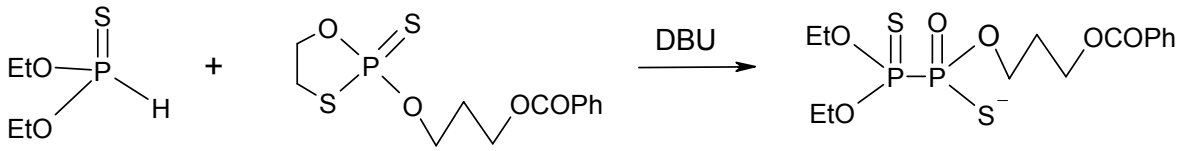
**ELECTRONIC SUPPLEMENTARY INFORMATION**




**8** **1b** **3d**

Scheme 1S. Synthesis of 3-*O*-benzoylpropyl ester of $P^2$-*O,O*-diethyl-$P^1$,$P^2$-dithiohypophosphoric acid.

**SYNTHESES:**

$P^1$-*O*-(3-*O*-Benzoylpropyl)-$P^2$-*O,O*-diethyl-$P^1$,$P^2$-dithiohypophosphate (**3d**): To a solution of 2-(3-*O*-benzoylpropyloxy)-2-thio-(1,3,2)-oxathiaphospholane (**1b**, 80 mg; 0.25 mmol) in dry MeCN (1 mL), *O,O*-diethyl *H*-thiophosphonate (**8**, 40 mg, 0.25 mmol) and DBU (34 μL, 0.27mmol) were added. After 12 h the product was formed in 57% (by $^{31}$P NMR; δ ($CD_3CN$) 86.01 ppm (d), 57.24 ppm (d), $^1J_{P\text{-}P}$ =348 Hz). The reaction mixture was concentrated to dryness and the product was isolated on a DEAE Sephadex A-25 column eluted with a TEAB (a gradient 0.05-0.6 M). The product (41 mg, 40%) was >92% pure by $^{31}$P NMR: δ ($D_2O$) 81.80 ppm (d), 58.49 ppm (d), $^1J_{P\text{-}P}$ =381 Hz. Finally, the product was converted into the sodium salt (DOWEX $Na^+$) and lyophylized (14 mg, 13%). FAB MS $C_{14}H_{22}O_6P_2S_2$, calc.m/z 412, $[M\text{-}H]^-$ found m/z 411.

Uridine *5'-O*-hypophosphate: To a stirred solution of 2',3'-*O,O*-diacetyluridine (75 mg, 0.23 mmol) and tetrabutylammonium hypophosphate tetrahydrate (300 mg, 0.69 mmol, 3 equivalents) in DMF (5 mL), a solution of DCC (160 mg, 0.8 mmol, 3.5 equivalents) in DMF (0.4 mL) was added, while the temperature of the mixture was kept at 4 °C. After 1h, the cooling bath was removed and the mixture was stirred for 12 h at room temperature. Then water (20 mL) was added and the resulting suspension was filtered using a Schott funnel. Water was evaporated at reduced pressure and the residue was treated with concentrated $NH_4OH_{aq}$ for 2 h. Uridine *5'-O*-hypophosphate was isolated using ion-exchange chromatography on DEAE Sephadex A-25, with a linear gradient 0.05 to 0.6 M TEAB buffer. Final purification was done using an ion-exchange HPLC column (Nucleogen, DEAE 60-7, 125x4 mm) with a linear gradient 0.05 to 0.35 M TEAB buffer (retention time 6.35 min.). The product was converted into

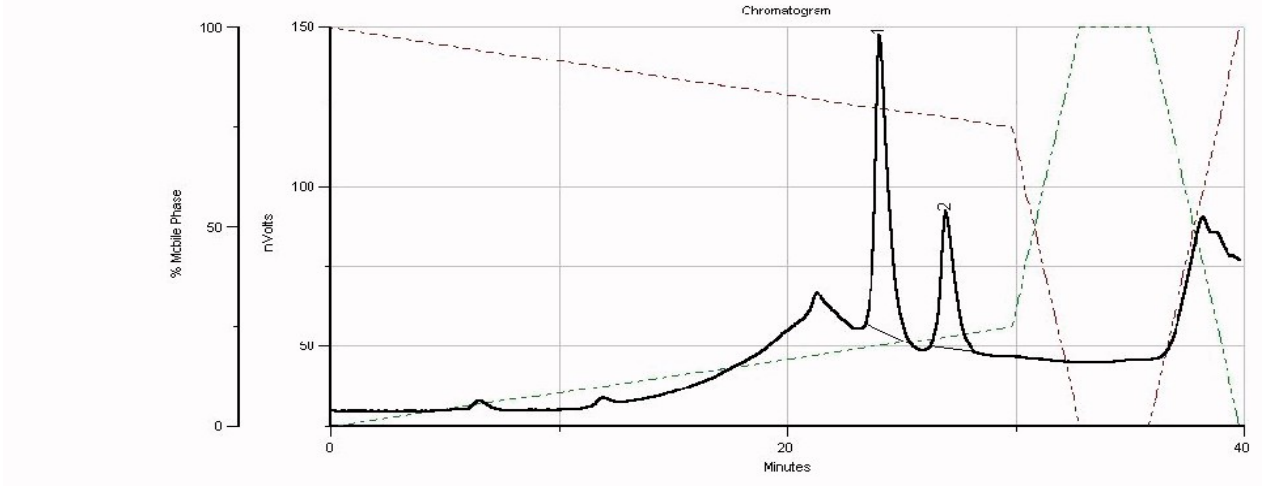
the sodium salt (DOWEX $Na^+$) and lyophilized (18 mg, three sodium counterions, 9%). $\delta$ $^{31}$P NMR ($D_2O$) 19.95 (d), 7,20 (d), $^1J_{P\text{-}P}$ =640 Hz; FAB MS m/z 453.0 $M^-$.

$N^6$-unprotected-2',3'-*O,O*-diacetyladenosine: To a solution of 5'-*O*-DMT-adenosine (1.43 g, 2.5 mmol) in acetonitrile (13 ml), N-methyl imidazole (435 µL, 5.5 mmol) was added, followed by acetic anhydride (566 µL, 6 mmol) and pyridine (3.4 mL). After 1 h stirring at room temperature, the mixture was evaporated to dryness and extracted (three times) using chloroform and saturated $NaHCO_3$. The organic layer was dried over anhydrous magnesium sulfate and evaporated to dryness. The residue was dissolved in methylene chloride (10 mL) and *p*-toulenesulfonic acid was added (ca. 600 mg). The mixture got red and after three hours methanol (4 mL) was added, followed by pyridine (0.5 mL), so colourless mixture was obtained. The mixture was concentrated under reduced pressure and the product was isolated (77%, 677 mg) on a silica gel column eluted with $CHCl_3$:MeOH (from 100:0 to 95:5, v/v). FAB MS $C_{14}H_{17}N_5O_6$, calc.m/z 351, $[M+H]^+$ found m/z 352, $[M-H]^-$ found m/z 350.

$N^6$-unprotected 5'-*O*-(2-thio-1,3,2-oxathiaphospholane)-2',3'-*O,O*-diacetyladenosine (**16**): Into a solution of $N^6$-unprotected-2',3'-*O,O*-diacetyladenosine (0.352 g; 1 mmol) in anhydrous pyridine (5 mL), elemental sulfur was added (0.32 g; 10 mmol) followed by 2-chloro-1,3,2-oxathiaphospholane (170 mg; 1.2 eq., added dropwise). The mixture was stirred overnight at room temperature and then evaporated to dryness. The residue was dissolved in acetonitrile (5 ml), excess sulfur was filtered off and the filtrate was concentrated. The product was isolated (314 mg, 64%) on a silica gel column eluted with $CHCl_3$:MeOH (from 100:0 to 95:5, v/v). $\delta$ $^{31}$P NMR ($CD_3CN$) 105.45 ppm (s), 105.64 ppm (s), relative intensity ca. 1:1; FAB MS $C_{16}H_{20}N_5O_7PS_2$, calc. m/z 489, $[M-H]^-$ found m/z=488.

Enzymatic phosphorylation of 5'-*O*-($P^1$-thiohypophosphate) derivatives of adenosine (**18**), uridine (**15**) and cytidine and 5'-*O*-hypophosphate derivative of adenosine (**10**) with the phosphoenolpyruvate/ pyruvate kinase system: A solution containing nucleoside 5'-*O*-($P^1$-thiohypophosphate), or nucleoside 5'-*O*-hypophosphate, at 1mM concentration, 10 mM phosphoenolpyruvate (PEP), 5 µg of pyruvate kinase (PK), (total volume 20 µl) was incubated at 37°C for 48h. The sample was heat-denatured (at 95°C for 5 min, cooled down to the room temperature and centrifuged at 13,000 rpm for 10 min. The supernatant was collected and analyzed by means of HPLC (a binary system consisting of two 306 pumps and a 151 UV/VIS detector, Gilson). Conditions: a C18 column, 250×4.6 mm, 5 µm; elution with a linear gradient of 0.1 M TEAB, pH 7.3 to 12% $CH_3CN$ in 0.1 M TEAB over 30 minuts. The figure below shows HPLC analysis for conversion **18** → **22**. Retention times: the remaining substrate (peak 1) – **18**-*fast*, 24.1 min; the product (peak 2)– adenosine 5'-*O*-($P^1$-thio-$P^1$,$P^2$-hypotriphosphate) **22** , 26.9 min.

| | Peak Name | R. Time | Area | Area % |
|---|---|---|---|---|
| 1 | - | 24.08 | 61422 | 67.1 |
| 2 | 2 | 26.96 | 30103 | 32.9 |

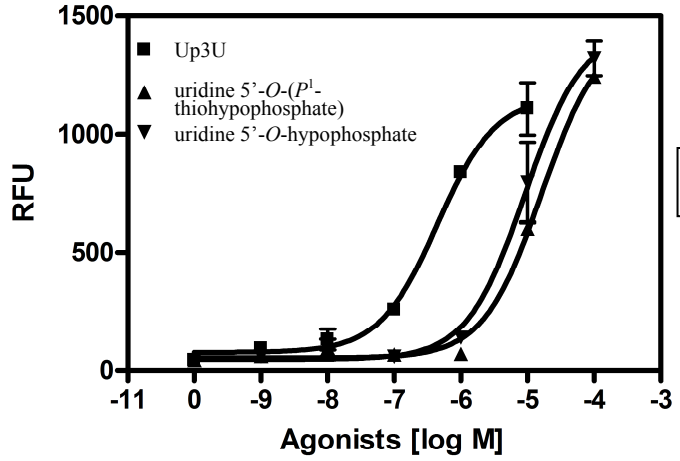



Measurement of inhibitory activity of nucleoside hypophosphates towards T7 RNA polymerase:
An AmpliScribe™ T7-*Flash*™ Transcription Kit (Epicentre® Biotechnologies, Madison, WI), a 192 nt DNA template (derived from pUCRz2 plasmid), and 22 nt and 42 nt (with the T7 polymerase promotor sequence) primers were used in a transcription experiment. A solution of transcription buffer (a total volume 20 µl) containing ATP, CTP, GTP and UTP (each at 0.9 mM concentration), ($\alpha$-$^{32}$P)-CTP (low pM concentration, 0.37 MBq), 10 mM DTT, 1 µg of DNA template, an investigated hypophosphate (**18**-*fast*, **18**-*slow*, **22** or **23**, at five concentrations ranging from 0.9 to 4.5 mM) and T7 RNA polymerase was incubated for 30 min at 37°C. The sample was heat-denatured (for 1 min at 95°C) and cooled down to the room temperature. Then, the DNA template was digested with RNase-free DNase I (1 MBU, 37°C for 15 min.) and the sample was heat-denatured (for 1 min at 95°C). The resulting mixture was analyzed by PAGE (10% gel, 7M urea). The gel was scanned by PhosphorImager (Storm 860, Molecular Dynamics) and the bands were quantified with ImageQuant 5.0 (Molecular Dynamics).

Calcium Assay by Fluorometric Imaging Plate Reader (FLIPR): Astrocytoma cells were grown overnight in 100 µl of medium in 96-well clear-bottom, black plates at 37°C at 5% $CO_2$ until they reached ~80% confluency at a density of 30,000 cells/well. After overnight incubation, the DMEM medium was replaced by calcium-4 dye (Molecular Devices, Sunnyvale, CA) and incubated for 1 h at room temperature. The calcium-4 assay kit was used as directed with no washing of cells. Cells were loaded with 40 µl of dye in each well and incubated for 1 h at room temperature. The compound plate was

prepared with various dilutions ($10^{-9}$, $10^{-8}$ ….$10^{-5}$ M) of compounds in Hank's balanced salt solution (HBSS) with 20 mM HEPES (pH 7.4). The change in calcium was measured by addition of $P2Y_6$ agonists to the dye and the change in calcium was measured by change in intracellular fluorescence. Samples were run in duplicate with a FLIPR$^{TETRA}$ (Molecular Devices) at room temperature. Cell fluorescence (excitation = 485 nm; emission = 525 nm) was monitored following exposure to a compound. Increases in intracellular calcium are reported as the maximum fluorescence value after exposure minus the basal fluorescence value before exposure (see a plot below).

**Calcium Assay by FLIPR in $P2Y_6$ Astrocytoma cells**




RFU – relative fluorescence units

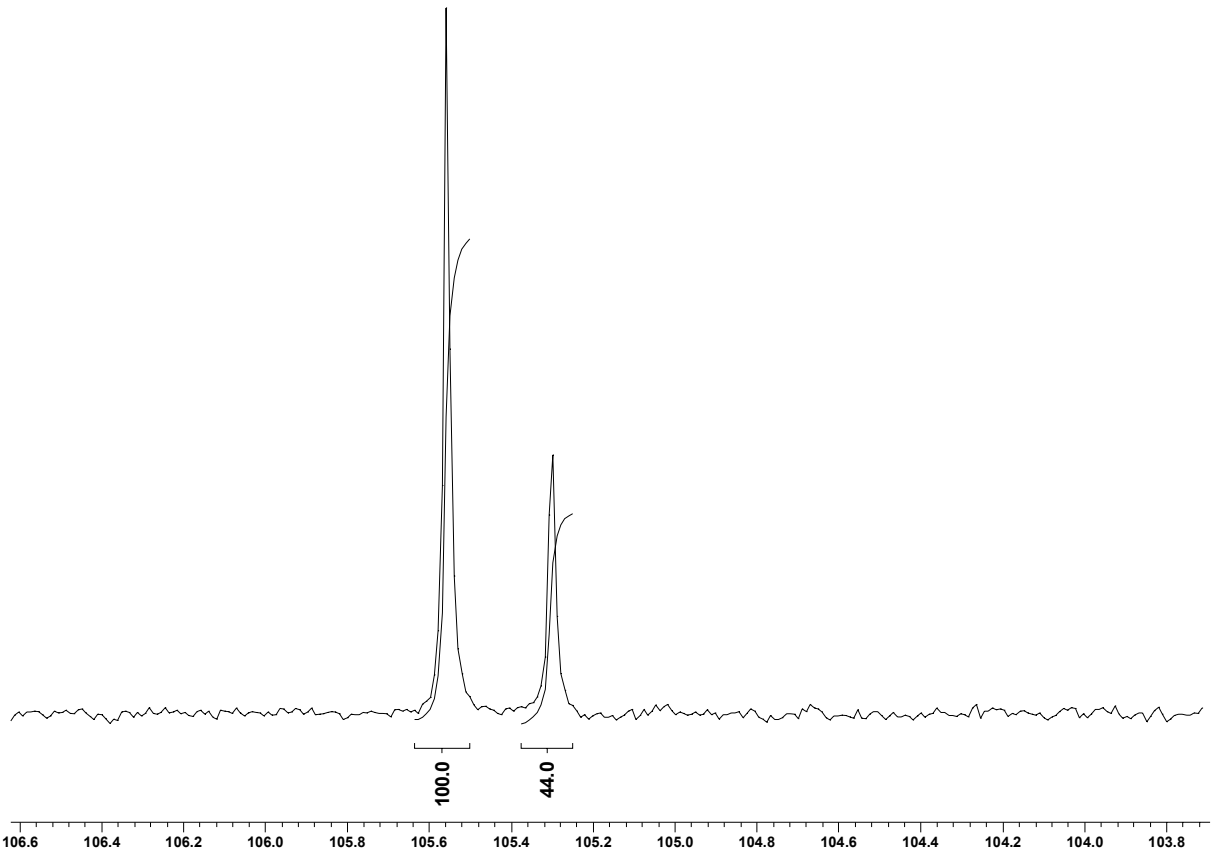
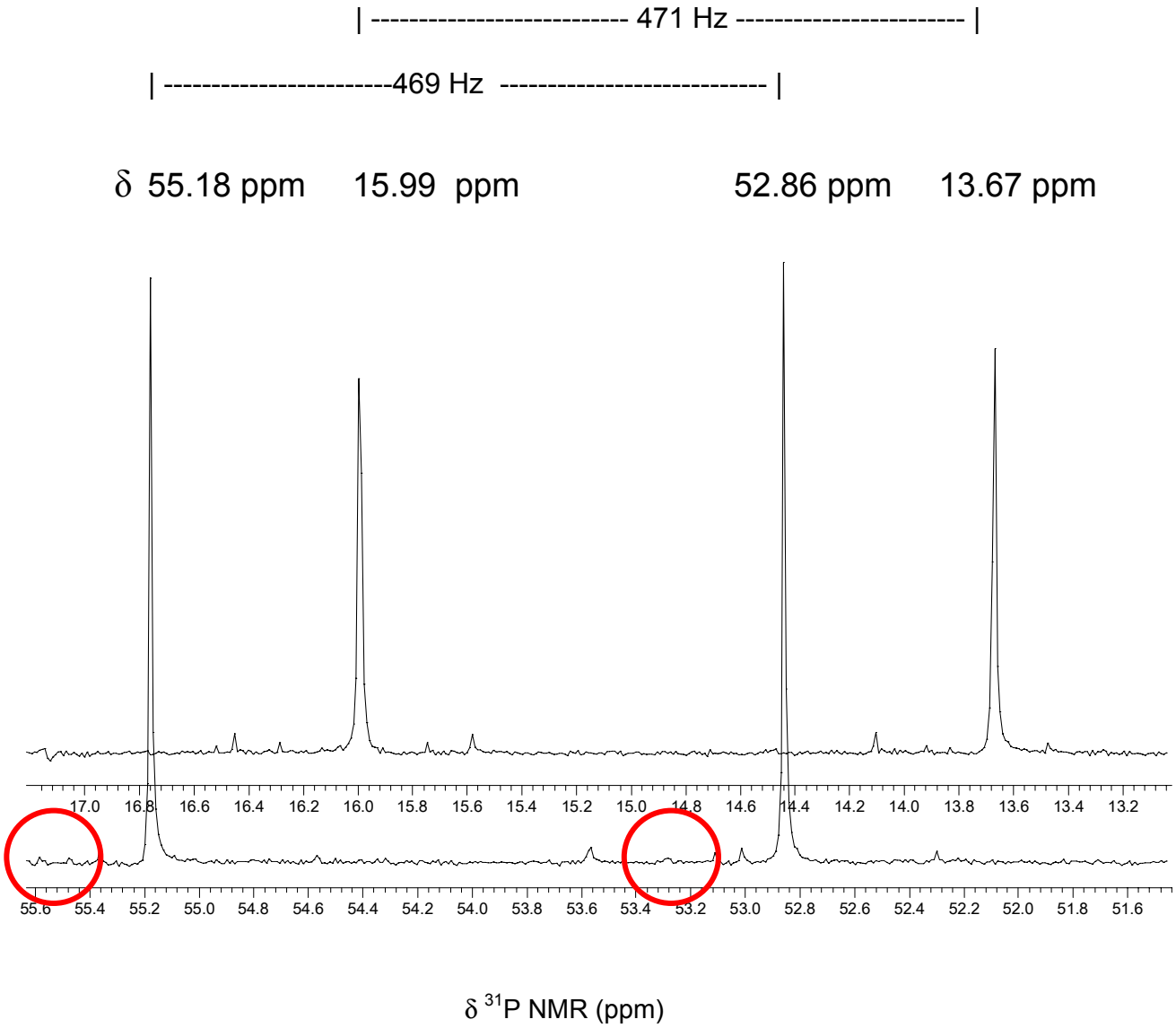



Figure 1S. $^{31}$P NMR spectrum for the 5'-*O*-DMT-$N^{iBu}$,$O^{DPC}$-deoxyguanosine-3'-*O*-(2-thio-1,3,2-oxathiaphospholane) (**19**); diastereomeric ratio *fast:slow* 100:44 ($S_P$:$R_P$)

Figure 2S. The regions of the $^{31}$P NMR proton decoupled spectrum (500 MHz) for the β-P atom (the upper line) and α-P atom (the bottom line) in 5'-*O*-DMT-$N^{iBu}$,$O^{DPC}$-deoxyguanosine-3'-*O*-($P^2$-*O,O*-diethyl-$P^1$-thiohypophosphate) **20**, obtained from diastereomerically pure **19** (100 % $S_P$). The red circles mark the regions where signals of α-P atom in the epimerized product are expected.



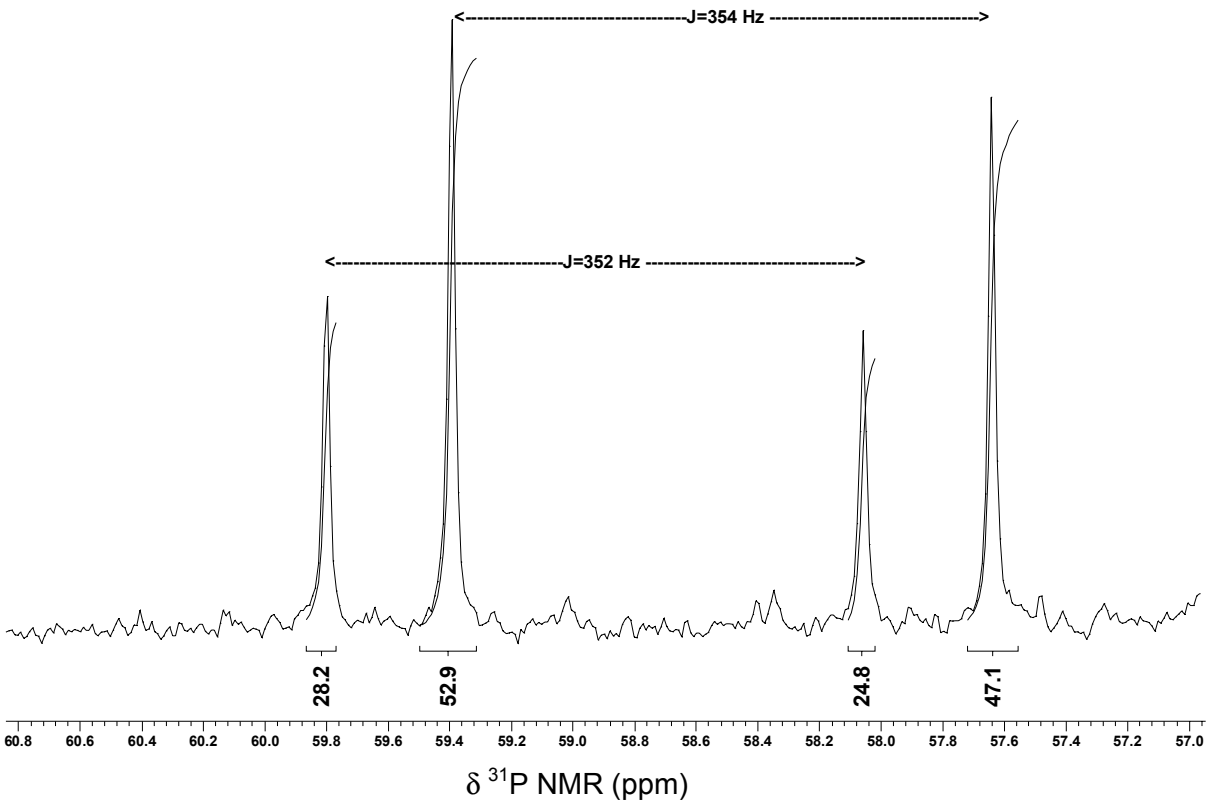



Figure 3S. A region of the $^{31}$P NMR proton decoupled spectrum for the α-P atoms in both P-diastereomers of 5'-*O*-DMT-$N^{iBu}$,$O^{DPC}$-deoxyguanosine-3'-*O*-($P^2$-*O,O*-diethyl-$P^1$,$P^2$-dithiohypophosphate) (**21**), obtained from **19** (diastereomeric ratio *fast:slow* 100:44 ($S_P$:$R_P$), see Figure 1S). Observed diastereomeric ratio 100:53.