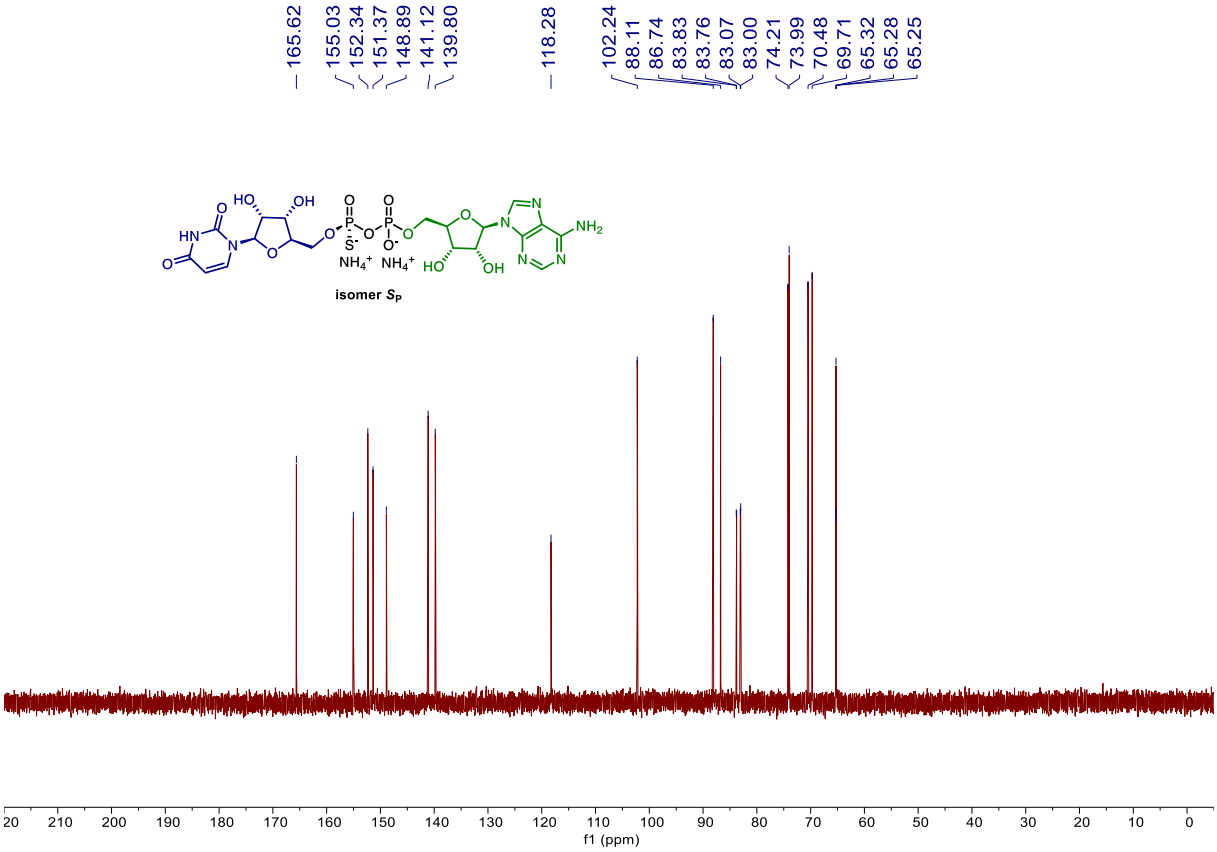


^{1}H NMR of compound (S_P)-36 (600 MHz, D_2O)



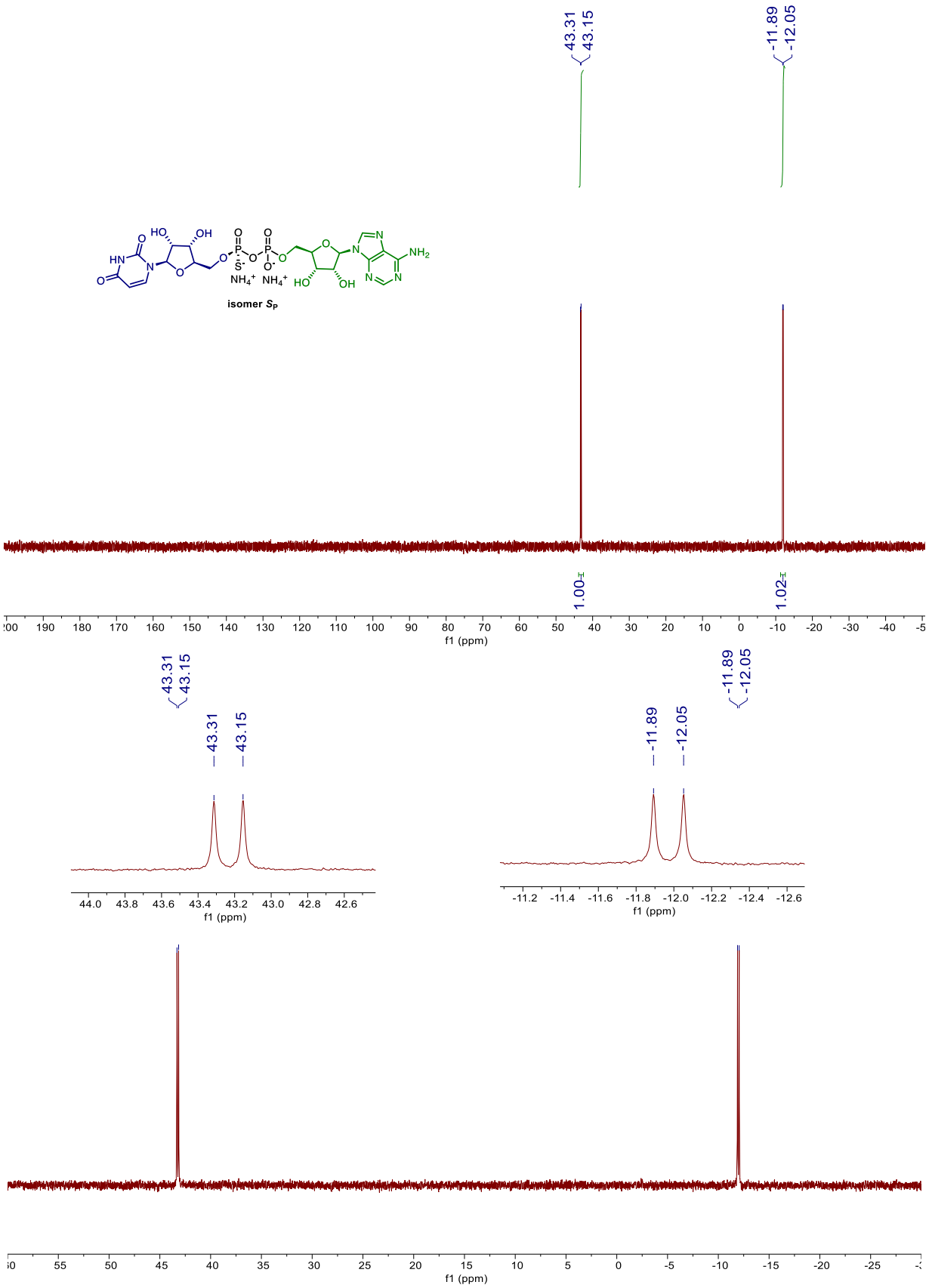
isomer S_P

^{13}C NMR of compound (S_P)-36 (150 MHz, D_2O)

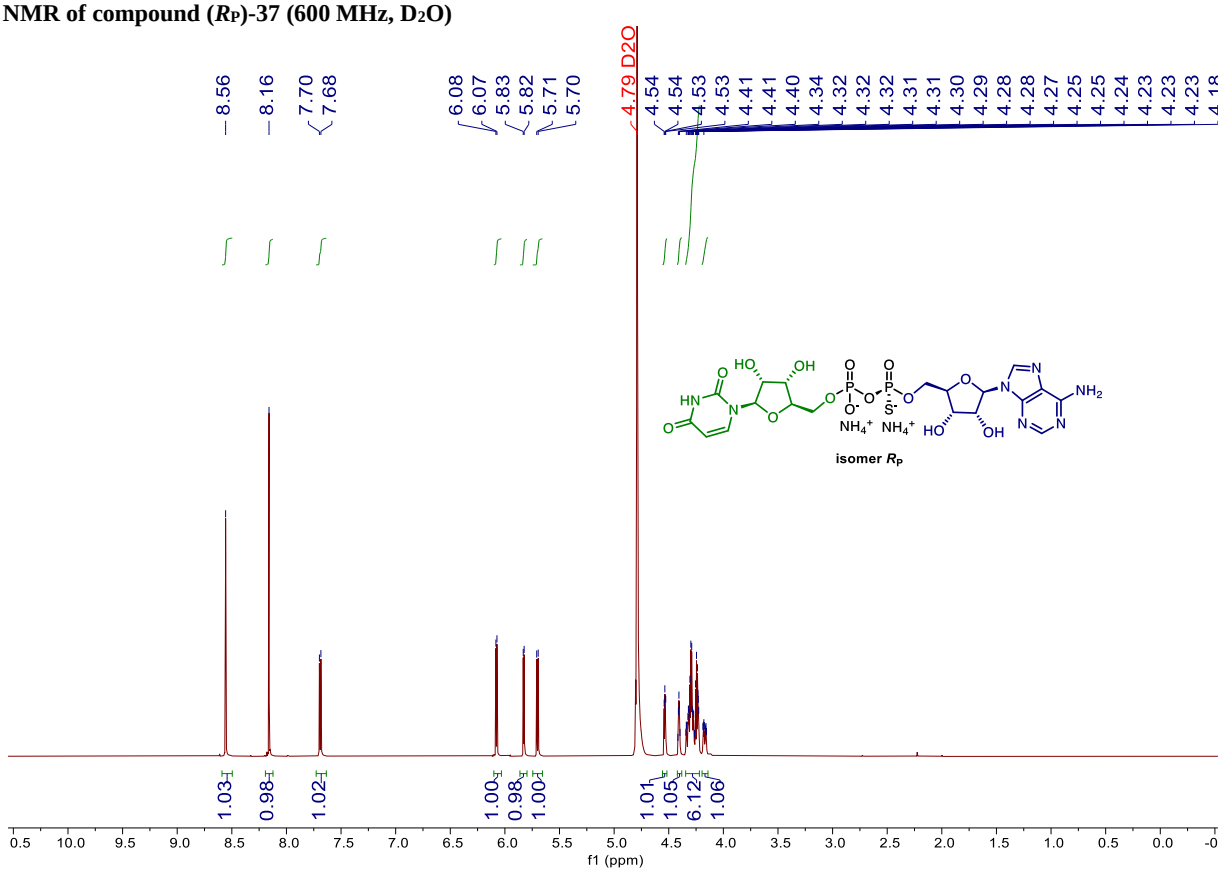


isomer S_P

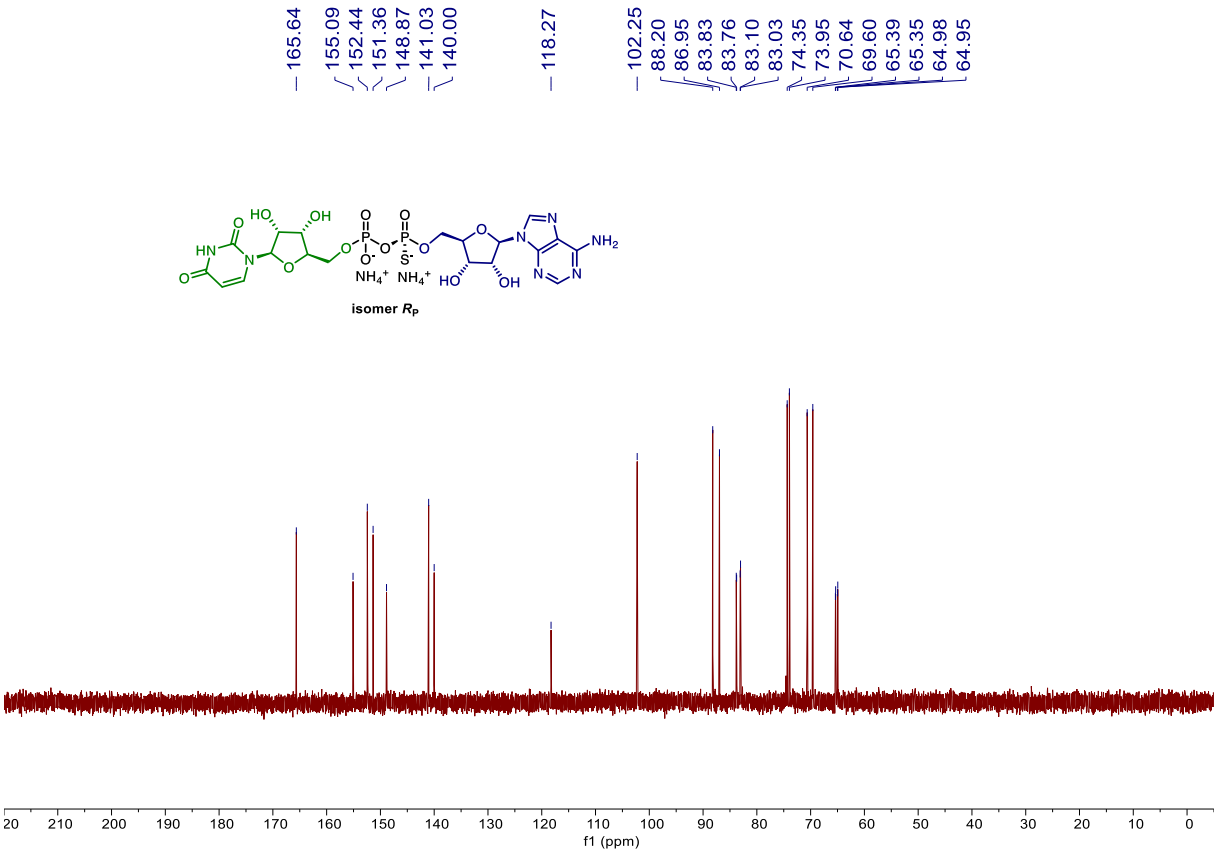
^{31}P NMR of compound (S_P)-36 (162 MHz, D_2O)



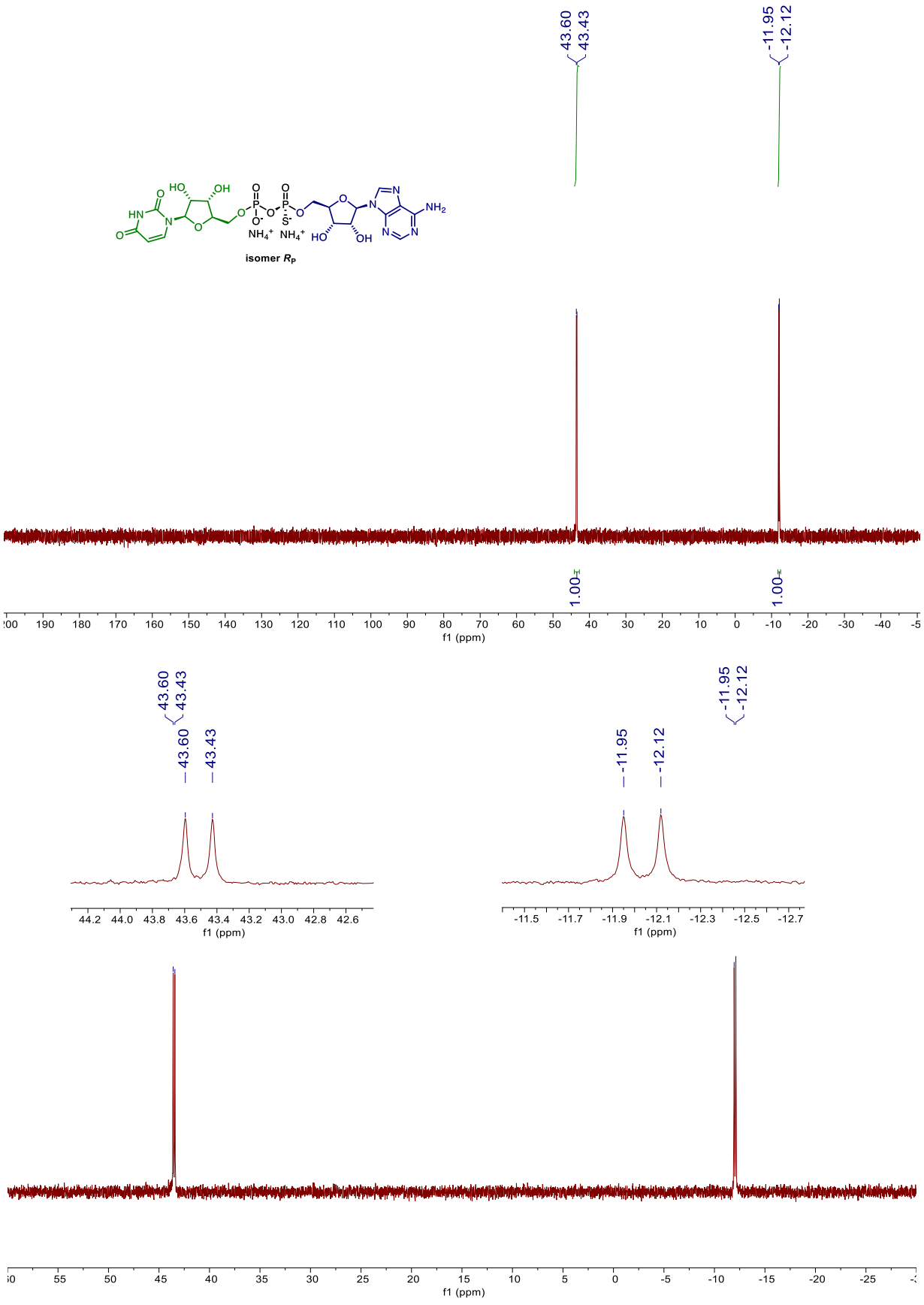
^{1}H NMR of compound (R_P)-37 (600 MHz, D_2O)



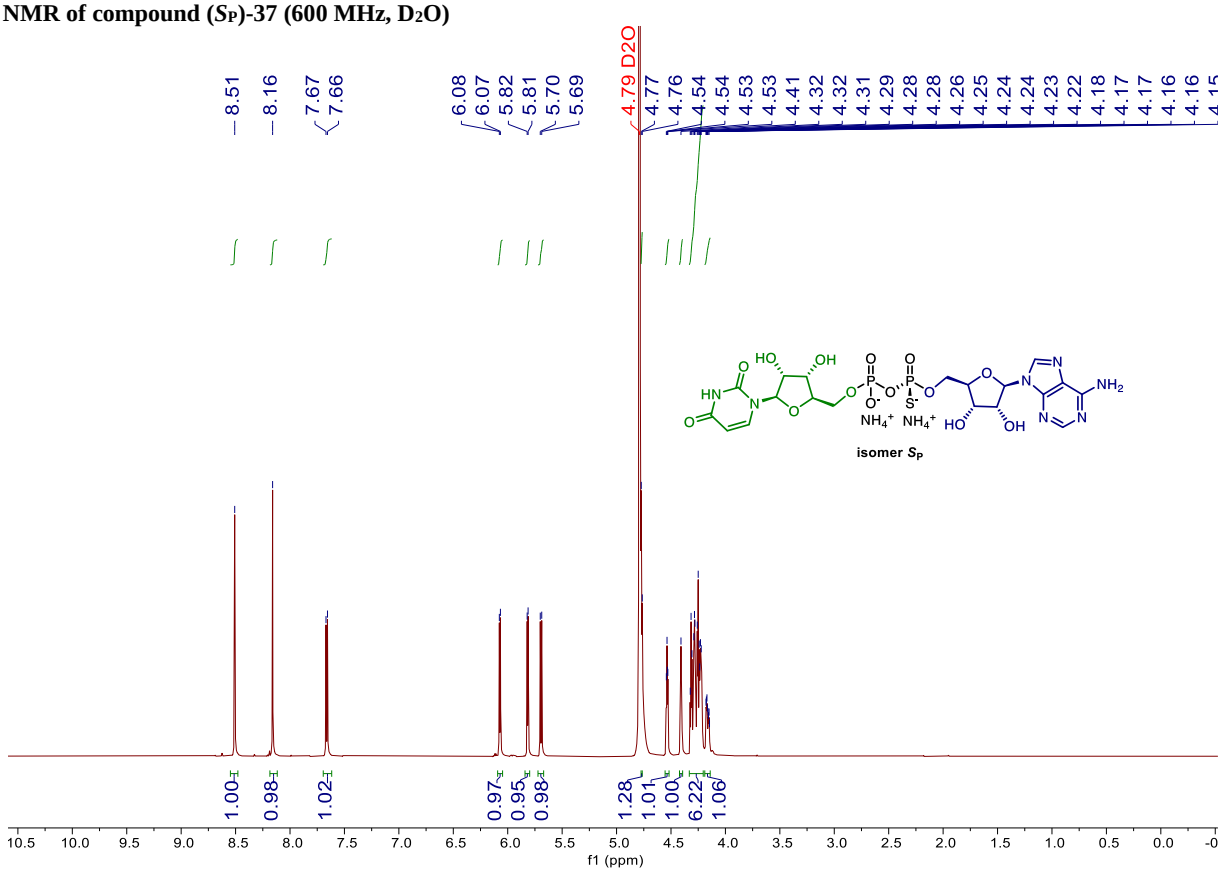
^{13}C NMR of compound (R_P)-37 (150 MHz, D_2O)



^{31}P NMR of compound (R_P)-37 (162 MHz, D_2O)

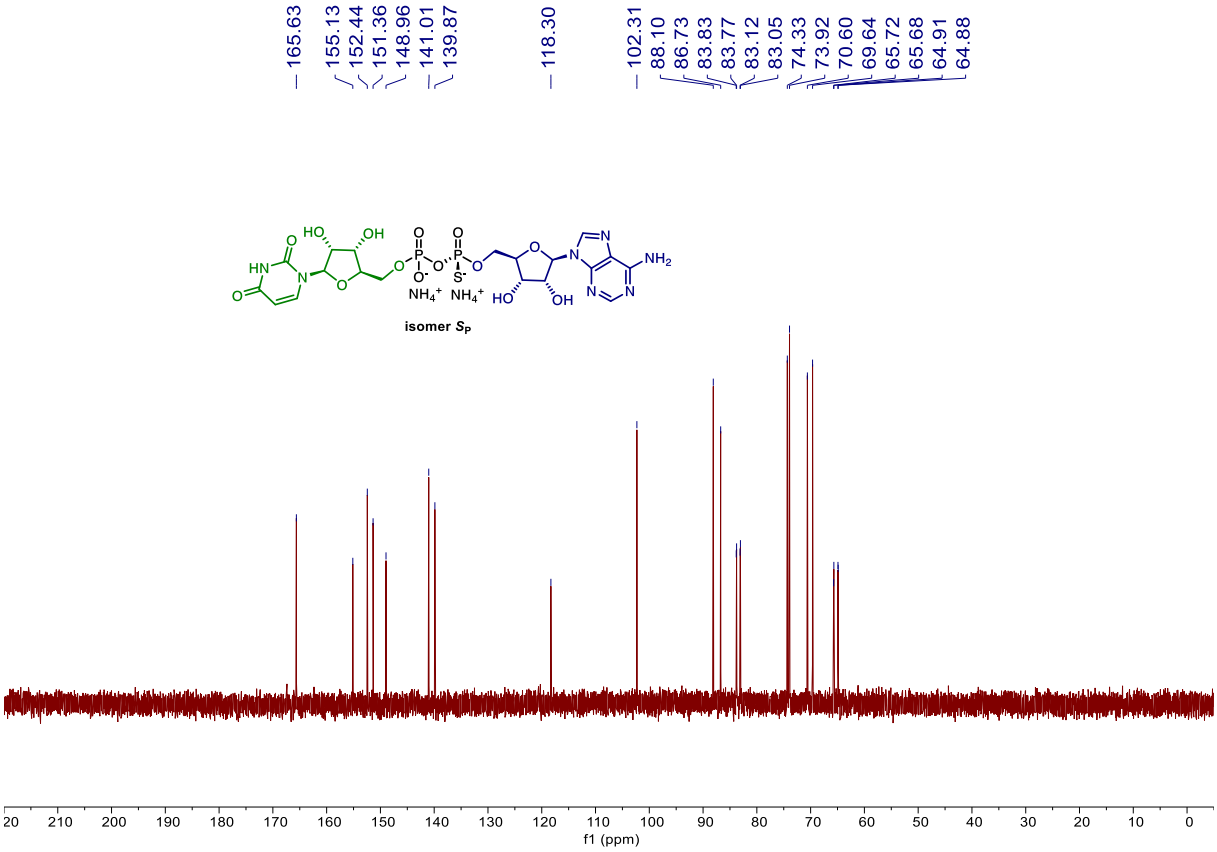


^{1}H NMR of compound (S_P)-37 (600 MHz, D_2O)



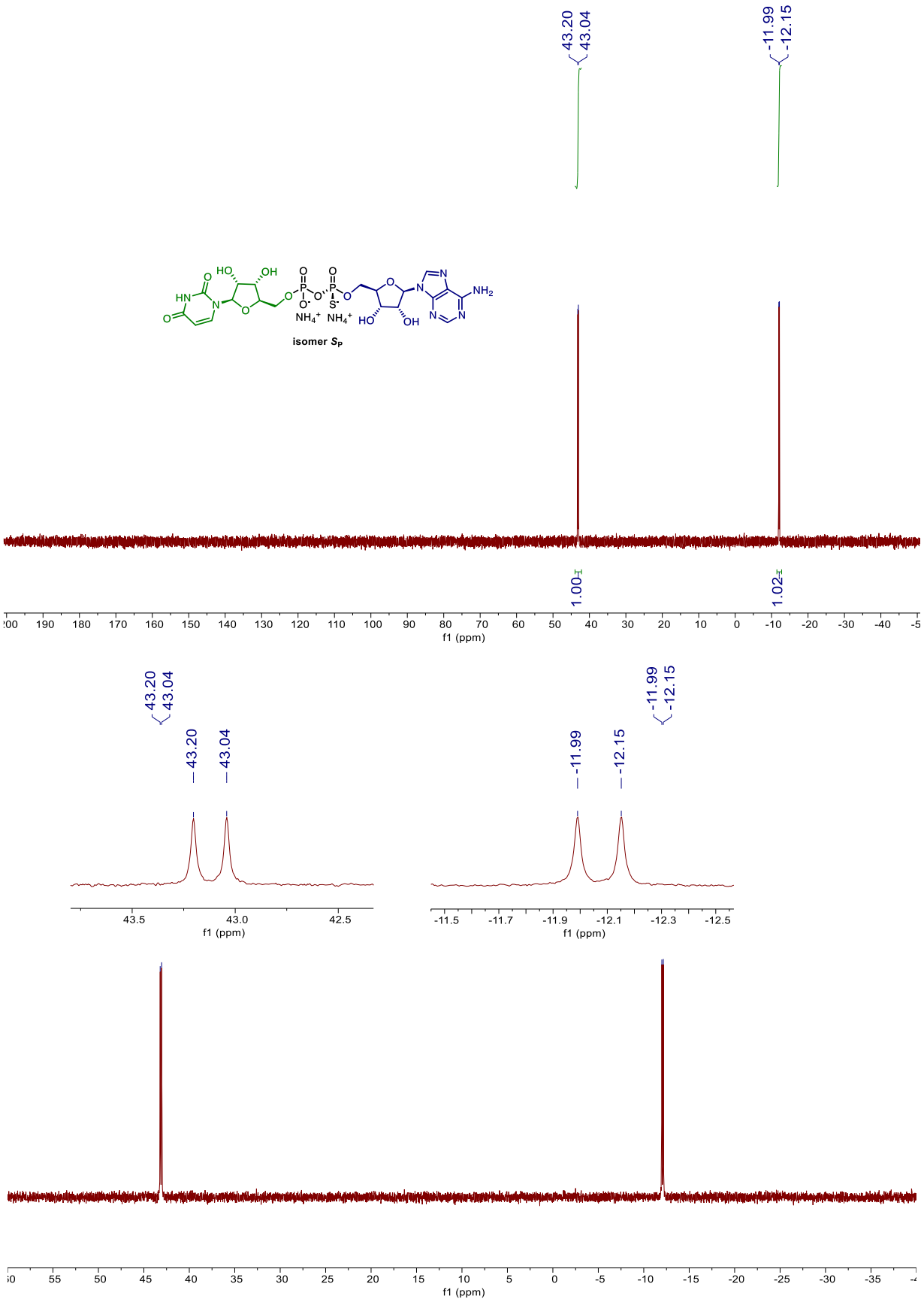
isomer S_P

^{13}C NMR of compound (S_P)-37 (150 MHz, D_2O)

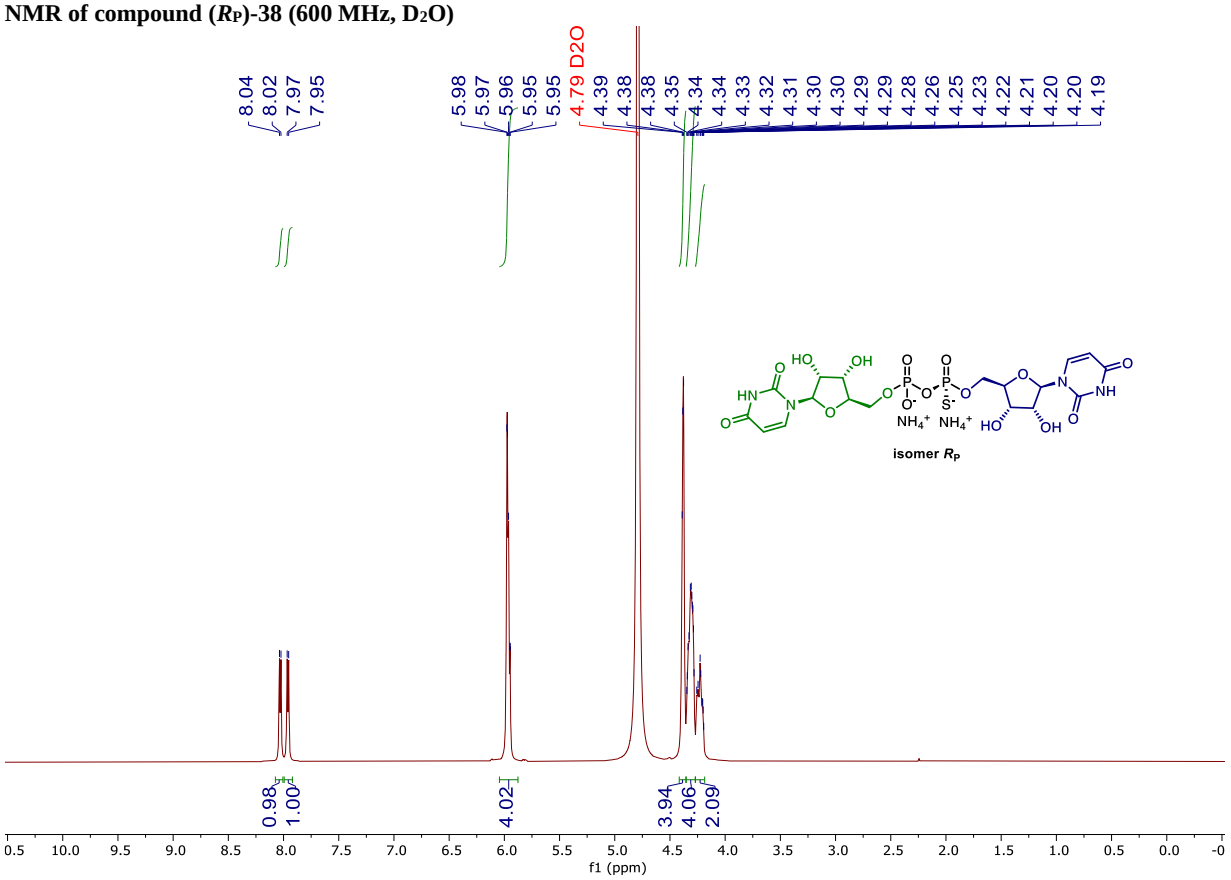


isomer S_P

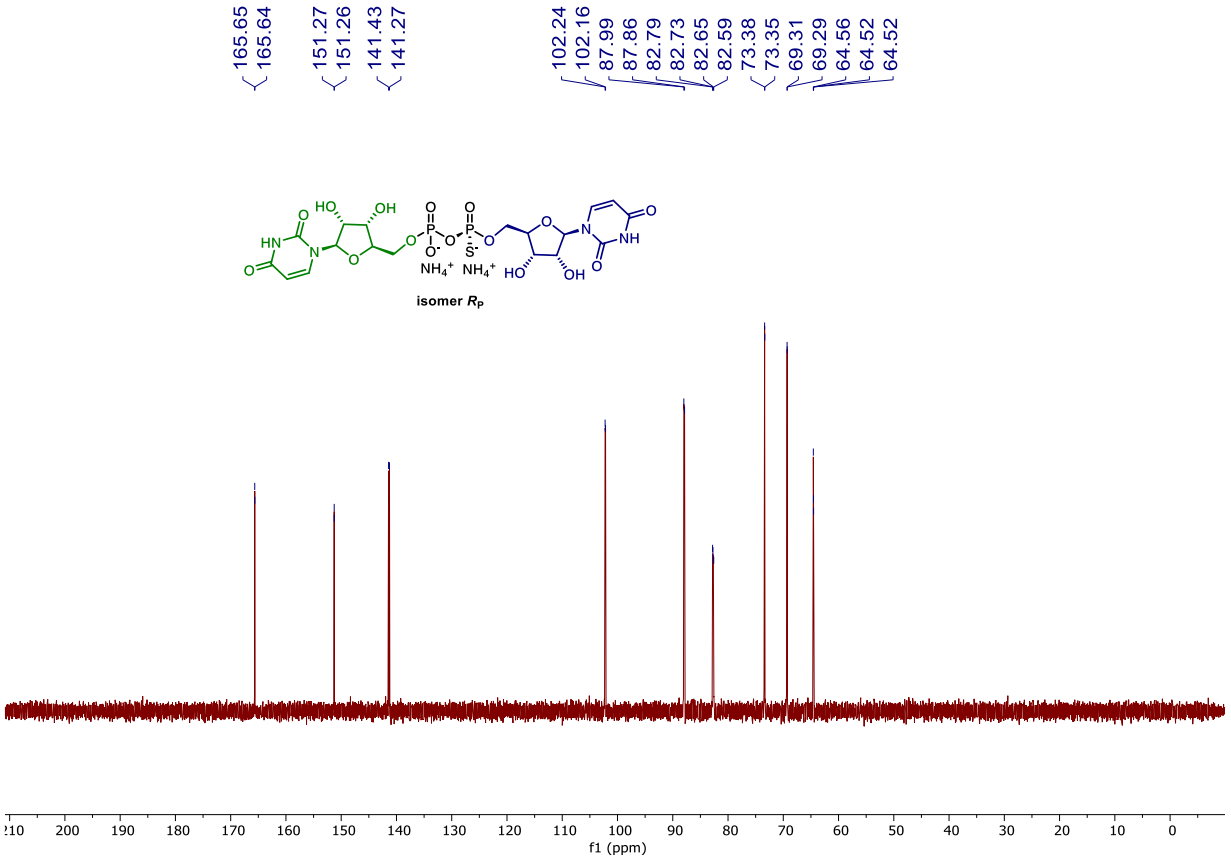
^{31}P NMR of compound (S_P)-37 (162 MHz, D_2O)



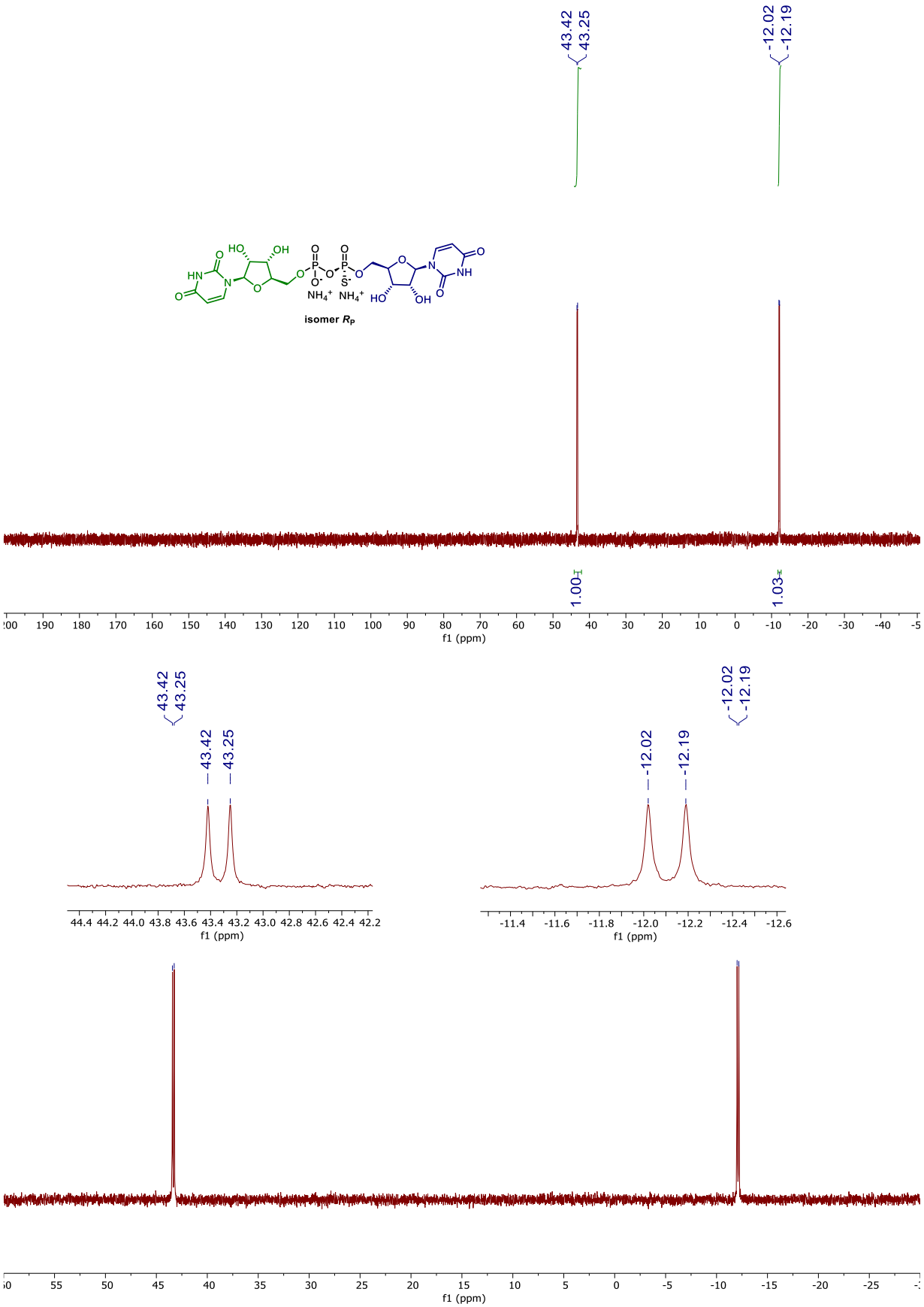
^{1}H NMR of compound (R_P)-38 (600 MHz, D_2O)



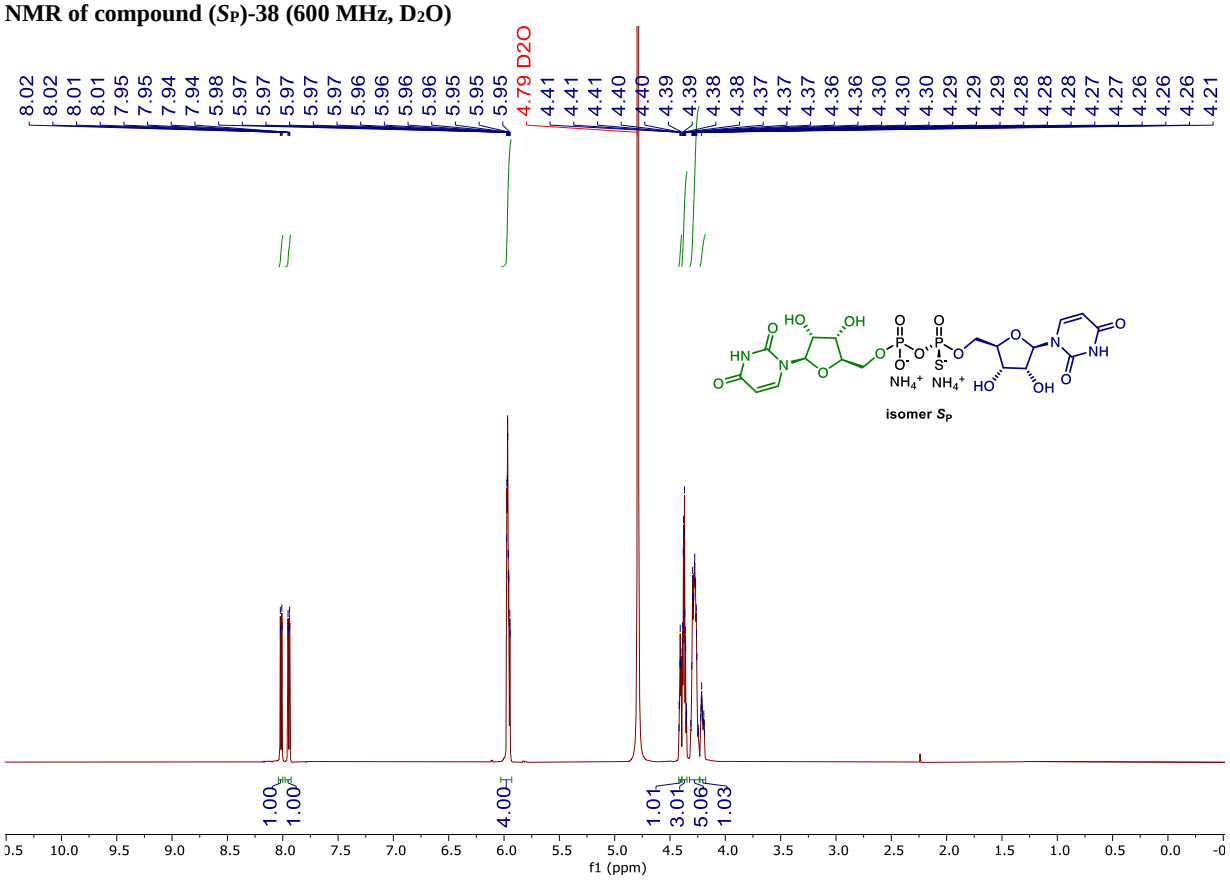
^{13}C NMR of compound (R_P)-38 (150 MHz, D_2O)



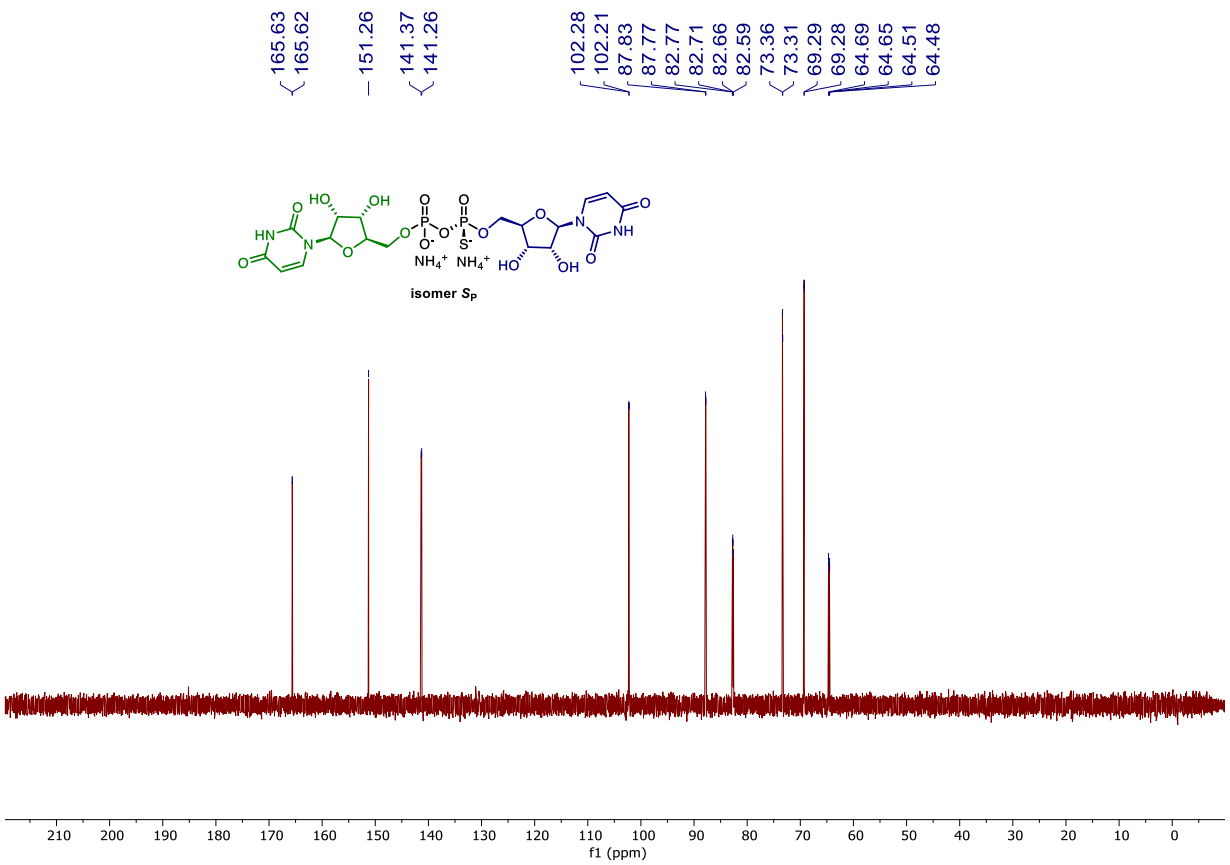
^{31}P NMR of compound (R_P)-38 (162 MHz, D_2O)



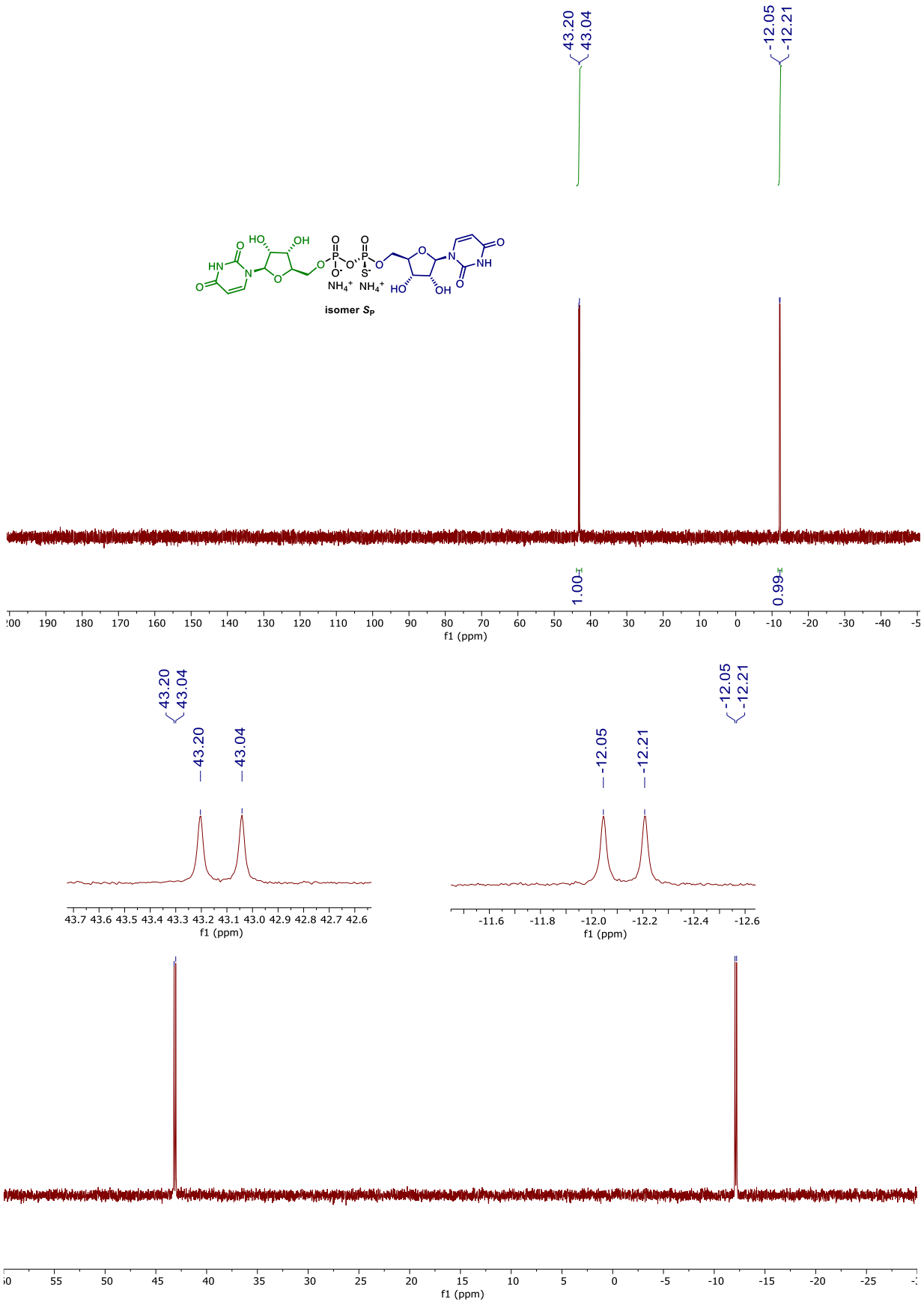
^{1}H NMR of compound (S_P)-38 (600 MHz, D_2O)



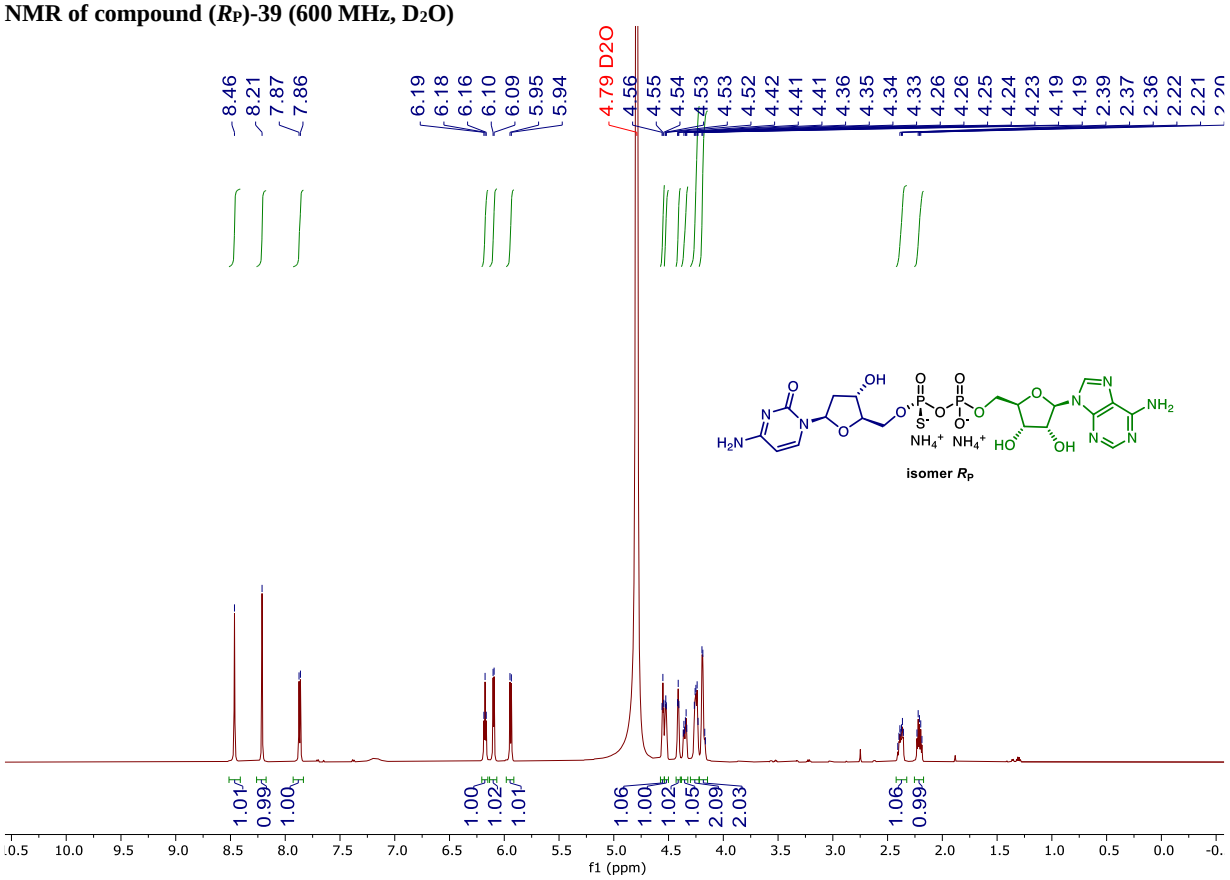
^{13}C NMR of compound (S_P)-38 (150 MHz, D_2O)



^{31}P NMR of compound (S_P)-38 (162 MHz, D_2O)

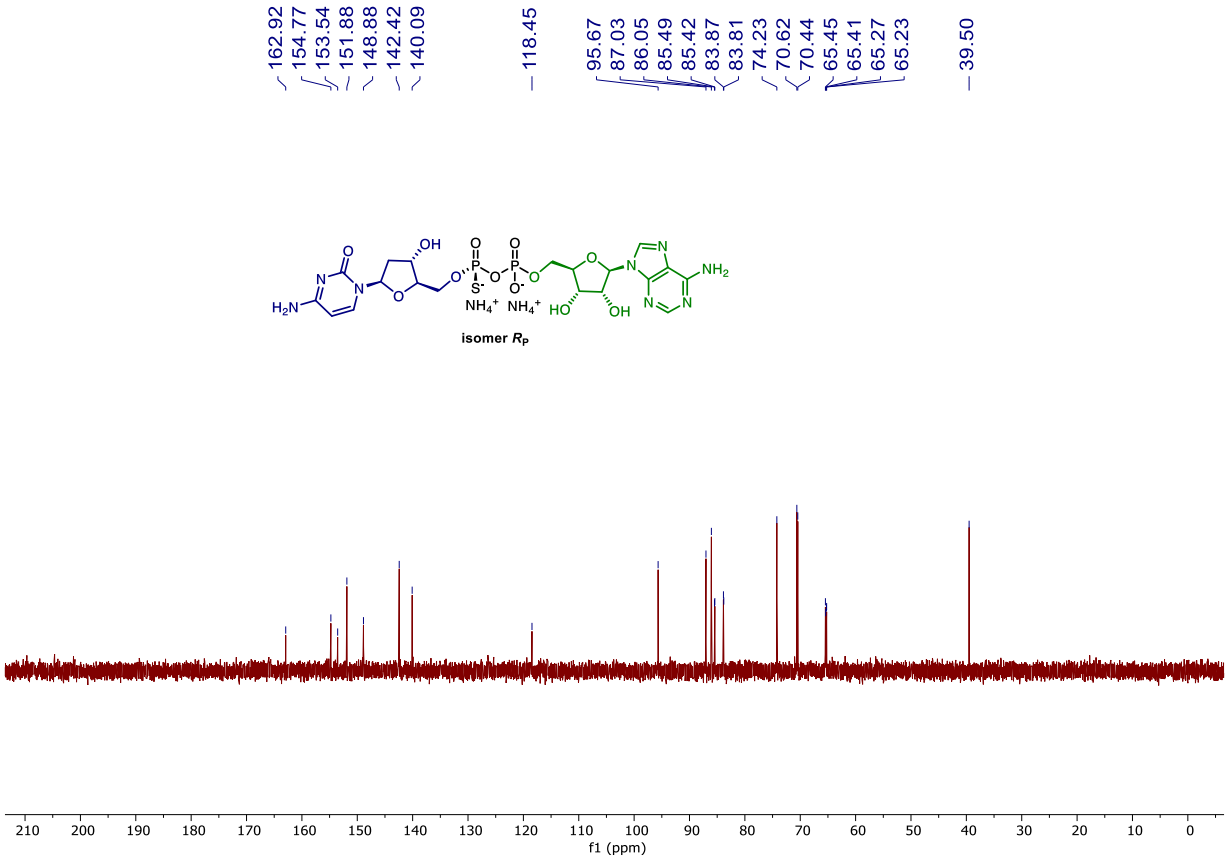


^{1}H NMR of compound (R_P)-39 (600 MHz, D_2O)



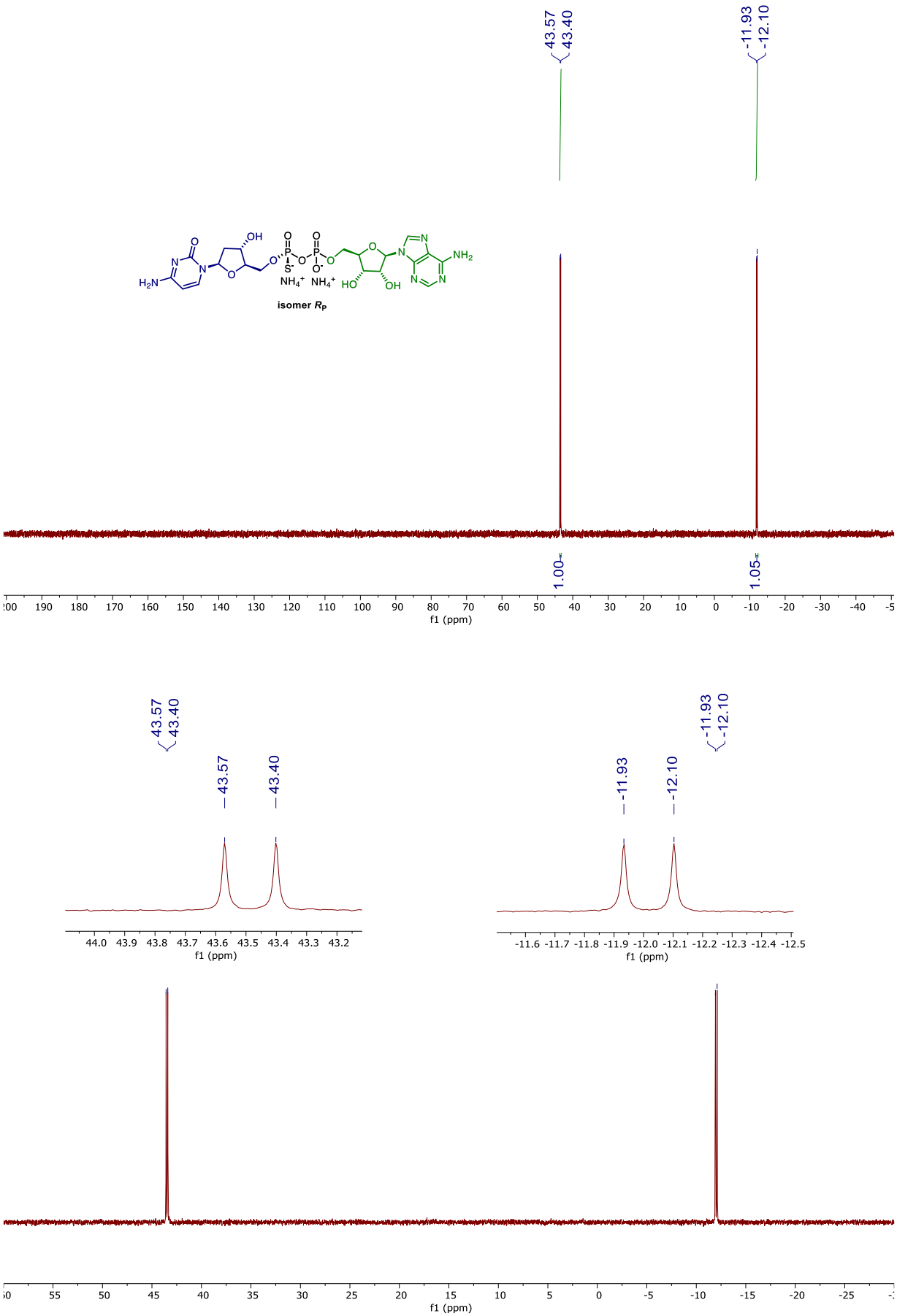
isomer R_P

^{13}C NMR of compound (R_P)-39 (150 MHz, D_2O)

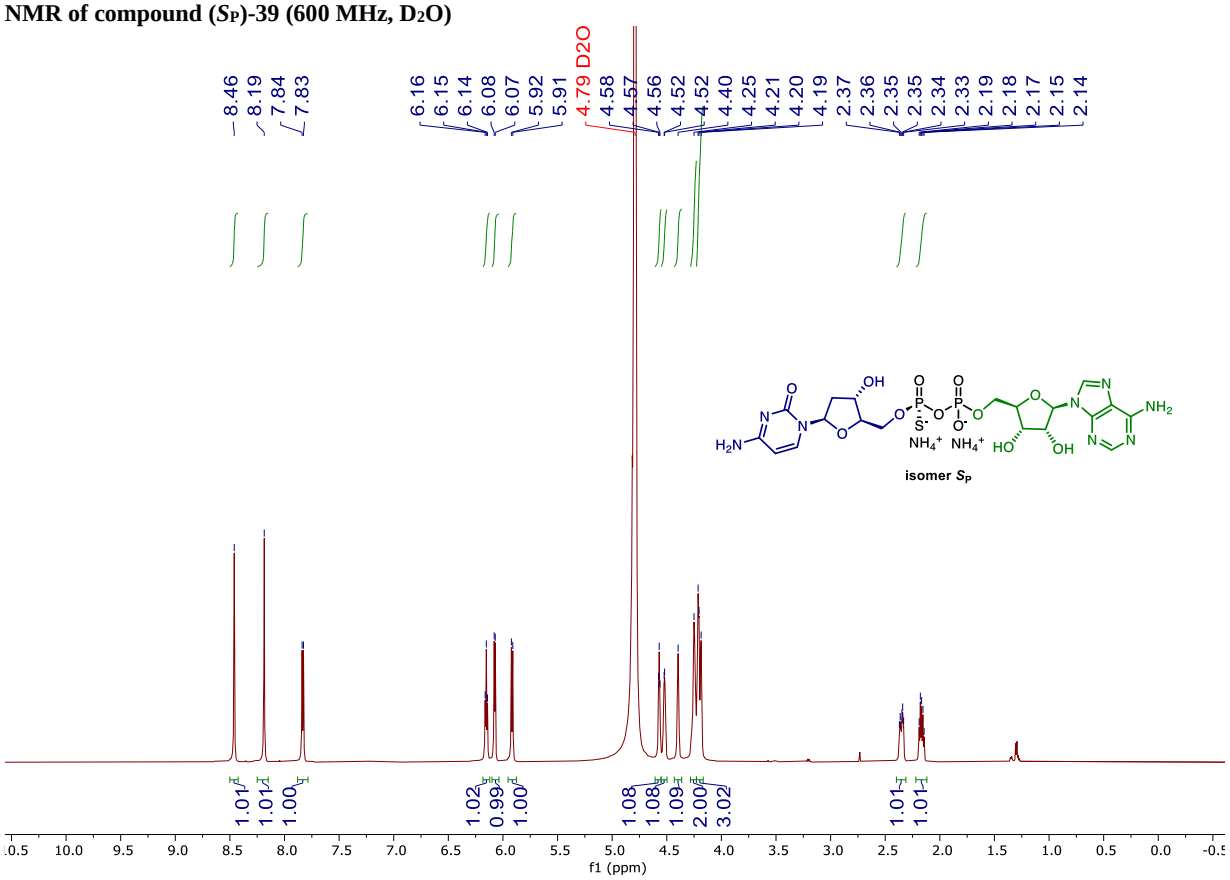


isomer R_P

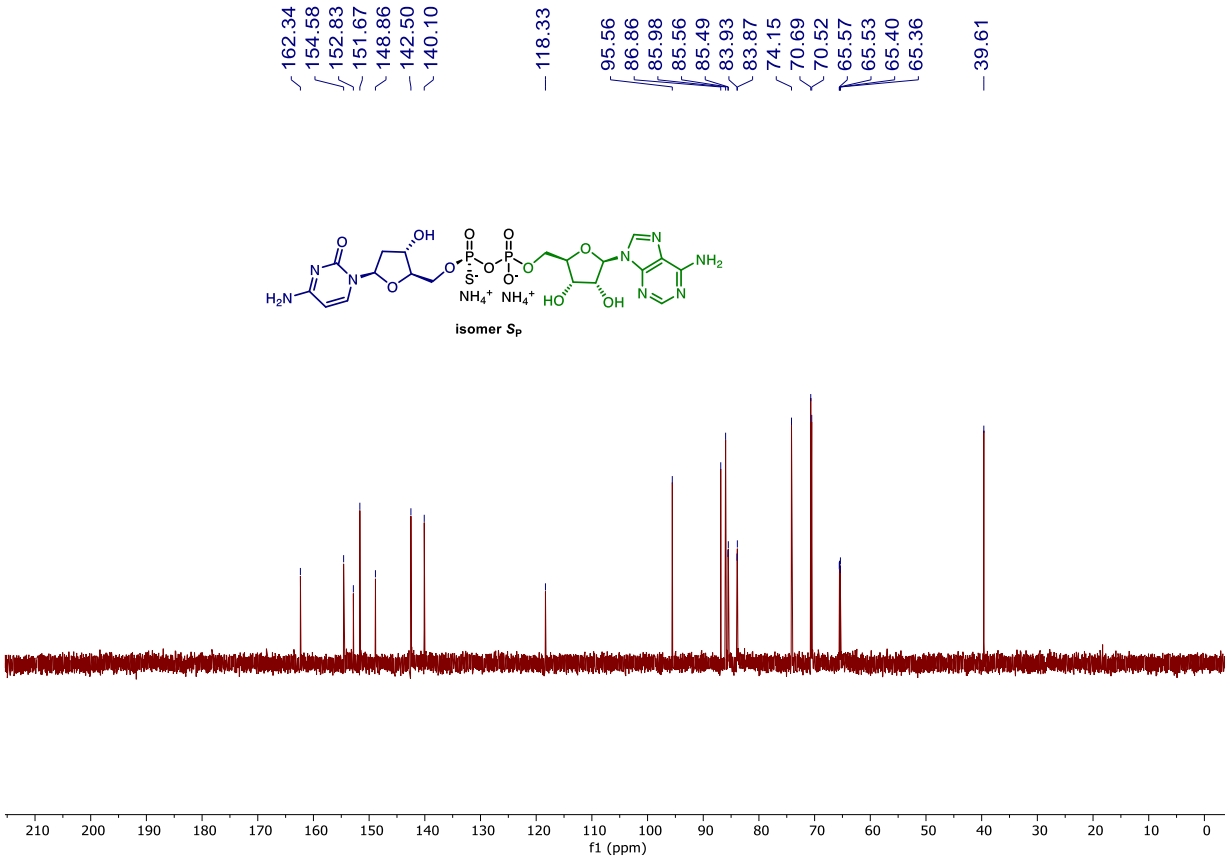
^{31}P NMR of compound (R_P)-39 (162 MHz, D_2O)



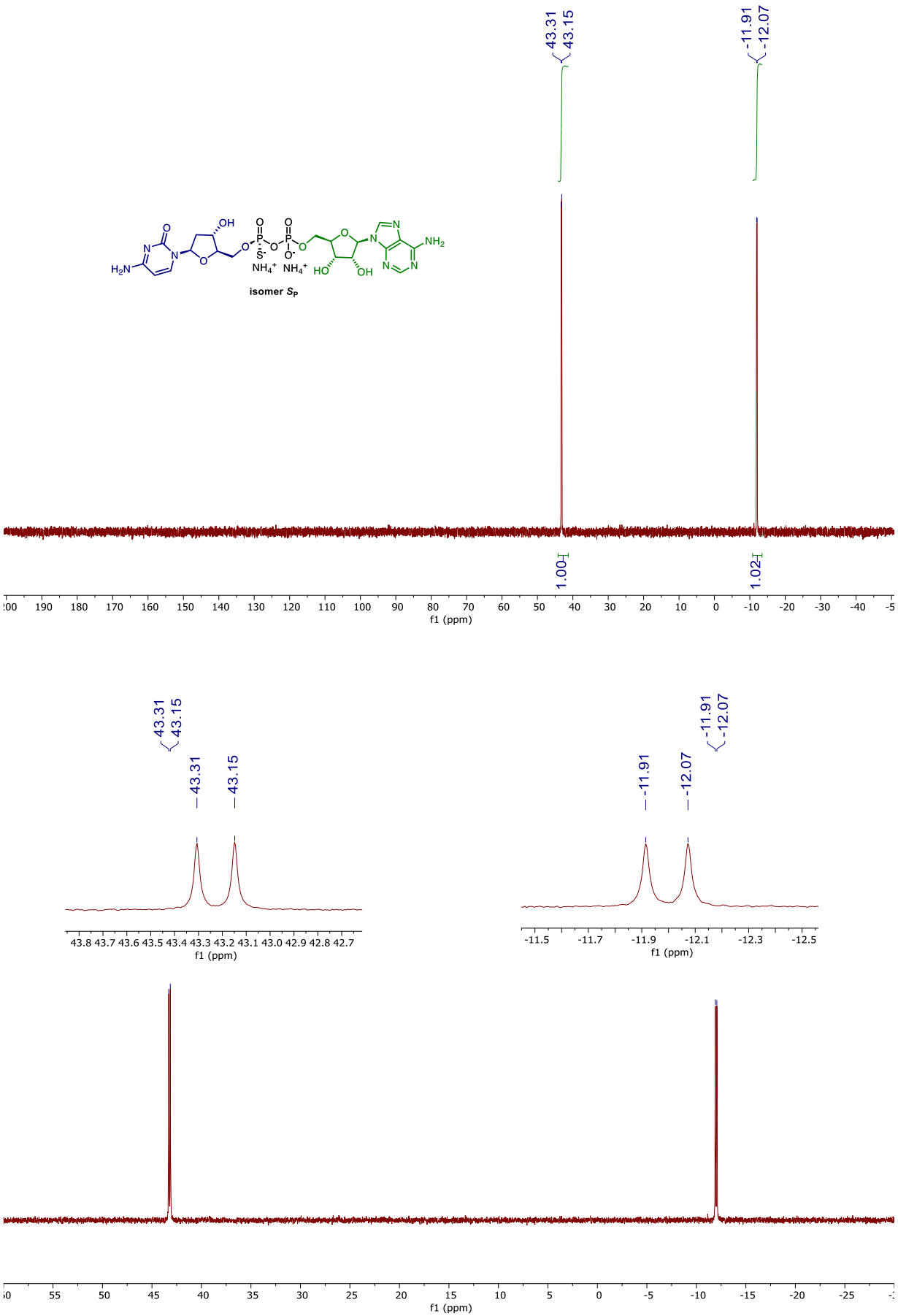
^{1}H NMR of compound (S_P)-39 (600 MHz, D_2O)



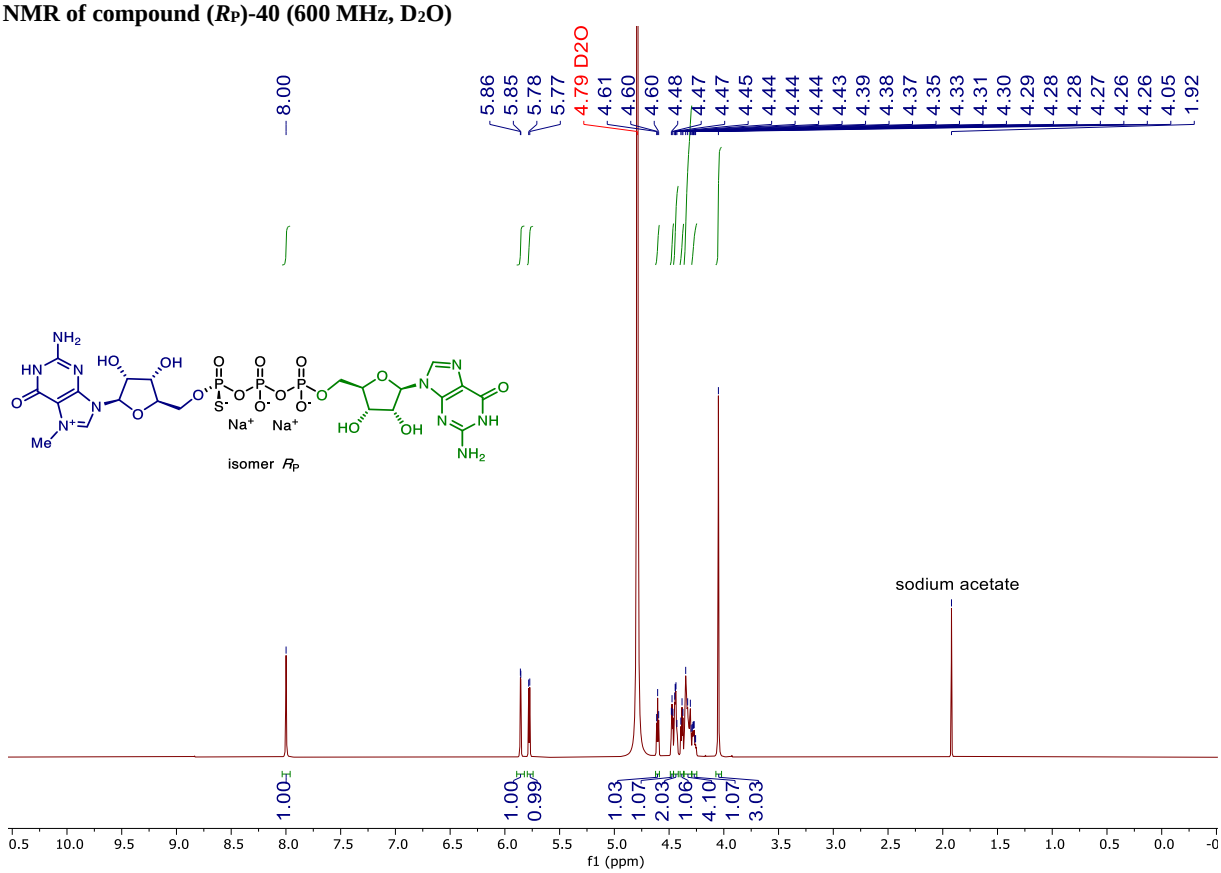
^{13}C NMR of compound (S_P)-39 (150 MHz, D_2O)



^{31}P NMR of compound (S_P)-39 (162 MHz, D_2O)

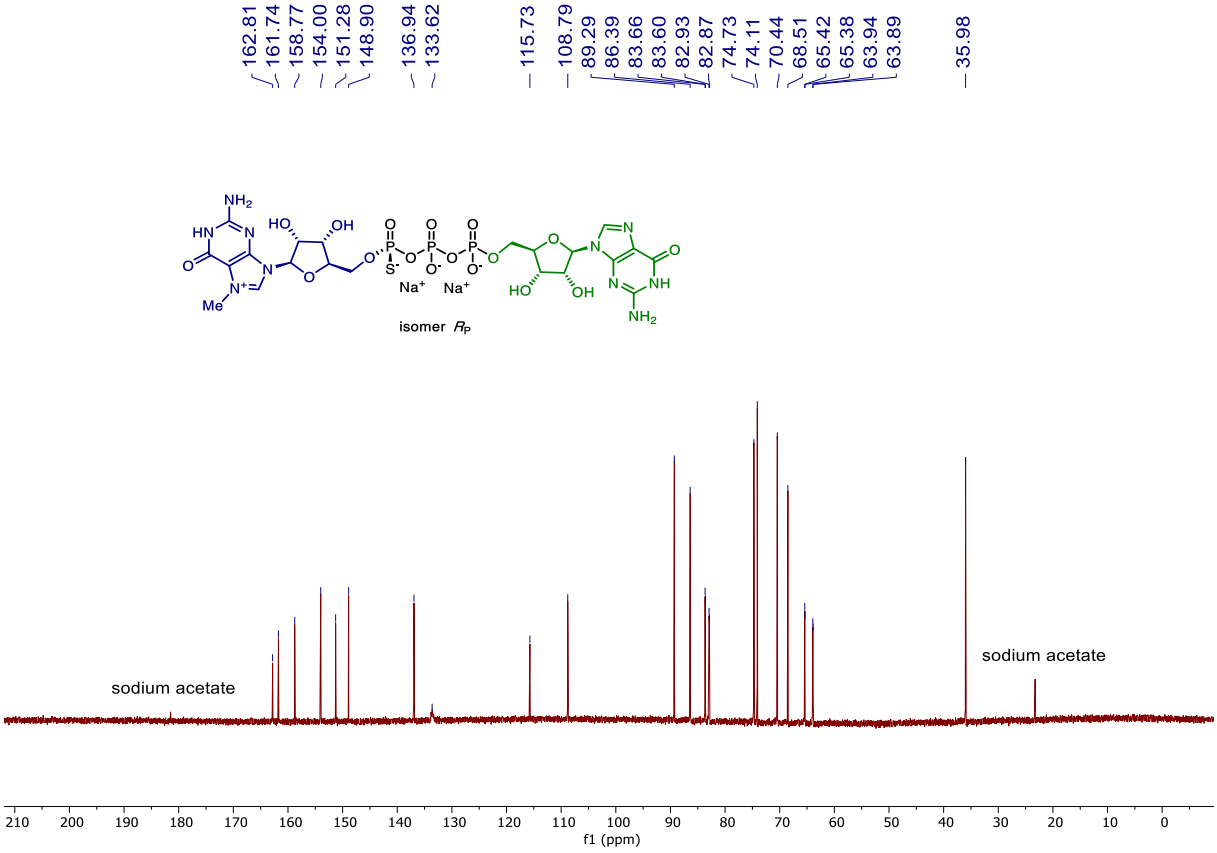


^{1}H NMR of compound (R_P)-40 (600 MHz, D_2O)



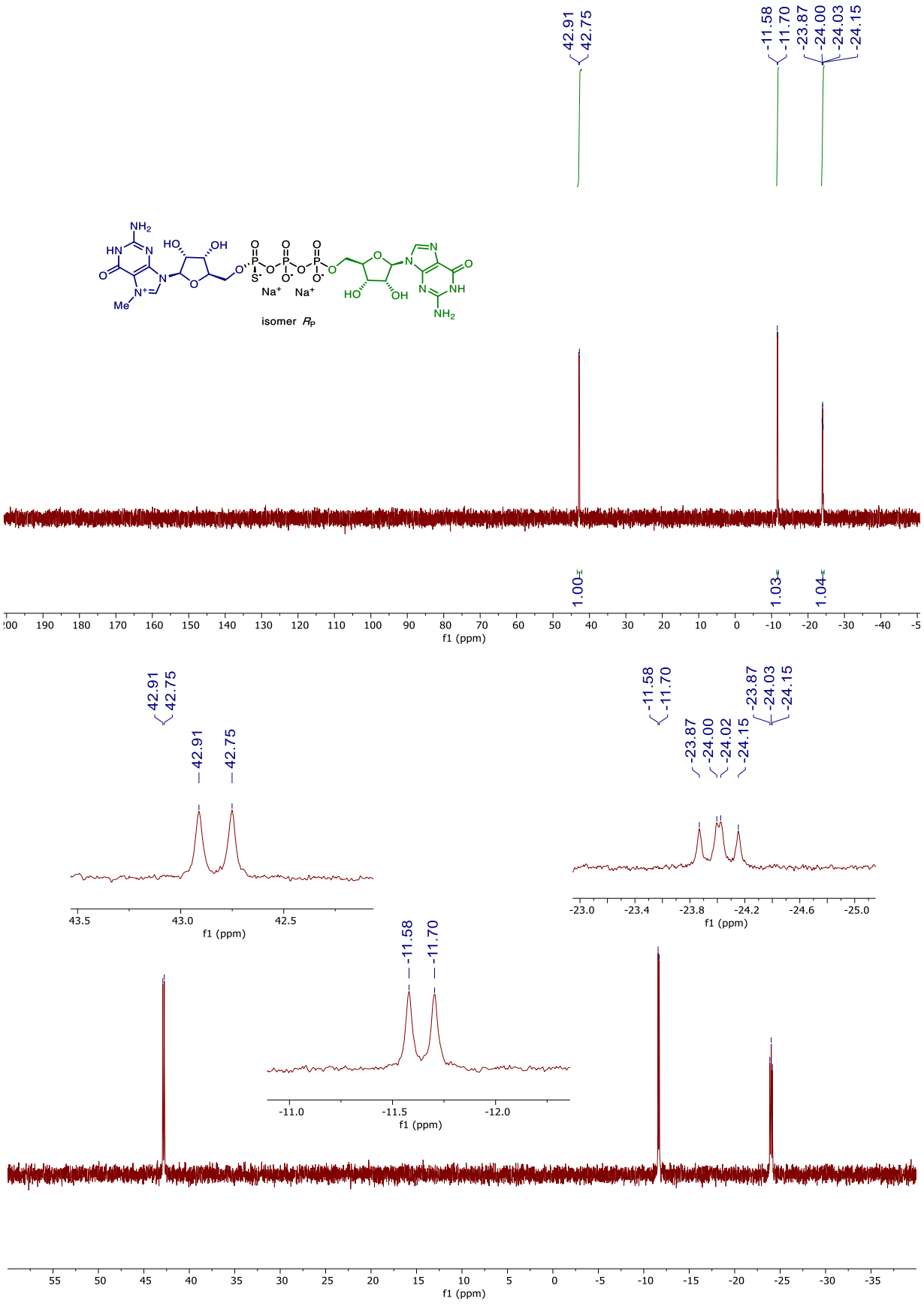
isomer R_P

^{13}C NMR of compound (R_P)-40 (150 MHz, D_2O)

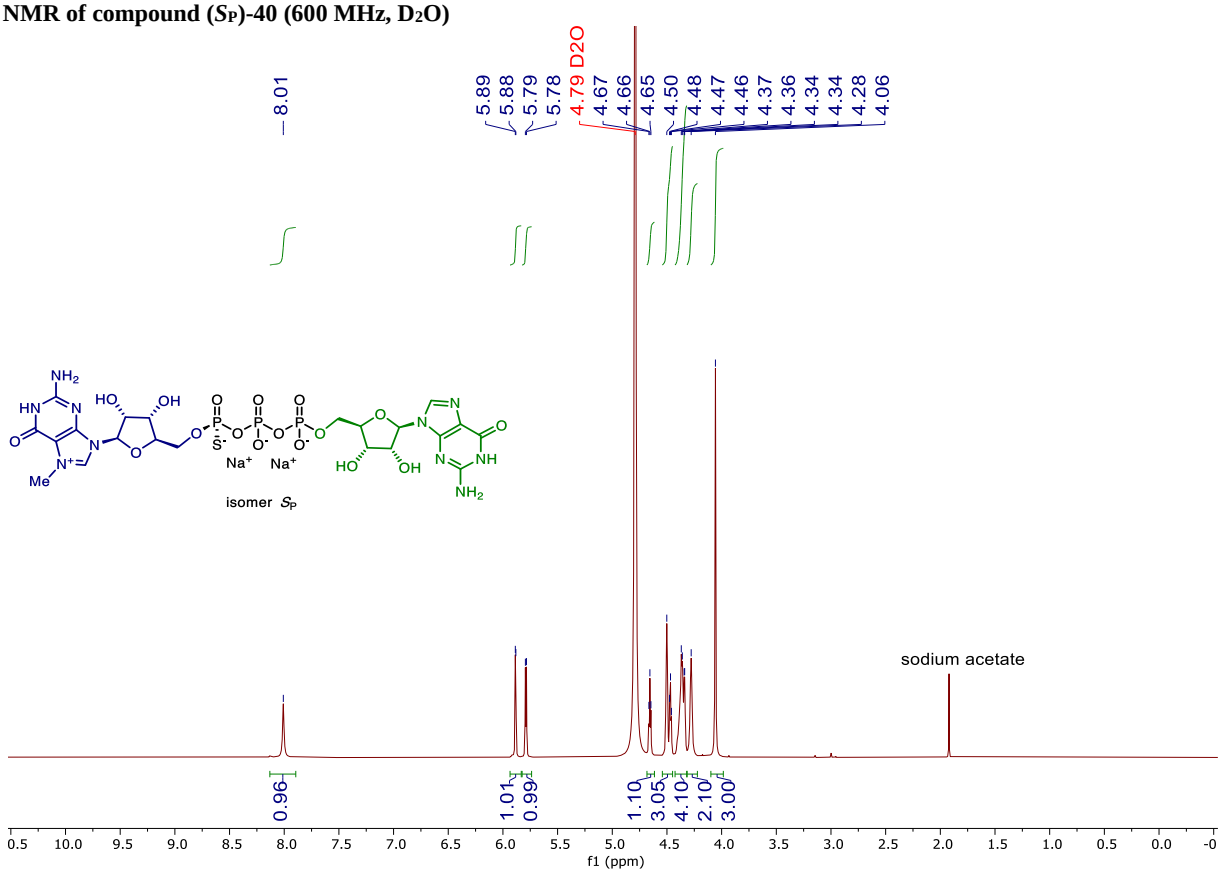


isomer R_P

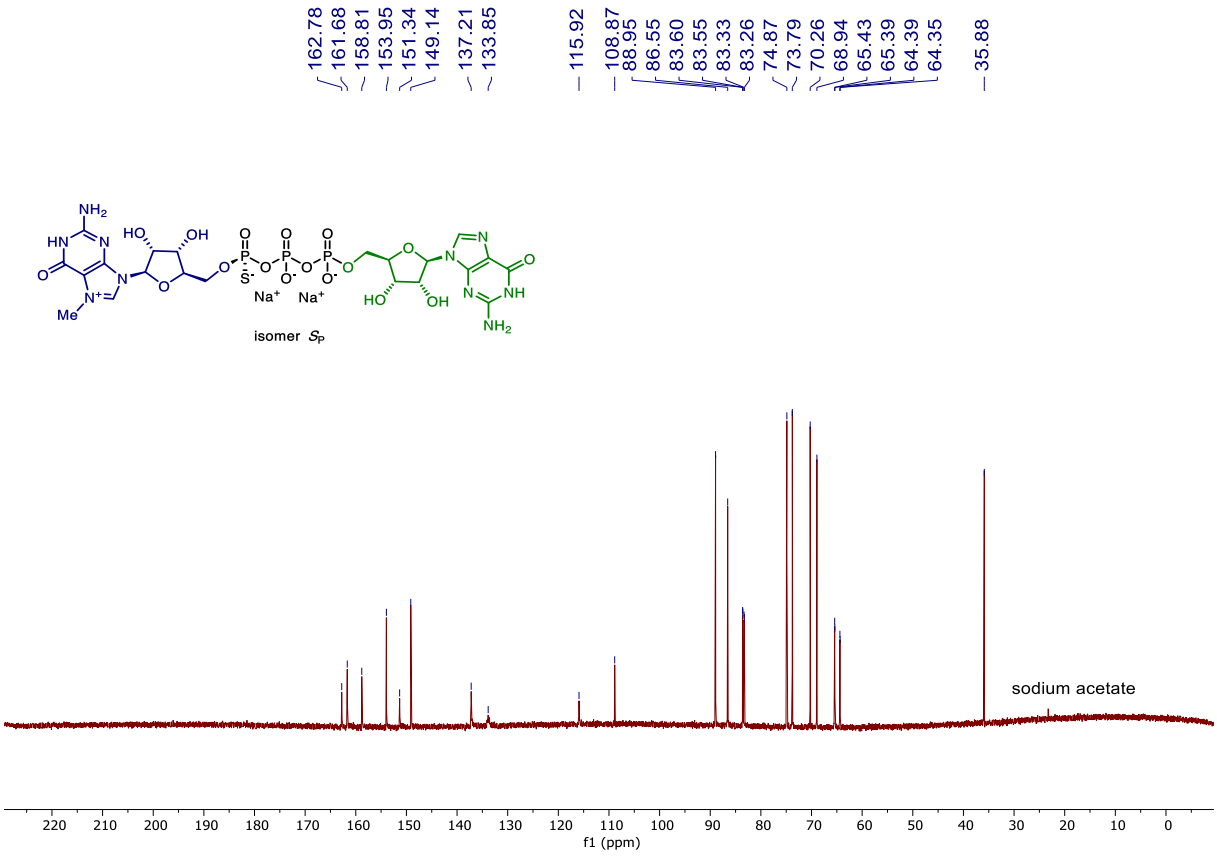
^{31}P NMR of compound (R_P)-40 (162 MHz, D_2O)



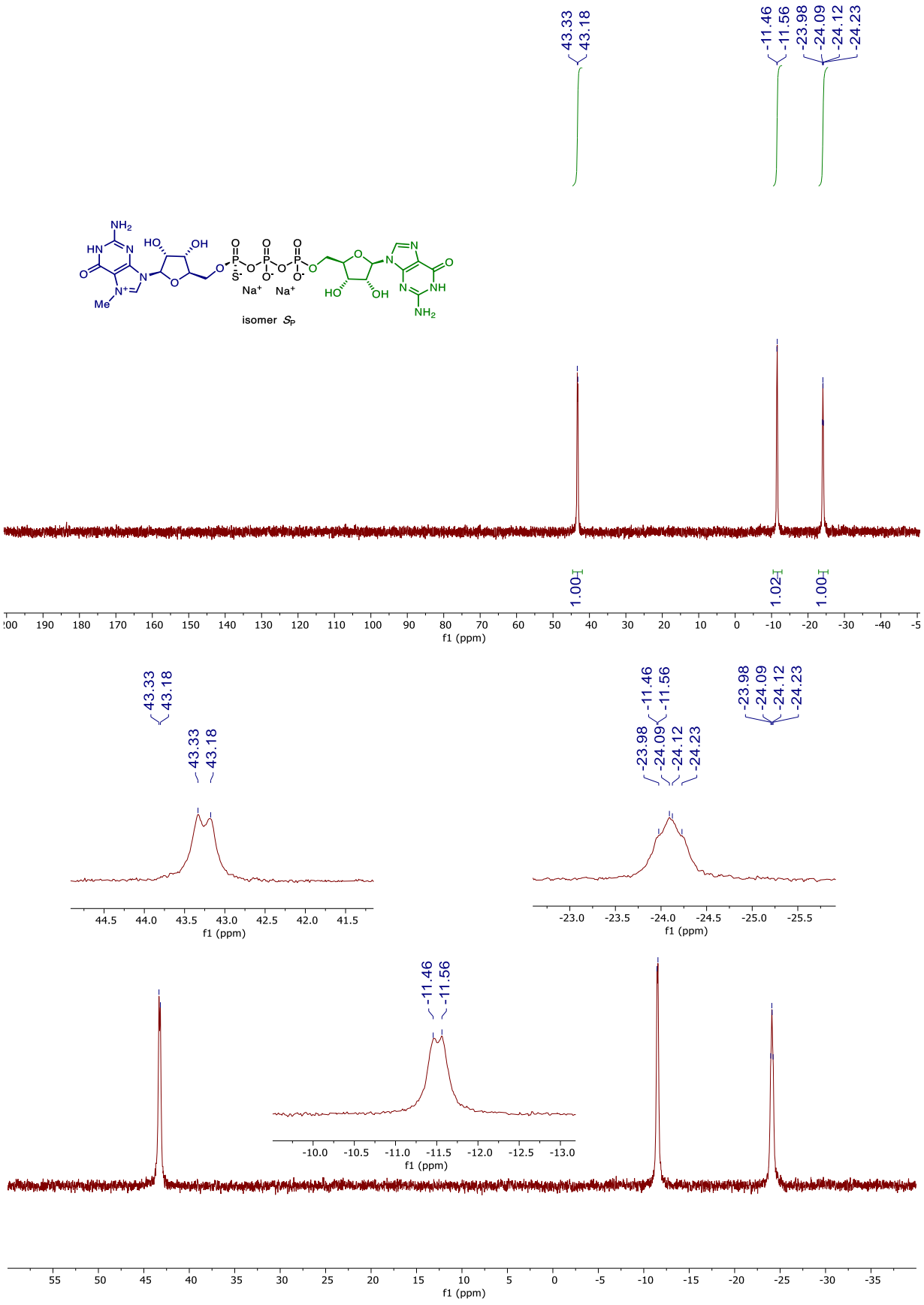
^{1}H NMR of compound (S_P)-40 (600 MHz, D_2O)



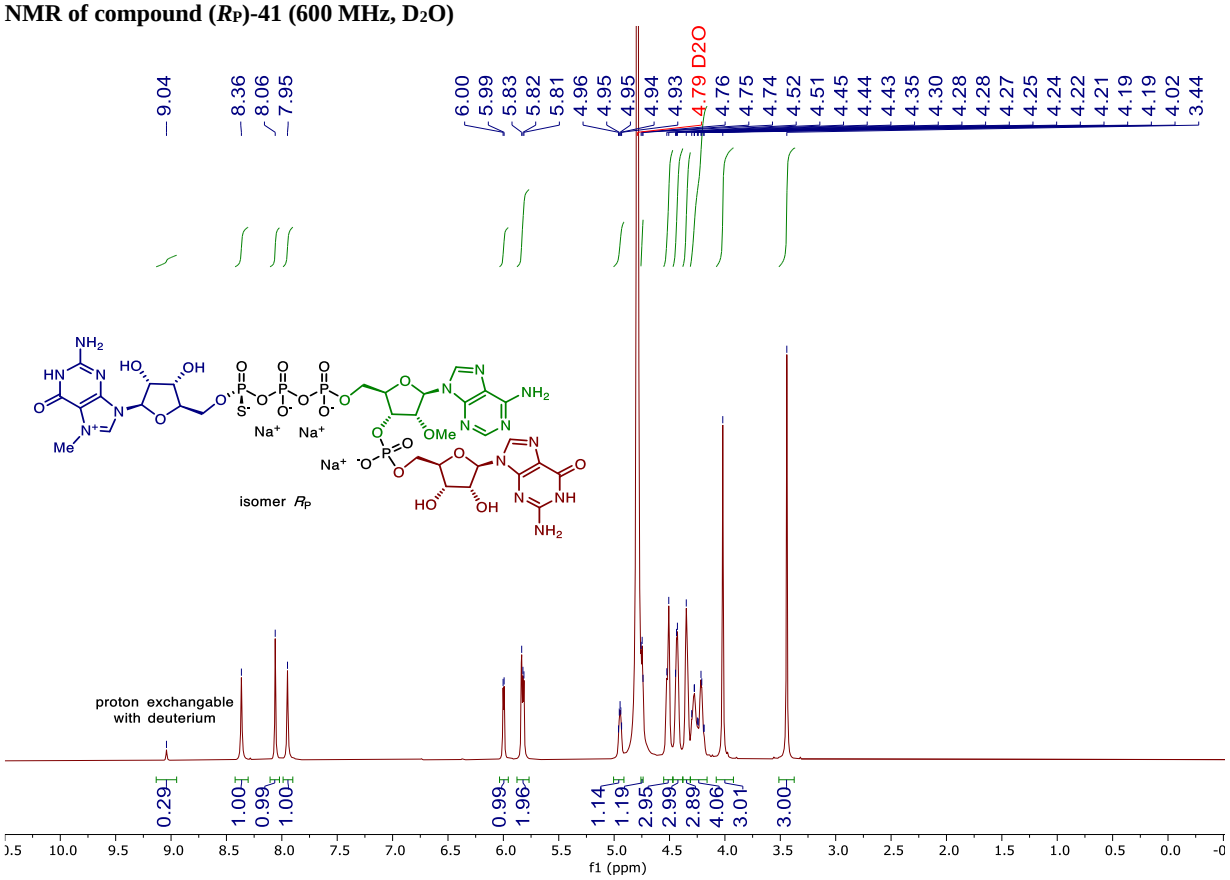
^{13}C NMR of compound (S_P)-40 (150 MHz, D_2O)



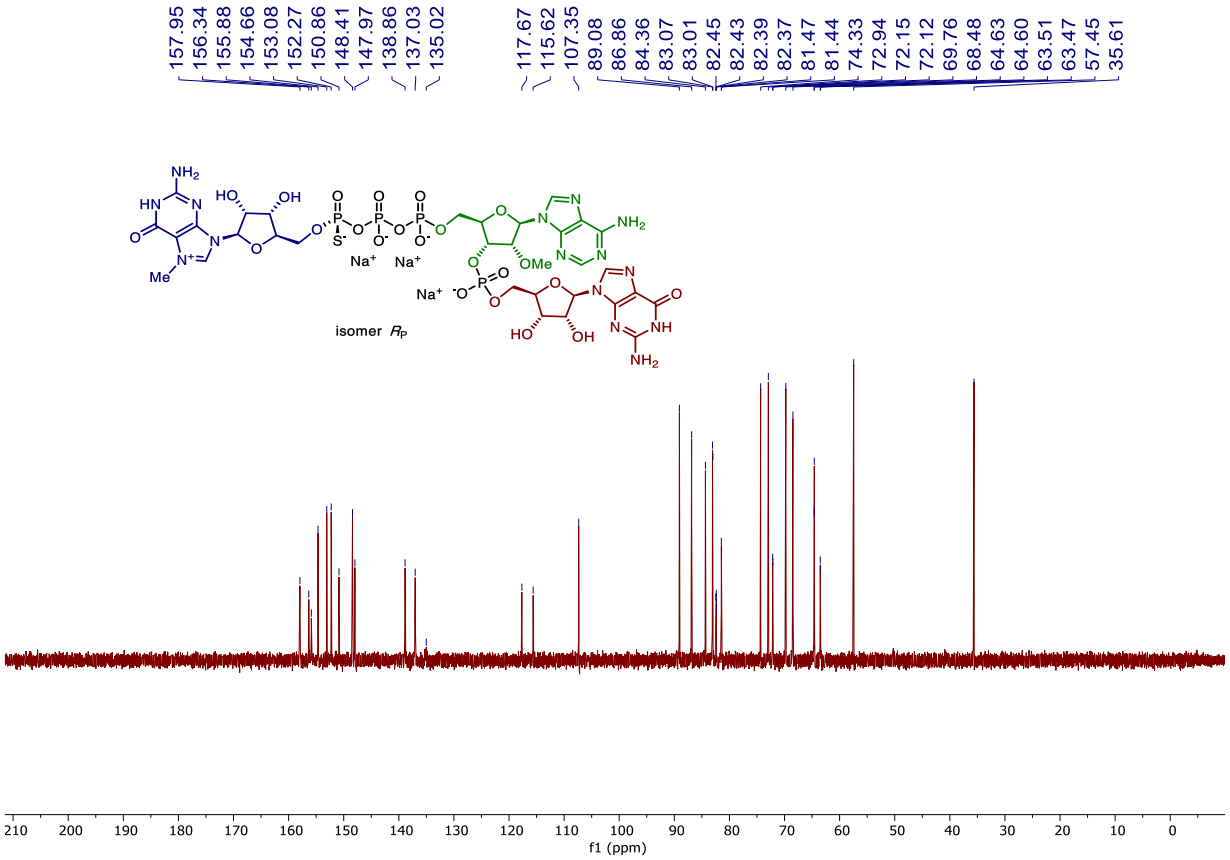
^{31}P NMR of compound (S_P)-40 (162 MHz, D_2O)



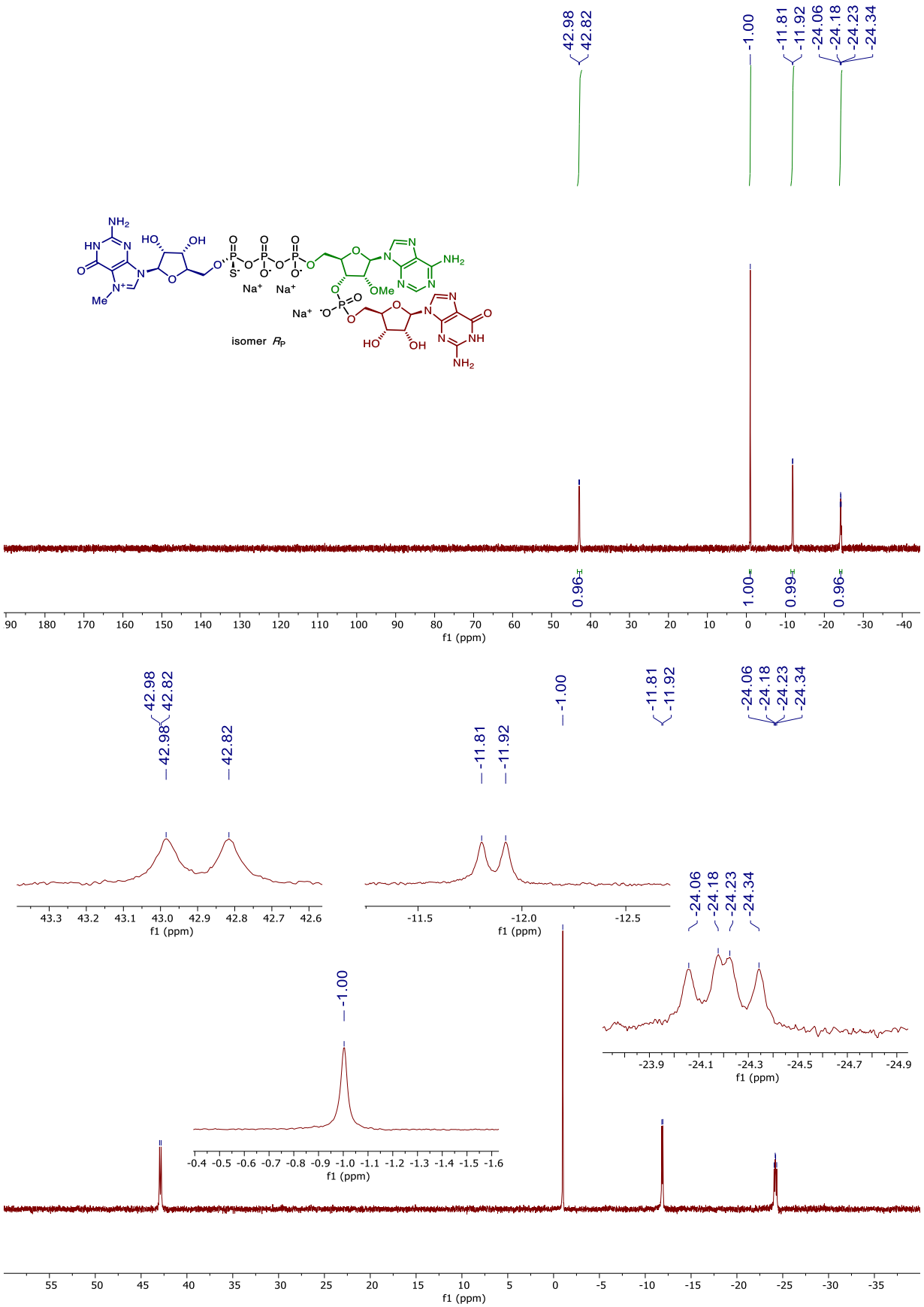
^{1}H NMR of compound (R_P)-41 (600 MHz, D_2O)



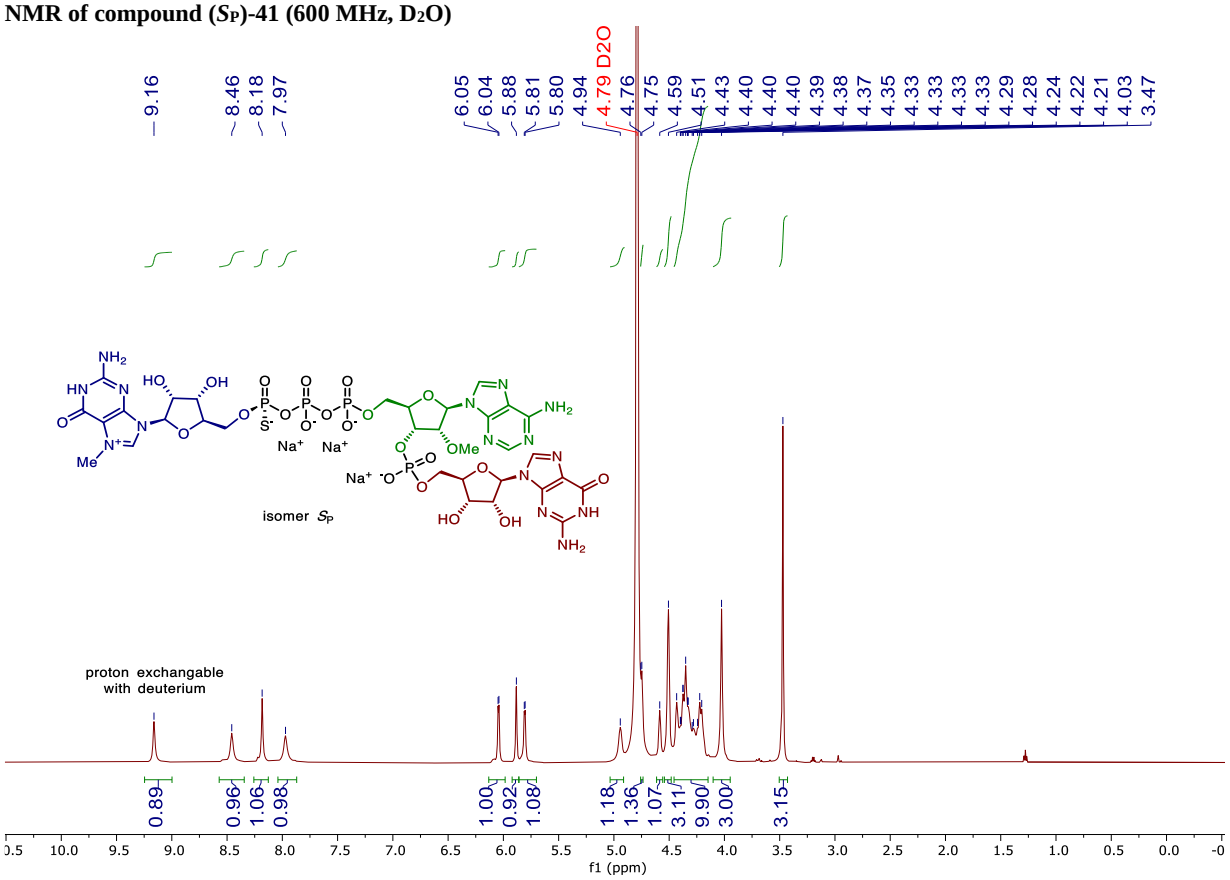
^{13}C NMR of compound (R_P)-41 (150 MHz, D_2O)



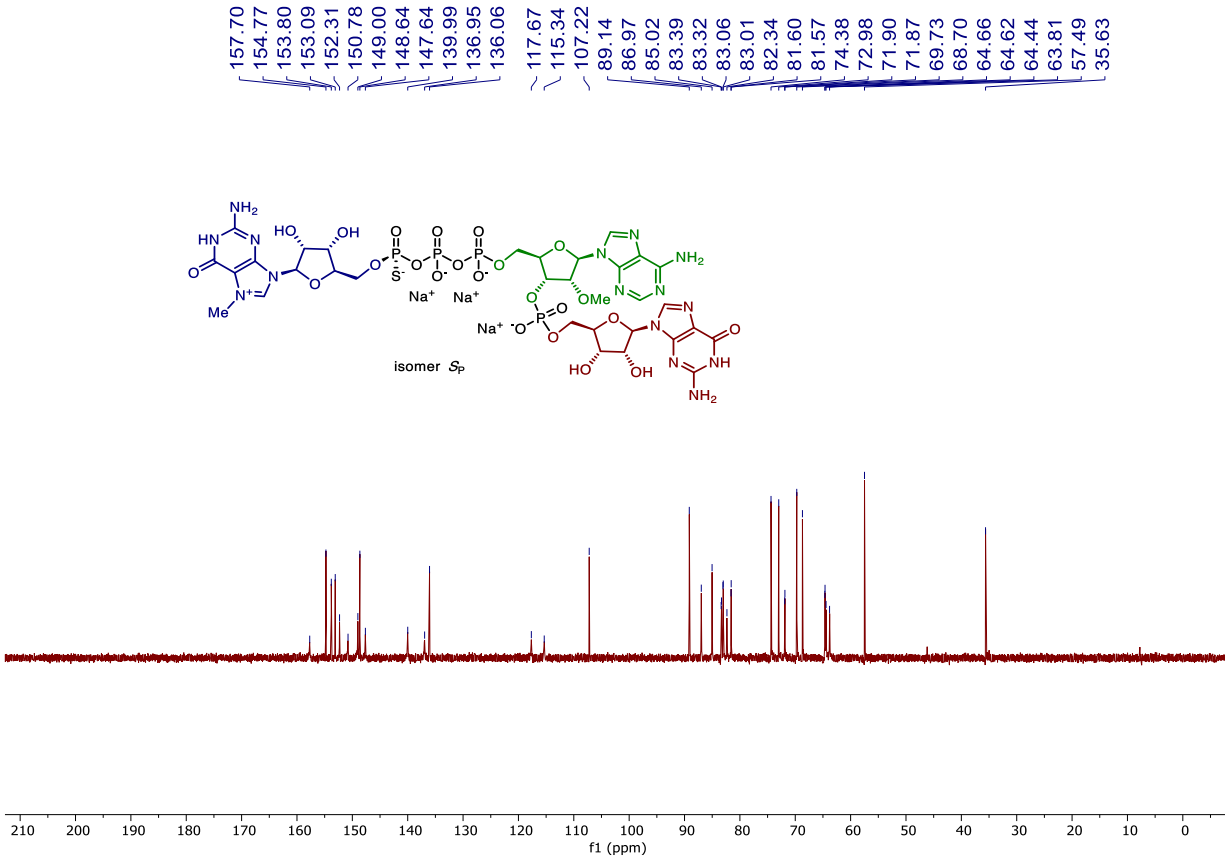
^{31}P NMR of compound (R_P)-41 (162 MHz, D_2O)



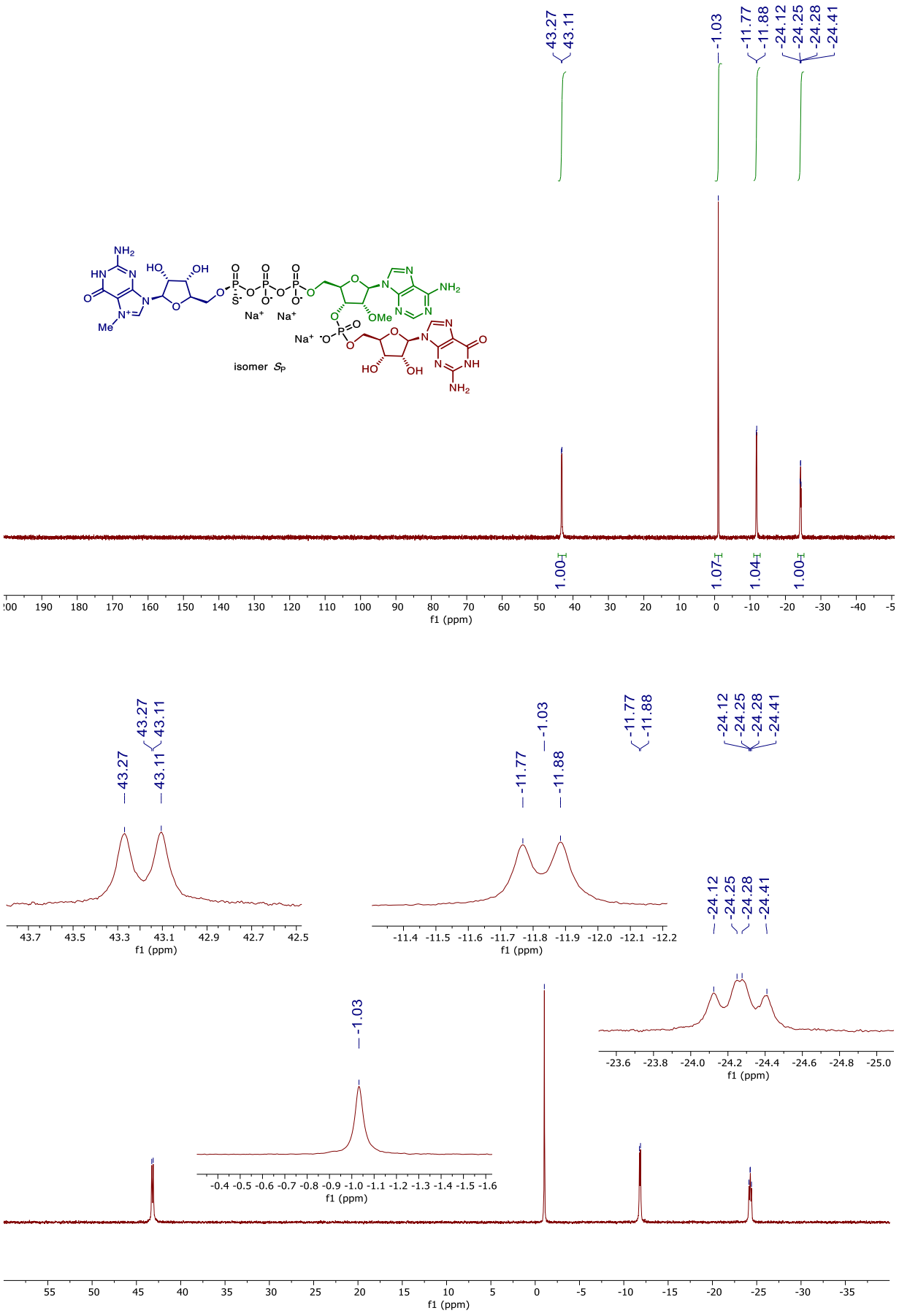
^{1}H NMR of compound (S_P)-41 (600 MHz, D_2O)



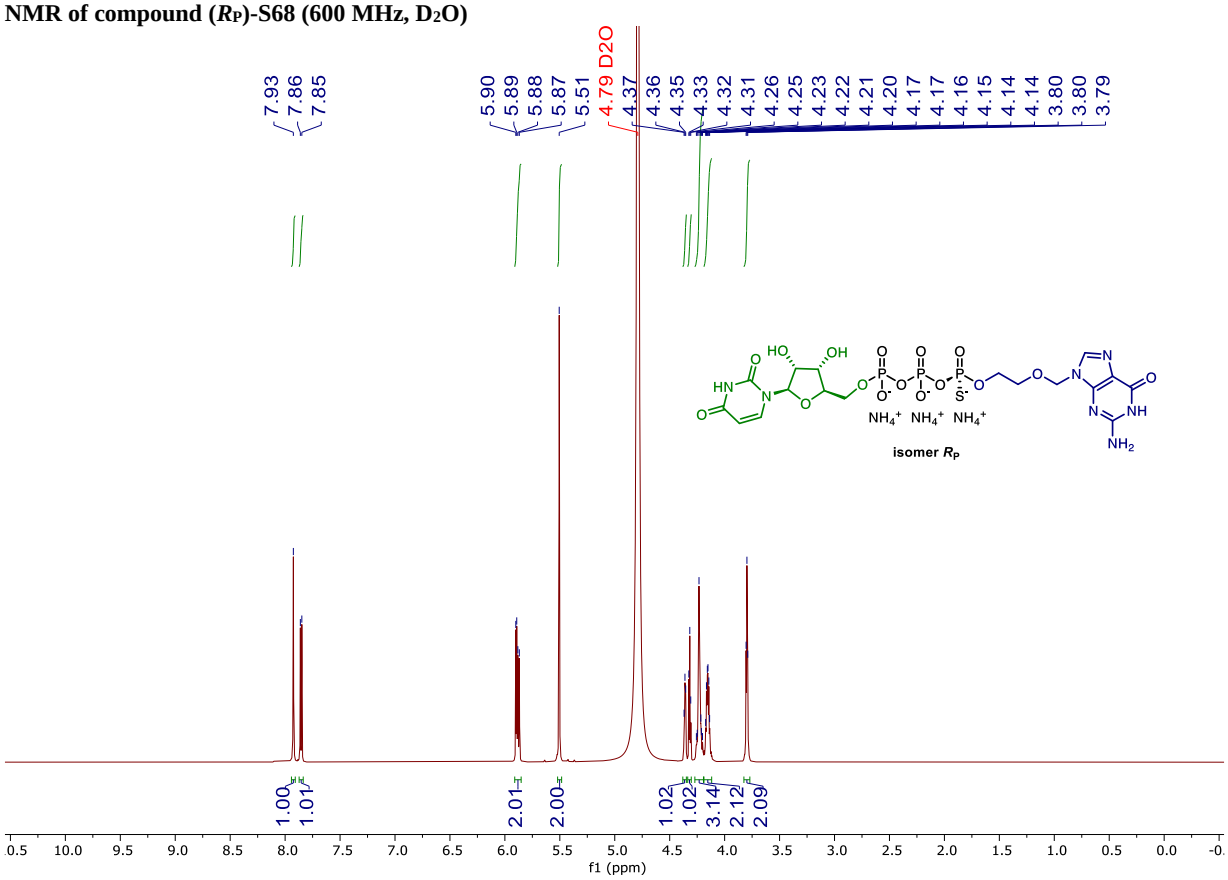
^{13}C NMR of compound (S_P)-41 (150 MHz, D_2O)



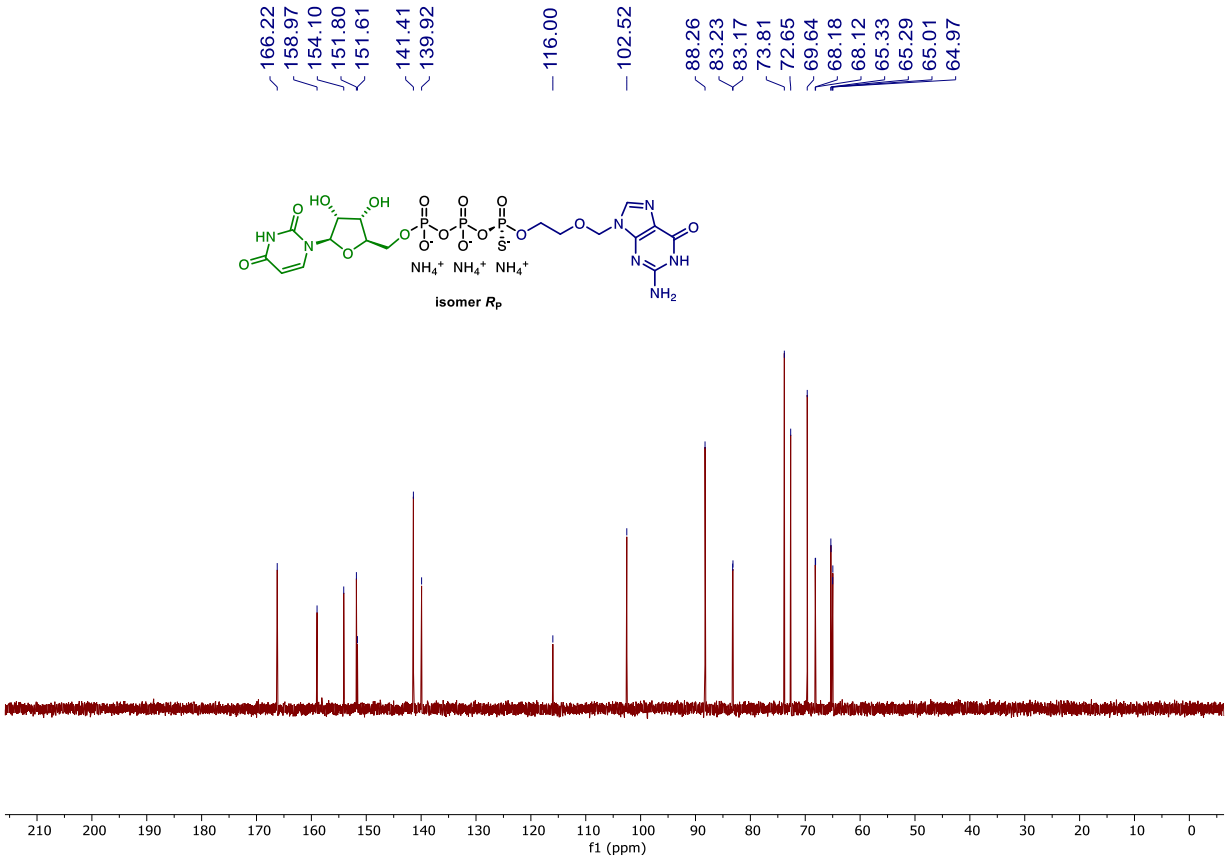
^{31}P NMR of compound (S_P)-41 (162 MHz, D_2O)



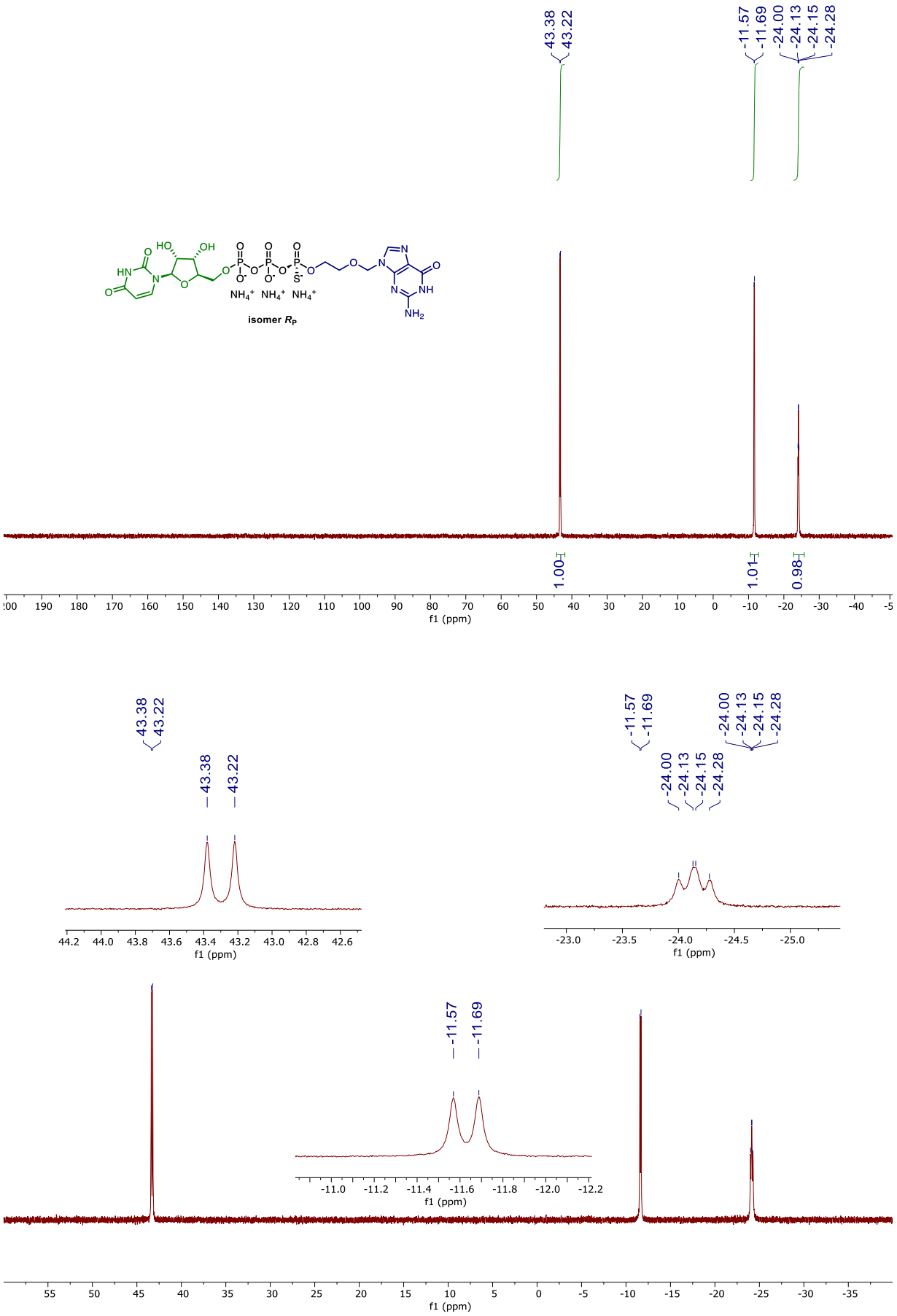
^{1}H NMR of compound (R_P)-S68 (600 MHz, D_2O)



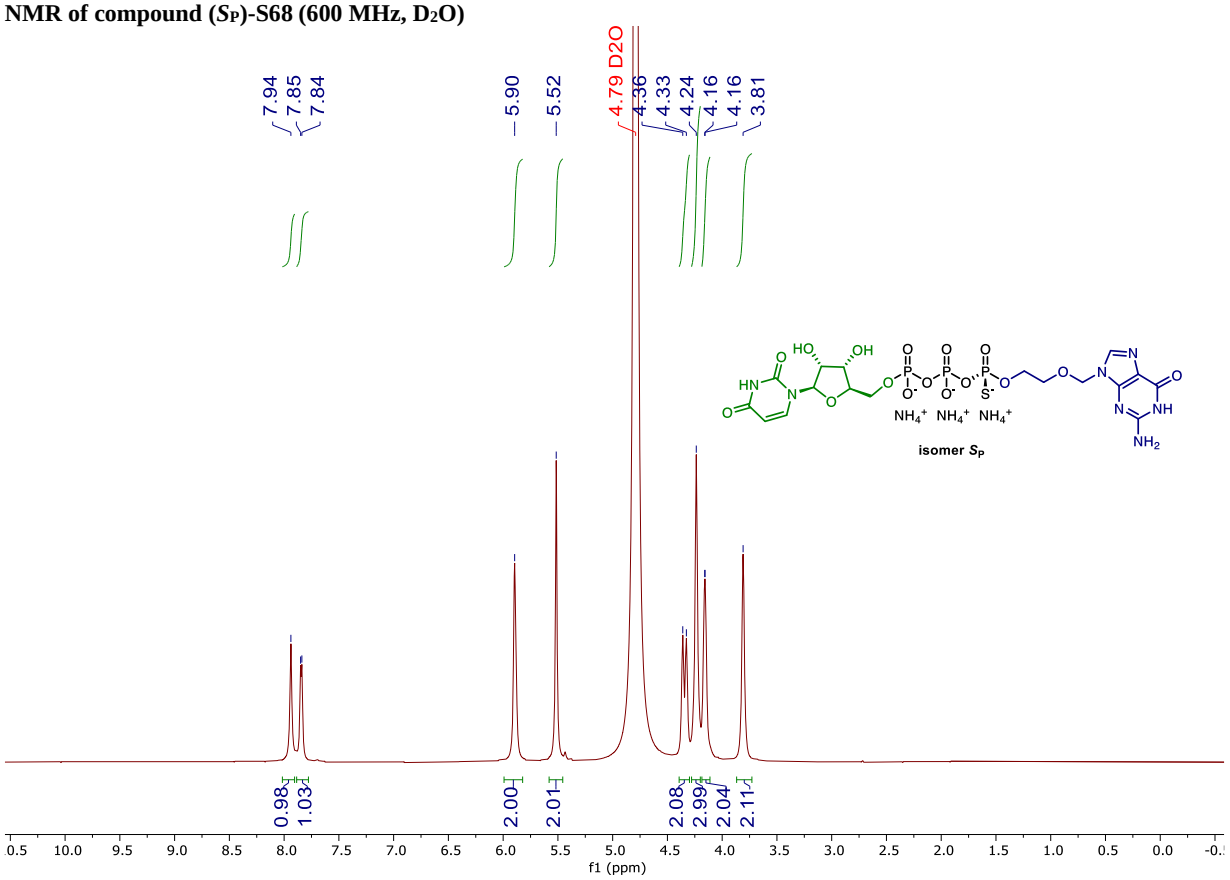
^{13}C NMR of compound (R_P)-S68 (150 MHz, D_2O)



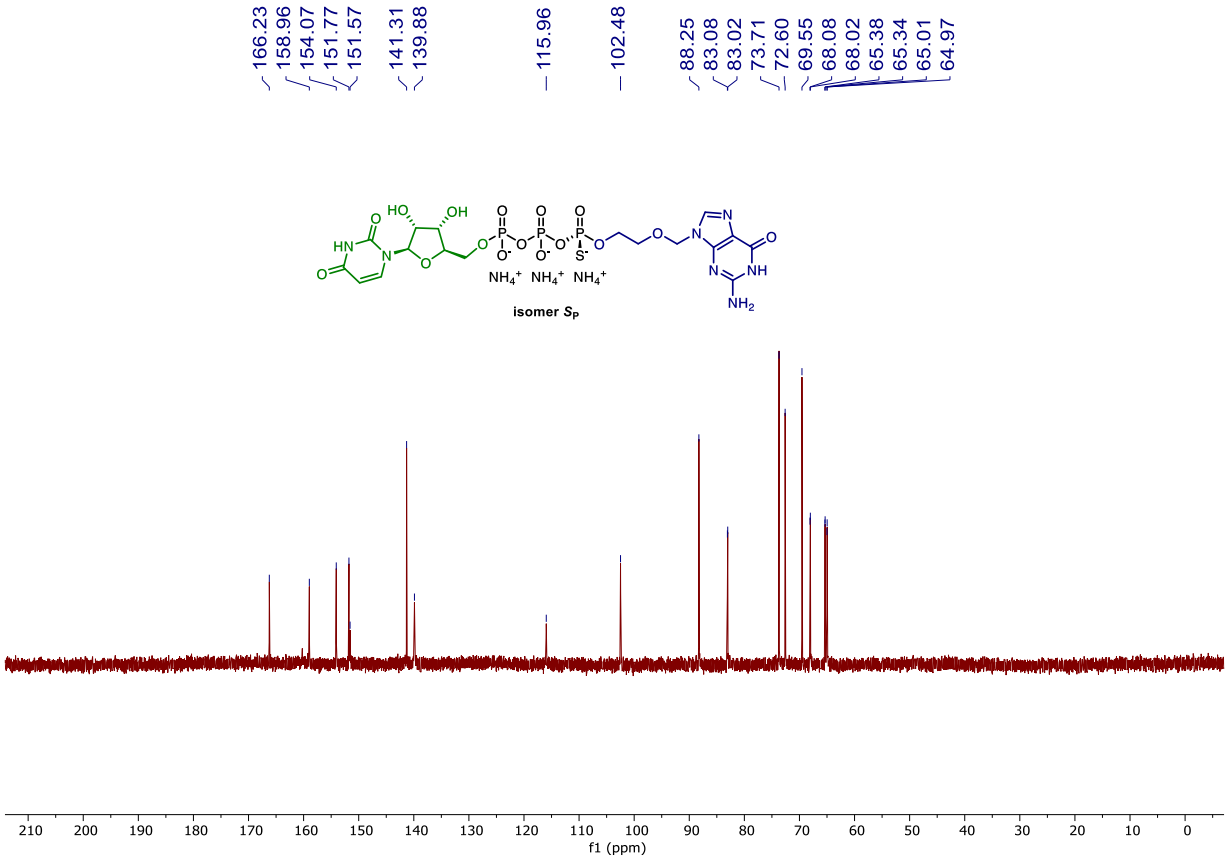
^{31}P NMR of compound (R_P)-S68 (162 MHz, D_2O)



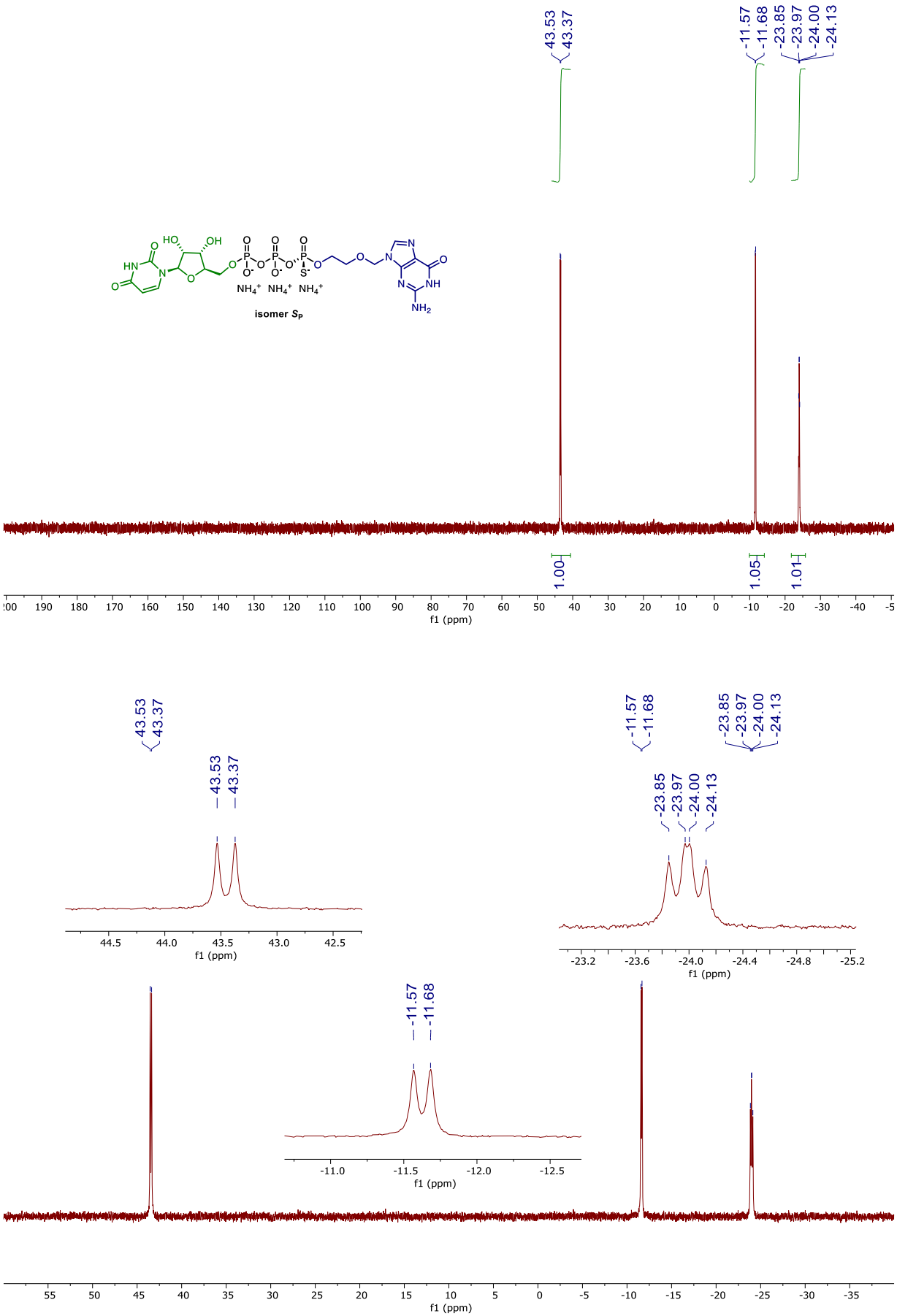
^{1}H NMR of compound (S_P)-S68 (600 MHz, D_2O)



^{13}C NMR of compound (S_P)-S68 (150 MHz, D_2O)



^{31}P NMR of compound (S_P)-S68 (162 MHz, D_2O)



15. References

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