Electronic Supplementary Material (ESI) for ChemComm.


**Supporting Information**

# 2'-(*R*)-fluorinated mC, hmC, fC and caC triphosphates are excellent substrates for DNA polymerases and TET-enzymes

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# 1. Content

# 2. General methods

Chemicals and absolute solvents for synthesis were purchased from *Sigma-Aldrich, Carbosynth, ABCR* or *Acros organics* and used without further purification. Solutions were concentrated *in vacuo* on a *Heidolph* rotary evaporator. The solvents for chromatography were of reagent grade and purified by distillation. Chromatographic purification of products was accomplished using flash column chromatography on *Merck* Geduran Si 60 (40 – 63 μm) silica gel (normal phase). Thin layer chromatography (TLC) was performed on *Merck* 60 (silica gel $F_{254}$) plates. Visualization of the developed chromatogram was performed using fluorescence quenching or staining solutions. $^{1}$H-, $^{13}$C-, $^{19}$F- and $^{31}$P-NMR spectra were recorded in deuterated solvents on *Varian Oxford 200, Bruker ARX 300, Varian VXR400S, Varian Inova 400, Bruker AMX 600* and *Bruker AMX 800* spectrometers and calibrated to the residual solvent peak. Multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, qi = quintet, m = multiplet, br. = broad. High-resolution ESI spectra were obtained on the mass spectrometers *Thermo Finnigan* LTQ FT-ICR. IR measurements were performed on *Perkin Elmer Spectrum BX FT-IR* spectrometer with a diamond-ATR (Attenuated Total Reflection) setup. Melting ranges of small molecules were measured on a Büchi B-540.

Acetonitrile for HPLC-purification of nucleoside standards were purchased from *VWR*. Acetonitrile of LC-MS grade was purchased from *Carl Roth GmbH + Co. KG*. Formic acid was purchased from *Fluka*, p.a. for mass spectrometry. Water was purified by a Milli-Q Plus system from *Merck Millipore*. Nuclease S1 (*Aspergillus oryzae*) was obtained from *Sigma Aldrich*, snake venom phosphodiesterase I (*Crotalus adamanteus*) from *USB corporation* and antarctic phosphatase from *New England Biolabs*.

dd$H_2O$ refers to double distilled water obtained by a Milli-Q® Plus water purification system from *Millipore* using a QPAK® 2 cartridge.

## 2.1. Enzymatic incorporation of 2'-(*R*)-F-xdC triphosphates

### 2.1.1. Primer Extension studies

To investigate optimal conditions for the incorporation of 2'-(*R*)-F-xdC triphosphates **11a-d** by polymerase chain reaction (PCR) first primer extension studies were performed using several DNA polymerases. The following ODNs were used:

| **Name** | **Sequence 5'→ 3'** |
|---|---|
| **ODN1** | GTA GTA GGA TGG GAG AGT GGT GGG AGG |
| **ODN2** | Fluorescein-CCT CCC ACC ACT CTC CCA TC |

The ODNs were hybridized and the resulting duplex was subsequently used for primer extension experiments. The general reaction set-up was:

| Component | Volume [µL] |
|---|---|
| **duplex (20 µM)** | **1** |
| **Buffer (5x)** | **2** |
| **DNA polymerase** | **0.5** |
| **2'-(*R*)-F-xdC (2mM)** | **2** |
| **ddH2O** | **4.5** |

The reaction was heated to 72 °C for 15 minutes and analyzed with a denaturing PAGE (20%) in 1x TBE at 40 °C and 35 mA. The fluorescent ODN was visualized using an Image Reader LAS 3000 (Fujifilm).

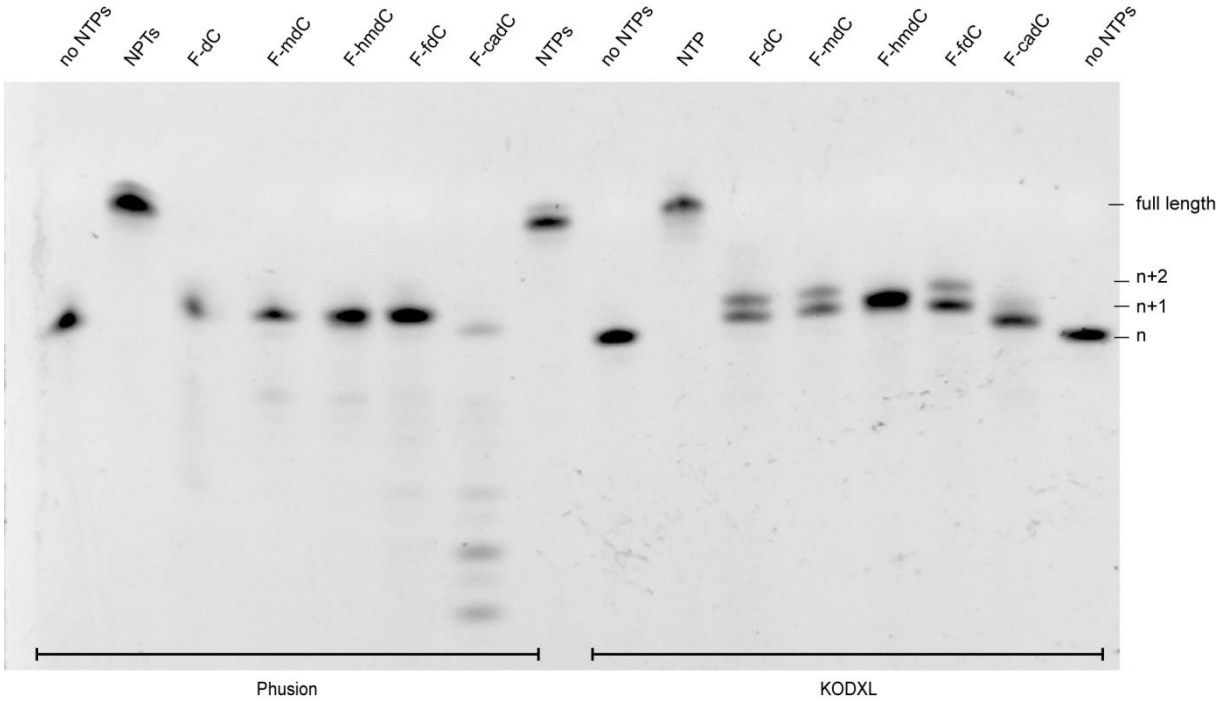



**Figure S1 – PAGE analysis of a primer extension experiment using 2'-(*R*)-F-xdC with Phusion and KODXL DNA Polymerase. Phusion DNA Polymerase seems to accept all but 2'-(*R*)-F-cadC as a substrate. KODXL yields n+1 and n+2 products for several 2'-(*R*)-F-xdC. The n+2 product may result due to the lack of a proof reading exonuclease activity of KODXL.**

Next full length elongation was investigated by also adding dA, dT and dG triphosphates to the reaction mixture (NTPs).

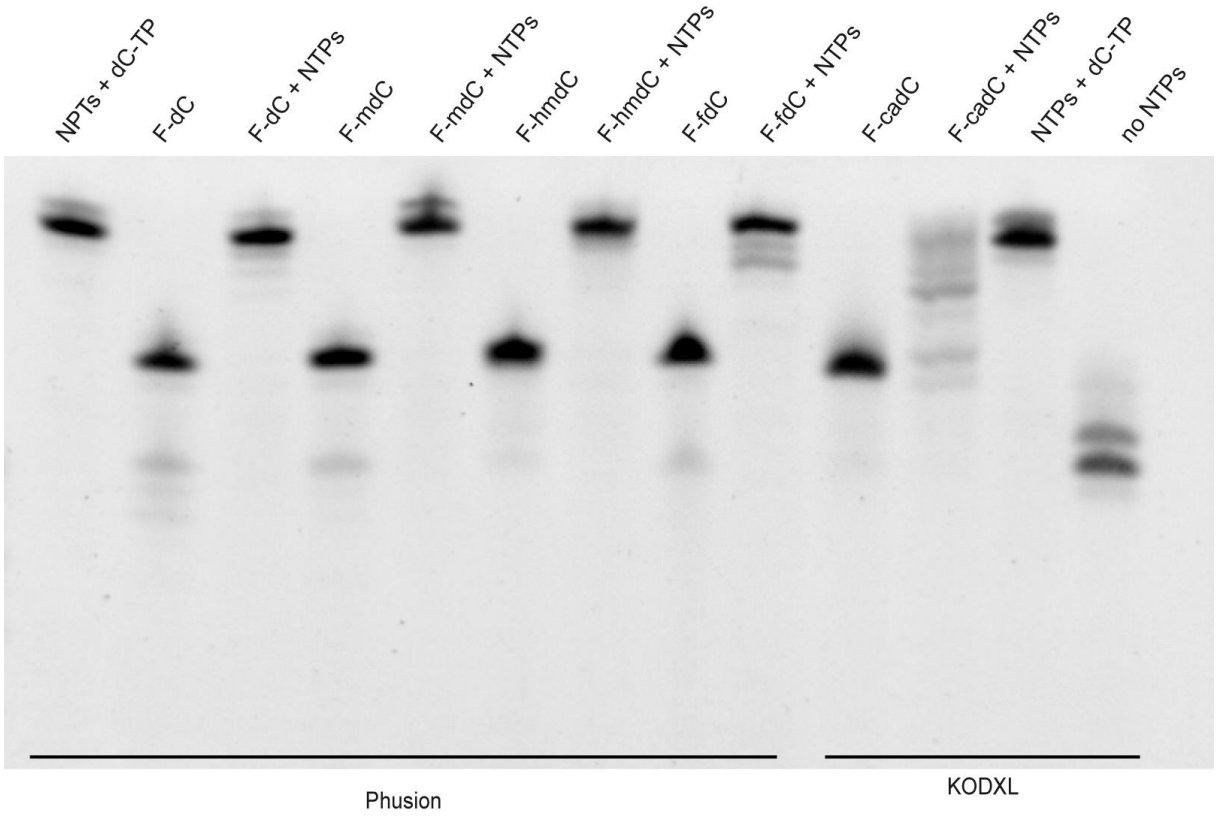



**Figure S2: PAGE analysis of a primer extension experiment using 2'-(*R*)-F-xdC triphosphates +/- NTPs and Phusion/KODXL Polymerase. All but 2'-(*R*)-F-cadC elongation yielded in full length products. The reactions labeled 2'-(*R*)-F-xdC+NTP contained all NTPs but dC-TP.**

As the 2'-(*R*)-F-cadC triphosphate did not yield full length products several Polymerases were screened for 2'-(*R*)-F-cadC. NTPs refer to all but dC-triphosphate.

| Lane | Polymerase |
|---|---|
| **1** | control |
| **2** | dC + NTPs + Vent |
| **3** | Vent |
| **4** | Vent + NTPs |
| **5** | Vent exo (-) |
| **6** | Vent exo (-) + NTPs |
| **7** | Therminator |
| **8** | Therminator + NTPs |
| **9** | OneTaq |
| **10** | OneTaq + NTPs |
| **11** | KODXL |
| **12** | KODXL + NTPs |
| **13** | Phusion |
| **14** | Phusion + NTPs |
| **15** | Q5 |
| **16** | Q5 + NTPs |
| **17** | Taq |
| **18** | Taq + Ntps |
| **19** | Neg control |
| **20** | dC + NTPs + Vent |

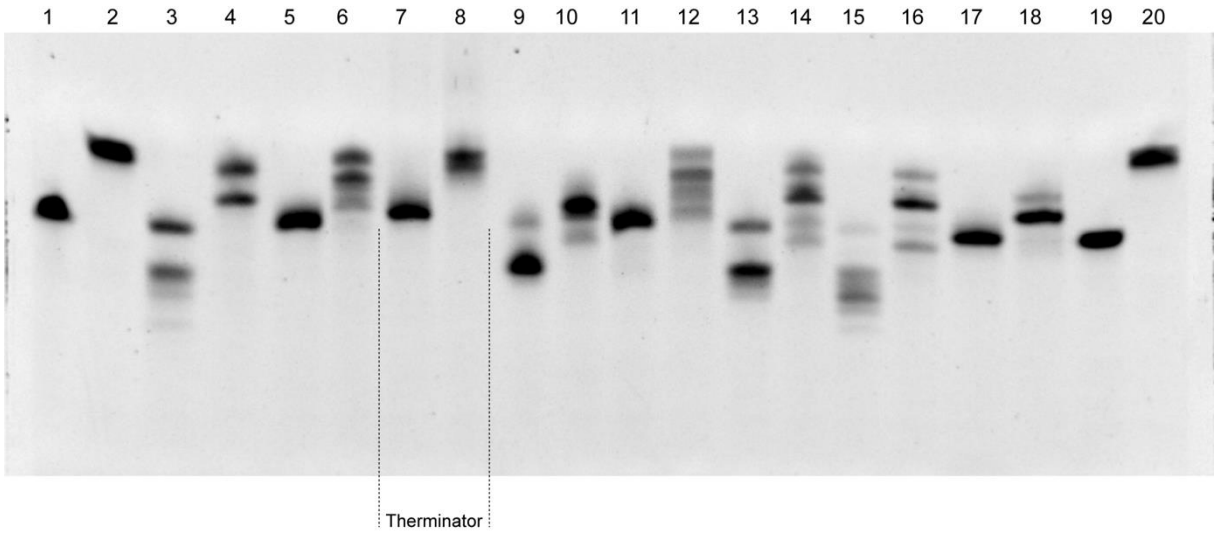



**Figure S3 –PAGE analysis of a screen of several DNA Polymerases for the incorporation of 2'-(*R*)-F-cadC. Lane 7 and 8 show the result for the Therminator Polymerase. The n+1 (lane 7) and full length product (lane 8) are visible.**

### 2.1.2. Polymerase Chain Reaction

For the PCR reaction a 81 bp OCT4 promoter fragment was used as a template. Primers and template (sequence is shown in **Figure 2A**) were purchased from Sigma-Aldrich.

Optimal conditions for PCR are as following. 50 µL of a solution containing 5 ng template, 0.2 mM of the natural triphosphates without dC triphosphate (dA, dG and dT triphosphate were purchased from *New England BioLabs*), 0.4 mM of the corresponding 2'-(*R*)-fluorinated triphosphate, 0.25 µM of both primers and 2 units of the corresponding polymerase (KOD XL was purchased from *Novagen*, Therminator and Phusion polymerase were purchased from *New England BioLabs*) in the corresponding buffer (as recommended by the supplier of the polymerase) were incubated according to the following protocol. For this purpose a Mastercycler Personal (*Eppendorf*) was used.

| Step | | time / s | T / °C |
|---|---|---|---|
| denaturing | | 120 | 98 |
| 30 cycles | denaturing | 20 | 98 |
| | annealing | 20 | 55 |
| | elongations | 25 | 72 |
| final elongation | | 300 | 72 |

The obtained PCR products were purified with Nucleo Spin® Kits (*Macherey-Nagel*) and analyzed by gel electrophoresis.

## 2.2. Gel electrophoresis

For analyzing the PCR-products 16 µL of the PCR-product (18.75 ng/µL) were mixed with 4 µL of 5 × loading buffer (*New England BioLabs*) and analyzed by 2% agarose gel (1 g agarose, 50 mL 1 × TBE-buffer (10.8 g tris-(hydroxymethyl)aminomethan, 0.7 g $Na_2EDTA{\cdot}2H_2O$, 5.5 g boric acid and 7 µL Peqgreen (*Peqlab*)) for staining that was run with a horizontal cell (Sub-Cell, *BioRad*) at 60 V in 0.5 × TBE buffer for 30 min. FastRuler Low Range DNA ladder (*ThermoScientific*) or 1 kb DNA ladder (*New England BioLabs*) were used as a marker and they were mixed with a synthesized 46 bp oligonucleotide (10 µM). A Raytest Image Documentation Analysis Imager was used for visualization.

## 3. Extinction coefficients of several nucleosides

Extinction coefficient for the corresponding nucleosides are as following:

| **Nucleoside** | $\varepsilon_{260\ nm}$ / L·mmol$^{-1}$·cm$^{-1}$ | $\lambda_{max}$ / nm | **Nucleoside** | $\varepsilon_{260\ nm}$ / L·mmol$^{-1}$·cm$^{-1}$ |
|---|---|---|---|---|
| 2'-(*R*)-F-dC | 7.2 | 270 | dC | 7.1 |
| 2'-(*R*)-F-mdC | 5.5 | 276 | mdC | 7.8 |
| 2'-(*R*)-F-hmdC | 6.1 | 273 | mdC | 8.7 |
| 2'-(*R*)-F-fdC | 8.1 | 282 | fdC | 11.3 |
| 2'-(*R*)-F-cadC | 3.9 | 280 | cadC | 7.1 |
| 2'-(*R*)-F-dU | 9.3 | 260 | dU | 9.4 |
| 2'-(*R*)-F-hmdU | 9.1 | 263 | | |
| 2'-(*R*)-F-dT | 8.4 | 266 | dT | 8.4 |

## 4. Enzymatic digestion

**ODN2** and **ODN3** in 35 µL $H_2O$ were digested as follows: An aqueous solution (7.5 µL) of 480 µM $ZnSO_4$, containing 42 units Nuclease S1, 5 units antarctic phosphatase, and specific amounts of labeled internal standards (see below) was added and the mixture was incubated at 37 °C for 3 h in a *Thermomixer comfort* (*Eppendorf*). After addition of 7.5 µL of a 520 µM $[Na]_2$-EDTA solution containing 0.2 units snake venom phosphodiesterase I, the sample was incubated for another 3 h at 37 °C. The total volume was 50 µL. The sample was then kept at -20 °C until the day of analysis. Samples were then filtered by using an AcroPrep™ Advance 96 filter plate 0.20 µm Supor® (*Pall Life Sciences*) and then analyzed by LC-MS/MS according to the below mentioned procedure.

## 5. LC-ESI-MS

LC-ESI-MS/MS analysis was performed using an *Agilent* 1290 UHPLC system and an *Agilent* 6490 triple quadrupole mass spectrometer coupled with the stable isotope dilution technique. DNA samples were digested to give a nucleoside mixture and spiked with specific amounts of the corresponding isotopically labeled standards before LC-MS/MS analysis (see below). Quantification of 2'-(*R*)-F-xdC nucleosides was performed by the use of external calibrations curves. The nucleosides were analyzed in the positive as well as in the negative ion selected reaction monitoring mode (SRM). In the positive ion mode $[M+H]^+$ species and in the negative ion mode $[M-H]^-$ species were measured. The specific MS/MS transitions which gave highest intensities during our method development are summarized in Table 1. MS/MS fragmentation patterns of these compounds were partly earlier reported by Cao et. al.[1], Wang et. al.[2] and cited references therein. For source dependent parameters see[3].

For compound-dependent parameters see **Supplementary Table 1**.

**Supplementary Table 1. Compound-dependent LC-MS/MS-parameters used for the analysis of genomic DNA. CE: collision energy, CAV: collision cell accelerator voltage, EMV: electron multiplier voltage. The nucleosides were analyzed in the positive ($[M+H)]^+$ species) as well as the negative ($[M-H]^-$ species) ion selected reaction monitoring mode (SRM).**

| compound | Precursor ion (m/z) | MS1 Resolution | Product Ion (m/z) | MS2 Resolution | Dwell time [ms] | CE (V) | CAV (V) | Polarity |
|---|---|---|---|---|---|---|---|---|
| **Time segment 1.5-3.3 min** | | | | | | | | |
| F-dC | 246.09 | Wide | 112.06 | Wide | 70 | 15 | 3 | Positive |
| F-hmdC | 276.10 | Wide | 142.06 | Wide | 50 | 10 | 3 | Positive |
| [$^{15}N_2$]-cadC | 274.08 | Wide | 158.03 | Wide | 40 | 5 | 5 | Positive |
| cadC | 272.09 | Wide | 156.04 | Wide | 40 | 5 | 5 | Positive |
| [$^{15}N_2$,$D_2$]-hmdC | 262.12 | Wide | 146.07 | Wide | 25 | 27 | 1 | Positive |
| hmdC | 258.11 | Wide | 142.06 | Wide | 25 | 27 | 1 | Positive |
| [$D_3$]-mdC | 245.13 | Wide | 129.09 | Wide | 50 | 60 | 1 | Positive |
| mdC | 242.11 | Wide | 126.07 | Wide | 50 | 60 | 1 | Positive |
| **Time segment 3.3-4.8 min** | | | | | | | | |
| F-mdC | 260.10 | Wide | 126.07 | Wide | 160 | 15 | 3 | Positive |
| F-cadC | 290.08 | Wide | 156.04 | Wide | 80 | 5 | 5 | Positive |
| F-hmdU | 275.07 | Wide | 255.06 | Wide | 80 | 3 | 7 | Negative |
| F-dU | 245.06 | Wide | 225.06 | Wide | 80 | 3 | 5 | Negative |
| **Time segment 4.8-12 min** | | | | | | | | |
| F-fdC | 274.08 | Wide | 140.05 | Wide | 70 | 15 | 3 | Positive |
| F-dT | 259.07 | Wide | 239.07 | Wide | 70 | 3 | 5 | Negative |
| F-fdU | 273.05 | Wide | 253.05 | Wide | 30 | 3 | 5 | Negative |
| dT | 243.10 | Wide | 127.05 | Wide | 50 | 5 | 5 | Positive |
| [$^{15}N_2$]-fdC | 258.09 | Wide | 142.04 | Wide | 30 | 5 | 5 | Positive |
| fdC | 256.09 | Wide | 140.05 | Wide | 30 | 5 | 5 | Positive |

## 6. TET1cd oxidation assay

The HEK293T cells (ATCC) were cultivated at 37 °C in water saturated, $CO_2$-enriched (5%) atmosphere. RPMI (10% FBS) was used as growing medium. When reaching a confluence of 70% to 80% the cells were passaged. The transfection was performed in p150-petridishes. After seeding (5 million cells each), the cells were incubated as previously described cultivation conditions for 24 h to reach a confluence of 60% to 80%. 10 µg of the TET1cd construct and 30 µL of the transfection reagent jetPRIME® purchased from *Polyplus Transfection* were used as described by the manufacturer. The expression plasmid for GFP-Tet1cd was described previously[4]. To increase the transcription rate in transfected cells, 4 h after transfection the medium was removed and sodium butyrate (final conc. 4 mM) treated medium was added. After 48 h the cells were harvested and immediately used for nuclear extract preparation. The cells were centrifuged and lysed with 2 mL of RIPA buffer (Chromotek), supplemented with 250 U benzonase (Merck Millipore) and protease inhibitor (Roche). After 30 min on ice the suspension was centrifuged at 10000 x g and 4 °C for 15 min. The supernatant consisted of nuclear and cytoplasmic proteins. 100 µL of anti-GFP beads (Chromotek) were washed three times with wash buffer (10 mM Tris/Cl pH = 7.5, 150 mM NaCl; 0.5 mM EDTA) and then incubated for 1 h at 4°C with the supernatant. The GFP-Tet1cd loaded beads were washed with GFP wash buffer (Chromotek). Another two wash steps with 1 M NaCl solution containing 10 mM HEPES, pH = 7.5 were conducted followed by two wash steps with GFP wash buffer. The beads were centrifuged at 2500 x g,at 4 °C for 5 min and the supernatant was discarded. Next, the beads were split into two test tubes and the reaction buffer (50 mM HEPES pH = 7.5, 100 mM NaCl, 1 mM α-ketoglutarate, 2 mM Vitamin C, 1.2 mM ATP, 2.5 mM DTT, 0.1 mM $Fe^{(II)}(NH_4)_2(SO_4)_2 \cdot 6H_2O$) was added. As a control for the functionality of TET1cd, we used 500 pmol of an oligonucleotide containing mdC (5' – UUU UG**mdC** GGU UG – 3', see **Figure S1**). The reaction mixture was incubated at 37 °C for 3 h. After centrifugation (15000 rpm), the supernatant of the oxidation reaction was desalted using a desalting membrane and analyzed via MALDI-TOF/MS. For the investigation regarding the oxidation of 2'-(*R*)-F-mdC, 250 ng of the 2'-(*R*)-F-mdC-PCR-product (OCT4 promoter fragment, for the exact sequence see **Figure 2A**) was used and the reaction mixture incubated at 37 °C for 3 h. After centrifugation (15000 rpm), the supernatant of the 2'-(*R*)-F-mdC-reaction was digested and analysed by LC-MS/MS as described.[5] As a control, we furthermore incubated likewise 250 ng of the same 81 basepair long OCT4 promoter fragment containing mdC instead of 2-(*R*)-F-mdC with TET1cd enzyme.

**Supplementary Table 2: Product distribution mdC-PCR fragment after incubation with TET1cd and subsequent LC-MS/MS analysis**

| | |
|---|---|
| mdC | 92.7% |
| hmdC | 0% |
| fdC | 3.0% |
| cadC | 4.3% |

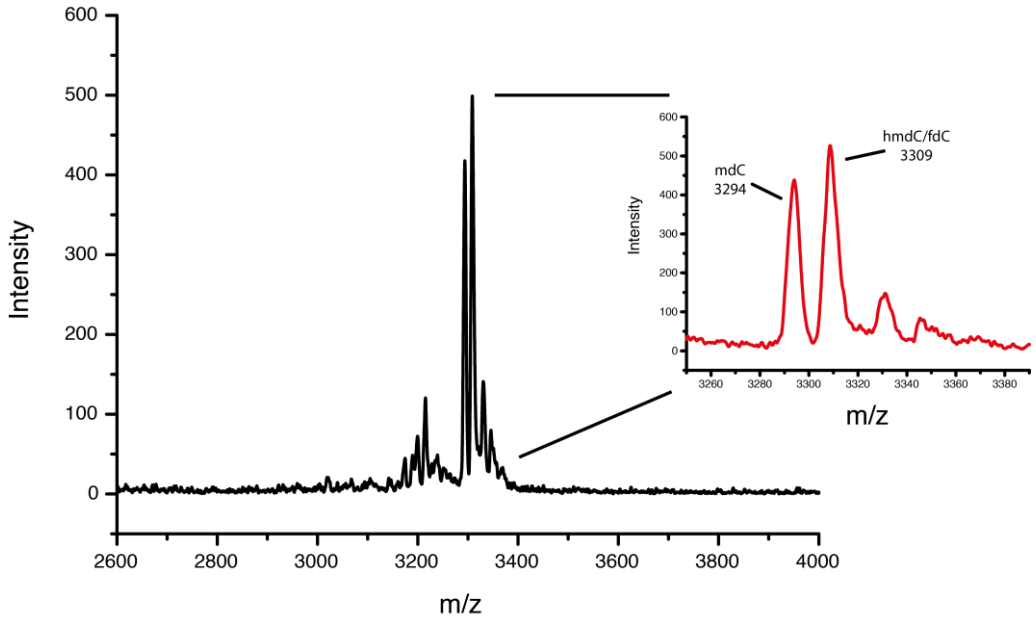
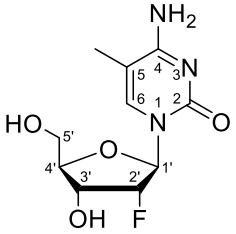



**Figure S2:** MALDI-TOF spectrum of the control of TET1cd oxidation assay with an oligonucleotide (5' – UUU UGmdC GGU UG – 3'). The formation of hmdC and fdC proves the oxidation activity of TET1cd.

# 7. Synthesis of fluorinated nucleosides and triphosphates

## 7.1. Synthesis of 5-Methyl-2'-deoxy-2'-(*R*)-fluoro-cytidine (7)




In a polypropylene tube 1.40 g of compound **12a** (2.87 mmol, 1.0 eq) were dissolved in 41 mL EtOAc (0.07 M). To the solution 1.16 mL pyridine (14.4 mmol, 5.0 eq) and 746 µL HF·pyridine (70% HF, 28.7 mmol, 10.0 eq) were added and the mixture was stirred at room temperature for 18 h. After complete conversion 2.87 mL TMSOMe (1 mL/mmol) were added and the suspension was stirred for 30 min. Subsequently, the suspension was centrifuged at 6000 rpm for 15 min, the supernatant was decanted and the residue was dissolved in dd$H_2O$. After lyophilization and purification by preparative, reversed phase HPLC 580 mg of compound **7** (2.24 mmol, 78%) were obtained as a colorless solid.

**TLC:** $R_f$ = 0.13 (DCM/MeOH 5:1).

**$^1$H-NMR (400 MHz, $D_2O$, ppm):** δ = 7.67 (s, 1H, 6-H), 6.00 (d, $^3J_{H-F}$ = 20.9 Hz, 1H, 1'-H), 5.14 (dd, $^2J_{H-F}$ = 53.1 Hz, $^3J$ = 5.7 Hz, 1H, 2'-H), 4.37 (ddd, $^3J_{H-F}$ = 22.2 Hz, $^3J$ = 8.9 Hz, $^3J$ = 4.7 Hz, 1H, 3'-H), 4.14 (dd, $^3J$ = 12.0 Hz, $^3J$ = 3.3 Hz, 1H, 4'-H), 4.04 (dd, $^2J$ = 13.0 Hz, $^3J$ = 2.4 Hz, 1H, 5'-H), 3.86 (dd, $^2J$ = 11.9 Hz, $^3J$ = 4.5 Hz, 1H, 5'-H), 1.97 (s, 3H, $CH_3$).

**$^{13}$C-NMR (101 MHz, $D_2O$, ppm):** δ = 166.2 (C4), 157.0 (C2), 139.1 (C6), 104.4 (C5), 93.7 (d, $^1J_{C-F}$ = 184.5 Hz, C2'), 89.7 (d, $^2J_{C-F}$ = 35.1 Hz, C1'), 82.1 (C4'), 67.8 (d, $^2J_{C-F}$ = 16.6 Hz, C3'), 59.8 (C5'), 12.2 ($CH_3$).

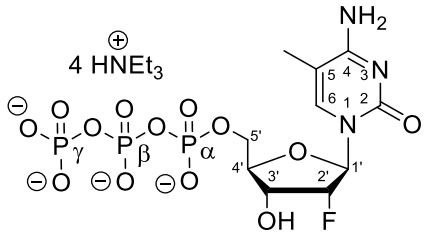
**$^{19}$F-NMR (376 MHz, $D_2O$, ppm):** δ = −200.3 (ddd, $^2J_{F-H}$ = 53.2 Hz, $^3J_{F-H}$ = 22.2 Hz, $^3J_{F-H}$ = 20.1 Hz).

**HRMS (ESI+):** calc. for $C_{10}H_{15}FN_3O_4^+$ $[M+H]^+$: 260.1041 found: 260.1042.

**IR (ATR):** ν ($cm^{-1}$) = 3345.3 (w), 1650.7 (s), 1495 (m), 1436 (m), 1350 (w), 1250 (m), 1105 (m), 1068 (w), 778 (w), 651 (w).

**Melting range:** 164-165 °C (decomposition).

## 7.2. 5-Methyl-2'-deoxy-2'-(*R*)-fluoro-cytidine-5'-triphosphate (as tetrakis (triethylammonium salt) (11a)




21 mg 2'-(*R*)-F-mdC (**7**, 80 µmol, 1.0 eq) and 132 mg bis(tributylammonium)pyrophosphate (240 µmol, 3.0 eq) were dried in high vacuum and 450 µL tributylamine (1.90 mmol, 23.7 eq) was dried over 3 Å molecular sieve for 15 h. Subsequently, bis(tributylammonium) pyrophosphate was dissolved in 405 µL DMF, tributylamine was added, the resulting emulsion mixed with a solution of 49 mg 2-Chloro-1,3,2-benzodioxaphosphorin-4-one (240 µmol, 3.0 eq) in 405 µL DMF and stirred at room temperature for 30 min. In the further course, this solution was mixed with dried 2'-(*R*)-F-mdC at 0 °C and was slowly warmed to room temperature. After 3 h, complete conversion was detected by TLC and subsequently, a iodine solution (20 mM $I_2$ in pyridine/dd$H_2O$ 9:1, ca. 1 mL) was added until the slightly brownish color of the solution retained for 15 min. Thereafter, 2.5 mL dd$H_2O$ and after 1 h 1.5 mL of an 3 M aq NaCl-solution were added. The solution was transferred into a polypropylene tube and rigorously shaken for 10 sec. 17 mL absolute ethanol was added and the crude product was precipitated for 40 min at −80 °C. Subsequently, the suspension was centrifuged (5 min, 6000 rpm), the supernatant was removed, the residue was dissolved in 1.5 mL buffer A and

lyophilized. After purification by preparative *reversed phase* HPLC (0-15% buffer B in 45 min) for three times, 6.7 µmol of compound **11a** (8%, determined by UV/VIS-spectroscopy) were obtained as a colorless solid.

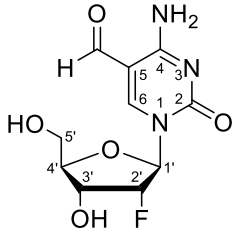
**TLC:** $R_f$ = 0.40 (DCM/MeOH 3:1).

**$^1$H-NMR (400 MHz, $D_2O$, ppm):** δ = 7.81 (s, 1H, 6-H), 5.97 (d, $^3J_{H\text{-}F}$ = 16.8 Hz, 1H, 1'-H), 5.02 (dd, $^2J_{H\text{-}F}$ = 52.7 Hz, $^3J$ = 4.3 Hz, 1H, 2'-H), 4.41 (ddd, $^3J_{H\text{-}F}$ = 23.0 Hz, $^3J$ = 8.5 Hz, $^3J$ = 4.6 Hz, 1H, 3'-H), 4.31 (ddd, $^2J$ = 12.2 Hz, $^3J_{H\text{-}P}$ = 4.0 Hz, $^3J$ = 1.9 Hz, 1H, 5'-H), 4.23-4.13 (m, 2H, 4'-H, 5'-H), 1.88 (s, 3H, $CH_3$).

**$^{31}$P-NMR (162 MHz, $D_2O$, ppm):** δ = −11.0 (d, $^2J_{P\text{-}P}$ = 19.9 Hz, γ-P), −11.8 (dt, $^2J_{P\text{-}P}$ = 20.0 Hz, $^3J_{P\text{-}H}$ = 4.0 Hz, α-P), −23.4 (t, $^2J_{P\text{-}P}$ = 20.0 Hz, β-P).

**HRMS (ESI−):** calc. for $C_{10}H_{16}FN_3O_{13}P_3^-$ [M−H]$^-$: 497.9886 found: 497.9886.

## 7.3. 5-Formyl-2'-deoxy-2'-(*R*)-fluoro-cytidine (9)




In a polypropylene tube 704 mg of compound **12c** (1.40 mmol, 1.0 eq) were dissolved in 20 mL EtOAc (0.07 M). To the solution 565 µL pyridine (7.00 mmol, 5.0 eq) and 364 µL HF·pyridine (70% HF, 14.0 mmol, 10.0 eq) were added and the mixture was stirred at room temperature for 20 h. After complete conversion 1.40 mL TMSOMe (1 mL/mmol) were added and the suspension was stirred for 30 min. Subsequently, the suspension was centrifuged at 6000 rpm for 15 min, the supernatant was decanted and the residue was dissolved in dd$H_2O$. After lyophilization of the crude product and purification by preparative, reversed phase HPLC, 380 mg of compound **9** (1.39 mmol, 98%) were obtained as a colorless solid.

**TLC:** $R_f$ = 0.13 (DCM/MeOH 5:1).

**$^1$H-NMR (400 MHz, $D_2O$, ppm):** δ = 9.51 (s, 1H, C(=O)-H), 8.88 (s, 1H, 6-H), 6.07 (d, $^3J_{H\text{-}F}$ = 17.7 Hz, 1H, 1'-H), 5.19 (dd, $^2J_{H\text{-}F}$ = 52.4 Hz, $^3J$ = 4.3 Hz, 1H, 2'-H), 4.37 (ddd, $^3J_{H\text{-}F}$ = 24.1 Hz, $^3J$ = 9.3 Hz, $^3J$ = 4.3 Hz, 1H, 3'-H), 4.27-4.19 (m, 1H, 4'-H), 4.13 (dd, $^2J$ = 13.2 Hz, $^3J$ = 2.3 Hz, 1H, 5'-H), 3.91 (dd, $^2J$ = 13.2 Hz, $^3J$ = 3.5 Hz, 1H, 5'-H).

**$^{13}$C-NMR (101 MHz, $D_2O$, ppm):** δ = 190.2 (C(=O)-H), 162.8 (C4), 155.0 (C2), 154.9 (C6), 105.9 (C5), 93.7 (d, $^1J_{C\text{-}F}$ = 185.2 Hz, C2'), 89.9 (d, $^2J_{C\text{-}F}$ = 34.9 Hz, C1'), 82.4 (C4'), 67.2 (d, $^2J_{C\text{-}F}$ = 16.8 Hz, C3'), 59.1 (C5').

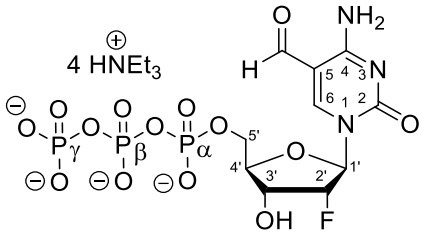
**$^{19}$F-NMR (376 MHz, $D_2O$, ppm):** δ = −201.6 (ddd, $^2J_{F-H}$ = 52.4 Hz, $^3J_{F-H}$ = 24.3 Hz, $^3J_{F-H}$ = 17.8 Hz).

**HRMS (ESI+):** calc. for $C_{10}H_{13}FN_3O_5^+$ $[M+H]^+$: 274.0834 found: 274.0835.

**IR (ATR):** ν ($cm^{-1}$) = 3412 (w), 3314 (w), 1638 (s), 1597 (s), 1419 (m), 1236 (m), 1096 (s), 1062 (s), 987 (m), 947 (m), 789 (s), 763 (m).

**Melting range:** 168-169 °C (decomposition).

## 7.4. 5-Formyl-2'-deoxy-2'(*R*)-fluoro-cytidine-5'-triphosphate (as tetrakis (triethylammonium salt) (11c)




21 mg 2'-(*R*)-F-mdC (**9**, 80 µmol, 1.0 eq) and 132 mg bis(tributylammonium)pyrophosphate (240 µmol, 3.0 eq) were dried in high vaccum and 450 µL tributylamine (1.90 mmol, 23.7 eq) was dried over 3 Å molecular sieve for 15 h. Subsequently, bis(tributylammonium) pyrophosphate was dissolved in 405 µL DMF, tributylamine was added, the resulting emulsion mixed with a solution of 49 mg 2-Chloro-1,3,2-benzodioxaphosphorin-4-one (240 µmol, 3.0 eq) in 405 µL DMF and stirred at room temperature for 30 min. In the further course, this solution was mixed with dried 2'-(*R*)-F-mdC at 0 °C and was slowly warmed to room temperature. After 3 h, complete conversion was detected by TLC and subsequently, a iodine solution (20 mM $I_2$ in pyridine/dd$H_2O$ 9:1, ca. 1 mL) was added until the slightly brownish color of the solution retained for 15 min. Thereafter, 2.5 mL dd$H_2O$ and after 1 h 1.5 mL of an 3 M aq NaCl-solution were added. The solution was transferred into a polypropylene tube and rigorously shaken for 10 sec. 17 mL absolute ethanol was added and the crude product was precipitated for 40 min at −80 °C. Subsequently, the suspension was centrifuged (5 min, 6000 rpm), the supernatant was removed, the residue was dissolved in 1.5 mL buffer A and lyophilized. After purification by preparative *reversed phase* HPLC (0-15% buffer B in 45 min) for three times, 2.4 µmol of compound **11c** (3%, determined by UV/VIS-spectroscopy) were obtained as a colorless solid.

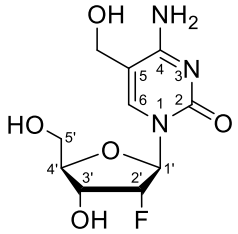
**TLC:** $R_f$ = 0.81 (DCM/MeOH 3:1).

**$^1$H-NMR (400 MHz, $D_2O$, ppm):** δ = 9.56 (s, 1H, C(=O)-H), 8.79 (s, 1H, 6-H), 5.98 (d, $^3J_{H\text{-}F}$ = 16.6 Hz, 1H, 1'-H), 5.05 (dd, $^2J_{H\text{-}F}$ = 52.6 Hz, $^3J$ = 4.8 Hz, 1H, 2'-H), 4.47-4.34 (m, 2H, 3'-H, 5'-H), 4.28-4.17 (m, 2H, 4'-H, 5'-H).

**$^{31}$P-NMR (162 MHz, $D_2O$, ppm):** δ = −11.0 (d, $^2J_{P\text{-}P}$ = 19.8 Hz, γ-P), −11.8 (dt, $^2J_{P\text{-}P}$ = 20.1 Hz, $^3J_{P\text{-}H}$ = 3.1 Hz, α-P), −23.4 (t, $^2J_{P\text{-}P}$ = 20.0 Hz, β-P).

**HRMS (ESI−):** calc. for $C_{10}H_{14}FN_3O_{14}P_3^-$ [M−H]$^-$: 511.9678 found: 511.9679.

## 7.5. 5-Hydroxymethyl-2'-deoxy-2'-(*R*)-fluoro-cytidine (8)




### 7.5.1. Procedure 1:

In a round bottom flask 25 mg of compound **9** (91 µmol, 1.0 eq) were dissolved in 4 mL MeOH (0.023 M) and 102 mg $CeCl_3 \cdot 7\ H_2O$ (274 µmol, 3.0 eq) were added. After complete dissolving of the reagent 3.4 mg $NaBH_4$ (91 µmol, 1.0 eq) were slowly added and stirred for 1 h at room temperature. After complete conversion 1 mL of a 1 M $HNEt_3OAc$-solution was added to neutralize the solution. After removal of the solvent *in vacuo* und purification by preparative *reversed phase* HPLC 24 mg of compound **8** (87 µmol, 96%) were obtained as a colorless solid.

### 7.5.2. Procedure 2:

In a polypropylene tube 1.00 g of compound **12b** (1.98 mmol, 1.0 eq) were dissolved in 28 mL EtOAc (0.07 M). To the solution 799 µL pyridine (9.90 mmol, 5.0 eq) and 515 µL HF·pyridine (70% HF, 19.8 mmol, 10.0 eq) were added and the mixture was stirred at room temperature for 24 h. After complete conversion 1.98 mL TMSOMe (1 mL/mmol) were added and the suspension was stirred for 30 min. Subsequently, the solution was concentrated to dryness and the crude product was purified via column chromatography (DCM/MeOH 5:1 → 3:1) and additionally by preparative, reversed phase HPLC to yield 336 mg of compound **8** (1.22 mmol, 62%) as a colorless solid.

**TLC:** $R_f$ = 0.13 (DCM/MeOH 5:1).

**$^1$H-NMR (400 MHz, $D_2O$, ppm):** δ =7.94 (s, 1H, 6-H), 6.01 (d, $^3J_{H\text{-}F}$ = 19.2 Hz, 1H, 1'-H), 5.16 (dd, $^2J_{H\text{-}F}$ = 52.9 Hz, $^3J$ = 4.6 Hz, 1H, 2'-H), 4.45 (s, 2H, C5'-$CH_2$), 4.37 (ddd, $^3J_{H\text{-}F}$ = 22.8 Hz, $^3J$ = 9.0 Hz, $^3J$ = 4.6 Hz, 1H, 3'-H), 4.16 (dd, $^3J$ = 8.9, Hz, $^3J$ = 3.0 Hz, 1H, 4'-H), 4.06 (dd, $^2J$ = 13.0 Hz, $^3J$ = 2.3 Hz, 1H, 5'-H), 3.87 (dd, $^2J$ = 13.1 Hz, $^3J$ = 4.1 Hz, 1H, 5'-H).

**$^{13}$C-NMR (101 MHz, $D_2O$, ppm):** δ = 165.3 (C4), 156.9 (C2), 140.9 (C6), 106.5 (C5), 93.7 (d, $^{1}J_{C\text{-}F}$ = 184.6 Hz, C2'), 89.8 (d, $^{2}J_{C\text{-}F}$ = 35.1 Hz, C1'), 82.1 (C4'), 67.7 (d, $^{2}J_{C\text{-}F}$ = 16.9 Hz, C3'), 59.6 (C5'), 57.7 (C5'-*C*$H_2$).

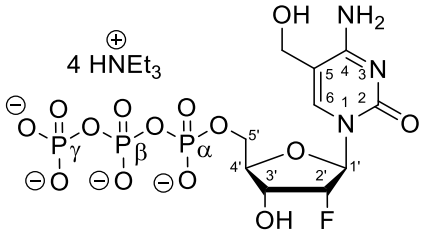
**$^{19}$F-NMR (376 MHz, $D_2O$, ppm):** δ = −201.2 (ddd, $^{2}J_{F\text{-}H}$ = 52.9 Hz, $^{3}J_{F\text{-}H}$ = 22.8 Hz, $^{3}J_{F\text{-}H}$ = 19.2 Hz).

**HRMS (ESI+):** calc. for $C_{10}H_{15}FN_3O_5^+$ $[M+H]^+$: 276.0990 found: 276.0991.

**IR (ATR):** ν ($cm^{-1}$) = 3476 (w), 3339 (w), 2890 (w), 1653 (s), 1489 (m), 1464 (m), 1299 (m), 1108 (s), 1069 (s), 786 (m).

**Melting range:** 169-170 °C (decomposition).

## 7.6. 5-Hydroxymethyl-2'-deoxy-2'-(*R*)-fluoro-cytidine-5'-triphosphate (as tetrakis (triethylammonium salt) (11b)




22 mg 2'-(*R*)-F-hmdC (**8**, 80 µmol, 1.0 eq) and 132 mg bis(tributylammonium)pyrophosphate (240 µmol, 3.0 eq) were dried in high vaccum and 450 µL tributylamine (1.90 mmol, 23.7 eq) was dried over 3 Å molecular sieve for 15 h. Subsequently, bis(tributylammonium) pyrophosphate was dissolved in 405 µL DMF, tributylamine was added, the resulting emulsion mixed with a solution of 49 mg 2-Chloro-1,3,2-benzodioxaphosphorin-4-one (240 µmol, 3.0 eq) in 405 µL DMF and stirred at room temperature for 30 min. In the further course, this solution was mixed with dried 2'-(*R*)-F-mdC at 0 °C and was slowly warmed to room temperature. After 3 h, complete conversion was detected by TLC and subsequently, a iodine solution (20 mM $I_2$ in pyridine/dd$H_2O$ 9:1, ca. 1 mL) was added until the slightly brownish color of the solution retained for 15 min. Thereafter, 2.5 mL dd$H_2O$ and after 1 h 1.5 mL of an 3 M aq NaCl-solution were added. The solution was transferred into a polypropylene tube and rigorously shaken for 10 sec. 17 mL absolute ethanol was added and the crude product was precipitated for 40 min at −80 °C. Subsequently, the suspension was centrifuged (5 min, 6000 rpm), the supernatant was removed, the residue was dissolved in 1.5 mL buffer A and lyophilized. After purification by preparative *reversed phase* HPLC (0-15% buffer B in 45 min) for three times, 7.2 µmol of compound **11b** (9%, determined by UV/VIS-spectroscopy) were obtained as a colorless solid.

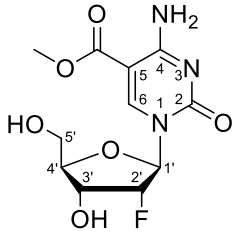
**TLC:** $R_f$ = 0.32 (DCM/MeOH 3:1).

**$^1$H-NMR (400 MHz, $D_2O$, ppm):** δ = 7.99 (s, 1H, 6-H), 5.96 (d, $^3J_{H-F}$ = 16.5 Hz, 1H, 1'-H), 5.02 (dd, $^2J_{H-F}$ = 52.1 Hz, $^3J$ = 4.8 Hz, 1H, 2'-H), 4.42 (ddd, $^3J_{H-F}$ = 19.3 Hz, $^3J$ = 8.9 Hz, $^3J$ = 4.3 Hz, 1H, 3'-H), 4.39 (s, 2H, C5-$CH_2$), 4.34 (ddd, $^2J$ = 12.5 Hz, $^3J_{H-P}$ = 3.8 Hz, $^3J$ = 2.5 Hz, 1H, 5'-H), 4.23-4.13 (m, 2H, 4'-H, 5'-H).

**$^{31}$P-NMR (162 MHz, $D_2O$, ppm):** δ = −11.0 (d, $^2J_{P-P}$ = 19.9 Hz, γ-P), −11.7 (dt, $^2J_{P-P}$ = 19.9 Hz, $^3J_{P-H}$ = 3.8 Hz, α-P), −23.4 (t, $^2J_{P-P}$ = 19.9 Hz, β-P).

**HRMS (ESI−):** calc. for $C_{10}H_{16}FN_3O_{14}P_3^-$ $[M-H]^-$: 513.9835 found: 513.9836.

## 7.7. 5-Methoxycarbonyl-2'-deoxy-2'-(*R*)-fluoro-cytidine (13)




In a polypropylene tube 1.18 g of compound **12d** (2.21 mmol, 1.0 eq) were dissolved in 32 mL EtOAc (0.07 M). To the solution 892 µL pyridine (11.1 mmol, 5.0 eq) and 574 µL HF·pyridine (70% HF, 22.1 mmol, 10.0 eq) were added and the mixture was stirred at room temperature for 24 h. After complete conversion 2.21 mL TMSOMe (1 mL/mmol) were added and the suspension was stirred for 30 min. Subsequently, the suspension was centrifuged at 6000 rpm for 15 min, the supernatant was decanted and the residue was dissolved in dd$H_2O$. After lyophilization of the crude product and purification by preparative, reversed phase HPLC, 600 mg of compound **13** (1.90 mmol, 86%) were obtained as a slightly yellowish solid.

**TLC:** $R_f$ = 0.33 (DCM/MeOH 10:1).

**$^1$H-NMR (400 MHz, DMSO-d6, ppm):** δ = 9.18 (s, 1H, 6-H), 8.03 (s, 1H, $NH_2$), 7.70 (s, 1H, $NH_2$), 5.90 (dd, $^3J_{H-F}$ = 17.1 Hz, $^3J$ = 5.1 Hz, 1H, 1'-H), 5.56 (s, 1H, 3'-OH), 5.33 (s, 1H, 5'-OH), 4.93 (dd, $^2J_{H-F}$ = 52.9 Hz, $^3J$ = 4.2 Hz, 1H, 2'-H), 4.22-4.08 (m, 1H, 3'-H), 4.98-4.82 (m, 2H, 4'-H, 5'-H), 3.81 (s, 3H, $OCH_3$), 3.61 (d, $^2J$ = 11.9 Hz, 1H, 5'-H).

**$^{13}$C-NMR (101 MHz, DMSO-d6, ppm):** δ = 165.2 (C(=O)-OH), 163.1 (C4), 153.0 (C2), 148.3 (C6), 94.8 (C5), 91.6 (d, $^1J_{C-F}$ = 188.4 Hz, C2'), 88.4 (d, $^2J_{C-F}$ = 33.8 Hz, C1'), 82.7 (C4'), 66.3 (d, $^2J_{C-F}$ = 17.2 Hz, C3'), 58.1 (C5'), 51.7 ($OCH_3$).

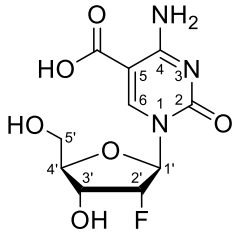
**$^{19}$F-NMR (376 MHz, DMSO-d6, ppm):** δ = −196.6 (ddd, $^2J_{F-H}$ = 53.4 Hz, $^3J_{F-H}$ = 26.7 Hz, $^3J_{F-H}$ = 17.2 Hz).

**HRMS (ESI+):** calc. for $C_{11}H_{15}FN_3O_6^+$ $[M+H]^+$: 304.0939, found: 304.0946.

**IR (ATR):** ν ($cm^{-1}$) = 3403 (w), 3302 (w), 1715 (s), 1650 (s), 1583 (m), 1417 (m), 1329 (m), 1074 (s), 798 (m).

**Melting range:** 202-204 °C (decomposition).

## 7.8. 5-Carboxy-2'-deoxy-2'-(*R*)-fluoro-cytidine (10)




In a round bottom flask 600 mg of compound **13** (1.90 mmol, 1.0 eq) were dissolved in 190 mL of a mixture of dd$H_2O$/MeCN (1:1, 0.01 M) and 550 mg LiOH (22.8 mmol, 12.0 eq) were added. The solution was stirred for 16 h at room temperature and subsequently, the pH of the solution was adjusted to pH = 4 with 2 N HCl. After removal of the solvent *in vacuo* and purification by preparative *reversed phase* HPLC 351 mg of compound **10** (1.22 mmol, 64%) were obtained as a colorless triethylammonium salt.

**TLC:** $R_f$ = 0.06 (DCM/MeOH 5:1).

**$^1$H-NMR (400 MHz, $D_2O$, ppm):** δ = 8.55 (s, 1H, 6-H), 6.05 (d, $^3J_{H\text{-}F}$ = 19.0 Hz, 1H, 1'-H), 5.17 (dd, $^2J_{H\text{-}F}$ = 52.7 Hz, $^3J$ = 4.6 Hz, 1H, 2'-H), 4.38 (ddd, $^3J_{H\text{-}F}$ = 22.8 Hz, $^3J$ = 9.0 Hz, $^3J$ = 4.6 Hz, 1H, 3'-H), 4.25-4.11 (m, 1H, 4'-H), 4.06 (dd, $^2J$ = 13.0 Hz, $^3J$ = 2.3 Hz, 1H, 5'-H), 3.88 (dd, $^2J$ = 11.9 Hz, $^3J$ = 4.5 Hz, 1H, 5'-H).

**$^{13}$C-NMR (101 MHz, $D_2O$, ppm):** δ = 170.3 (C(=O)-OH), 164.6 (C4), 155.6 (C2), 146.9 (C6), 102.9 (C5), 91.6 (d, $^1J_{C\text{-}F}$ = 188.4 Hz, C2'), 89.9 (d, $^2J_{C\text{-}F}$ = 35.3 Hz, C1'), 82.3 (C4'), 67.8 (d, $^2J_{C\text{-}F}$ = 16.8 Hz, C3'), 59.7 (C5').

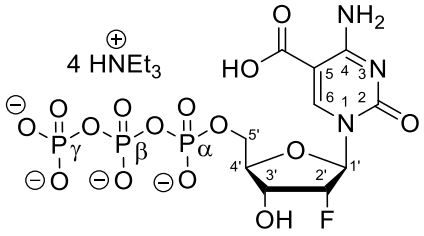
**$^{19}$F-NMR (376 MHz, $D_2O$, ppm):** δ = −200.8 (ddd, $^2J_{F\text{-}H}$ = 52.8 Hz, $^3J_{F\text{-}H}$ = 22.8 Hz, $^3J_{F\text{-}H}$ = 19.2 Hz).

**HRMS (ESI+):** calc. for $C_{10}H_{13}FN_3O_6^+$ $[M+H]^+$: 290.0783 found: 290.0785.

**IR (ATR):** ν ($cm^{-1}$) = 3248 (w), 1650 (s), 1559 (m), 1066 (m), 815 (w), 681 (w).

**Melting range:** 125-127 °C.

## 7.9. 5-Carboxy-2'-deoxy-2'-(*R*)-fluoro-cytidine-5'-triphosphate (as tetrakis(triethylammonium salt) (11d)




21 mg 2'-(*R*)-F-cadC (**10**, 80 µmol, 1.0 eq) and 132 mg bis(tributylammonium)pyrophosphate (240 µmol, 3.0 eq) were dried in high vaccum and 450 µL tributylamine (1.90 mmol, 23.7 eq) was dried over 3 Å molecular sieve for 15 h. Subsequently, bis(tributylammonium) pyrophosphate was dissolved in 405 µL DMF, tributylamine was added, the resulting emulsion mixed with a solution of 49 mg 2-Chloro-1,3,2-benzodioxaphosphorin-4-one (240 µmol, 3.0 eq) in 405 µL DMF and stirred at room temperature for 30 min. In the further course, this solution was mixed with dried 2'-(*R*)-F-mdC at 0 °C and was slowly warmed to room temperature. After 3 h, complete conversion was detected by TLC and subsequently, a iodine solution (20 mM $I_2$ in pyridine/dd$H_2O$ 9:1, ca. 1 mL) was added until the slightly brownish color of the solution retained for 15 min. Thereafter, 2.5 mL dd$H_2O$ and after 1 h 1.5 mL of an 3 M aq NaCl-solution were added. The solution was transferred into a polypropylene tube and rigorously shaken for 10 sec. 17 mL absolute ethanol was added and the crude product was precipitated for 40 min at −80 °C. Subsequently, the suspension was centrifuged (5 min, 6000 rpm), the supernatant was removed, the residue was dissolved in 1.5 mL buffer A and lyophilized. After purification by preparative *reversed phase* HPLC (0-15% buffer B in 45 min) for three times, 4.5 µmol of compound **11d** (6%, determined by UV/VIS-spectroscopy) were obtained as a colorless solid.

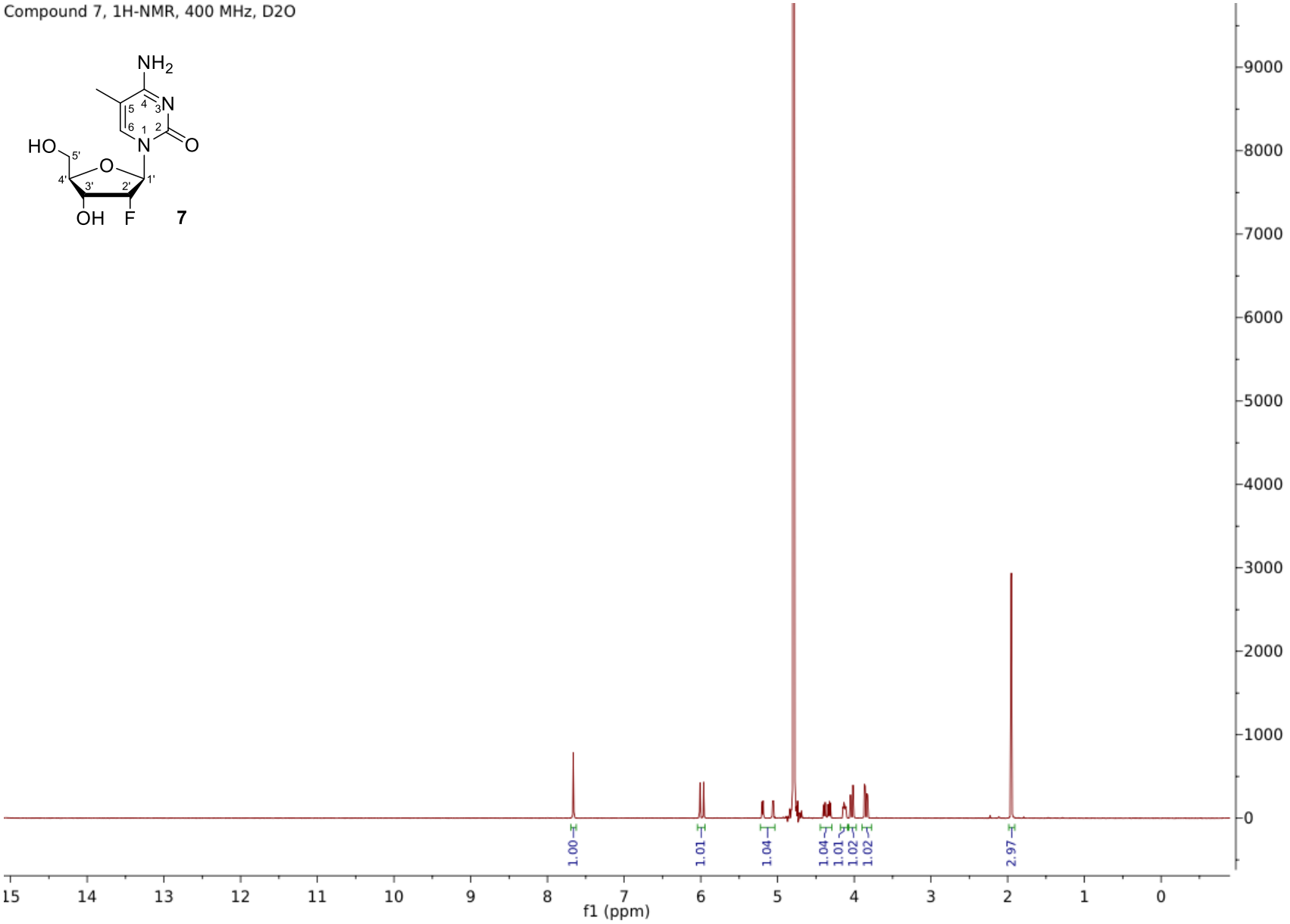
**TLC:** $R_f$ = 0.19 (DCM/MeOH 3:1).

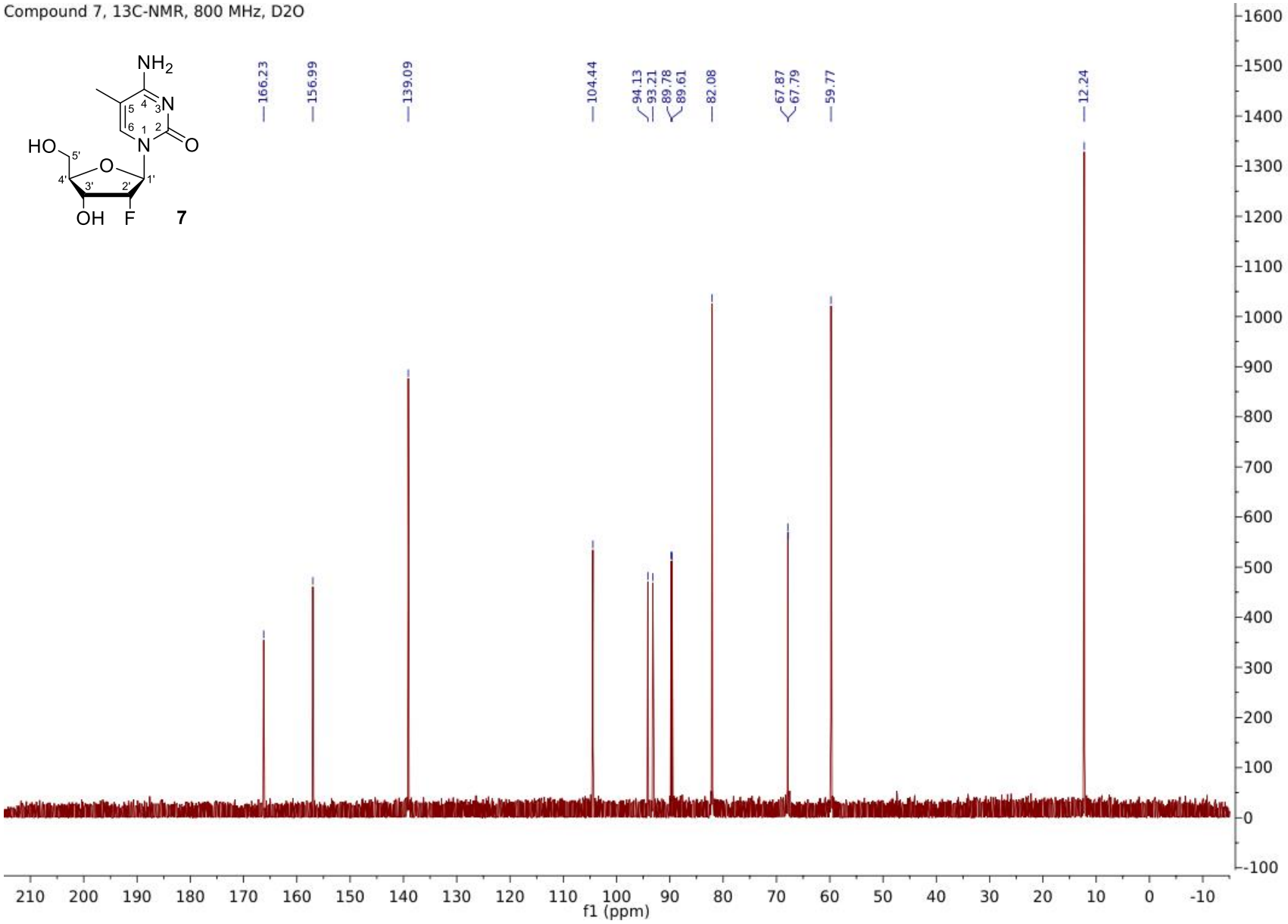
**$^1$H-NMR (400 MHz, $D_2O$, ppm):** δ = 8.38 (s, 1H, 6-H), 5.87 (d, $^3J_{H\text{-}F}$ = 20.3 Hz, 1H, 1'-H), 5.15 (dd, $^2J_{H\text{-}F}$ = 53.1 Hz, $^3J$ = 5.0 Hz, 1H, 2'-H), 4.40 (ddd, $^3J_{H\text{-}F}$ = 21.7 Hz, $^3J$ = 8.1 Hz, $^3J$ = 4.9 Hz, 1H, 3'-H), 4.27 (ddd, $^2J$ = 11.0 Hz, $^3J_{H\text{-}P}$ = 5.2 Hz, $^3J$ = 1.7 Hz, 1H, 5'-H), 4.22-4.10 (m, 2H, 4'-H, 5'-H).

**$^{31}$P-NMR (162 MHz, $D_2O$, ppm):** δ = −11.0 (d, $^2J_{P\text{-}P}$ = 19.8 Hz, γ-P), −11.4 (dt, $^2J_{P\text{-}P}$ = 19.8 Hz, $^3J_{P\text{-}H}$ = 5.2 Hz, α-P), −23.4 (t, $^2J_{P\text{-}P}$ = 19.7 Hz, β-P).

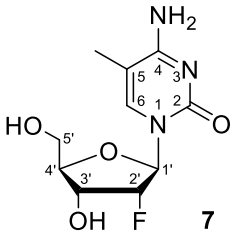
**HRMS (ESI−):** calc. for $C_{10}H_{14}FN_3O_{15}P_3^-$ $[M-H]^-$: 527.9627 found: 527.9628.

## 9. NMR spectra

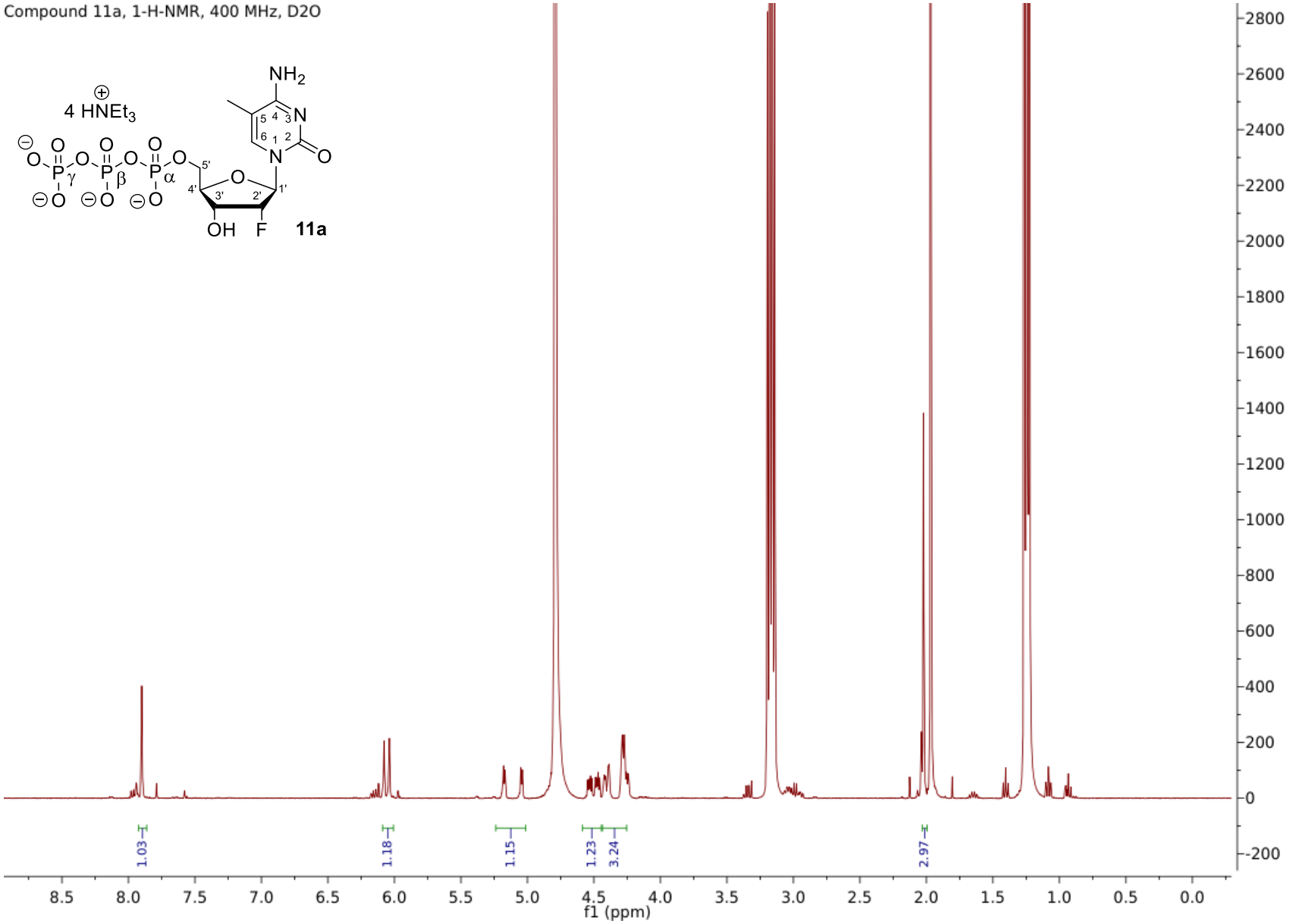



Compound 7, 13C-NMR, 800 MHz, D2O
NH2
N
O
HO
O
OH
F
7
166.23
156.99
139.09
104.44
94.13
93.21
89.78
89.61
82.08
67.87
67.79
59.77
12.24
f1 (ppm)

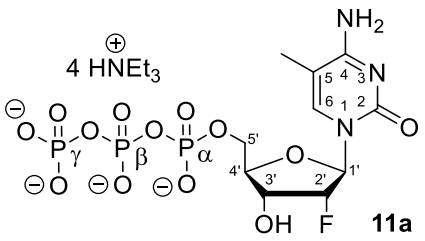
Compound 7, 19F-NMR, 400 MHz, D2O



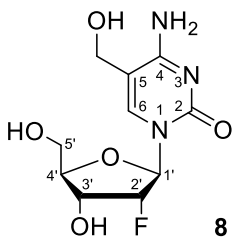

**7**


Compound 11a, 1-H-NMR, 400 MHz, D2O
4 HNEt3
NH2
OH
F
11a
1.03
1.18
1.15
1.23
3.24
2.97
f1 (ppm)

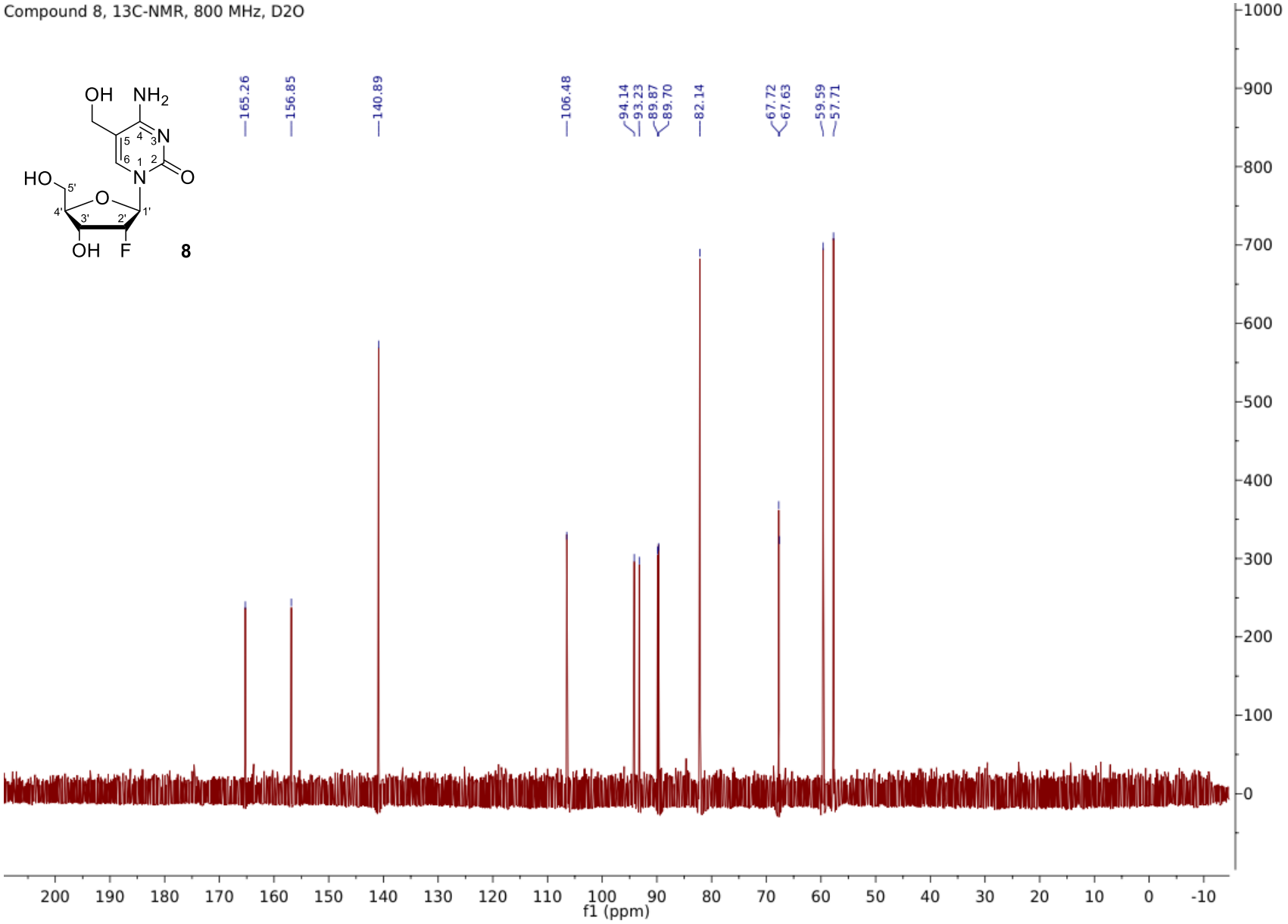
Compound 11a, 31P-NMR, 400 MHz, D2O



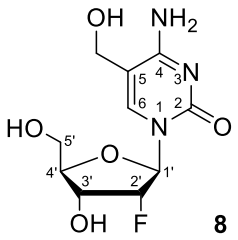
Compound 8, 1H-NMR, 400 MHz, D2O




**8**

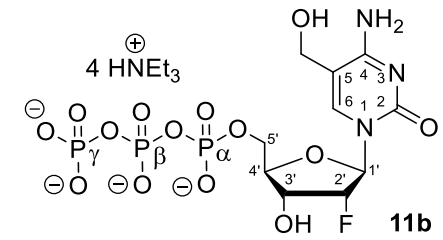

Compound 8, 13C-NMR, 800 MHz, D2O
OH
NH2
HO
O
OH
F
8
165.26
156.85
140.89
106.48
94.14
93.23
89.87
89.70
82.14
67.72
67.63
59.59
57.71
f1 (ppm)

Compound 8, 19F-NMR, 400 MHz, D2O



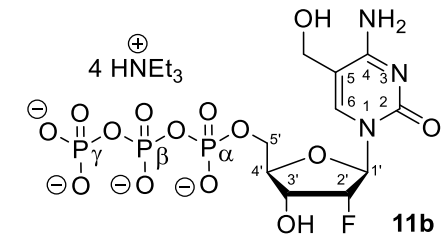

**8**

Compound 11b, 1H-NMR, 400 MHz, D2O

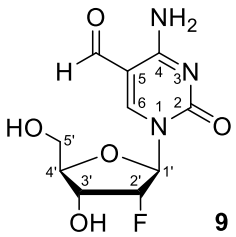



**11b**

Compound 11b, 1H-NMR, 400 MHz, D2O

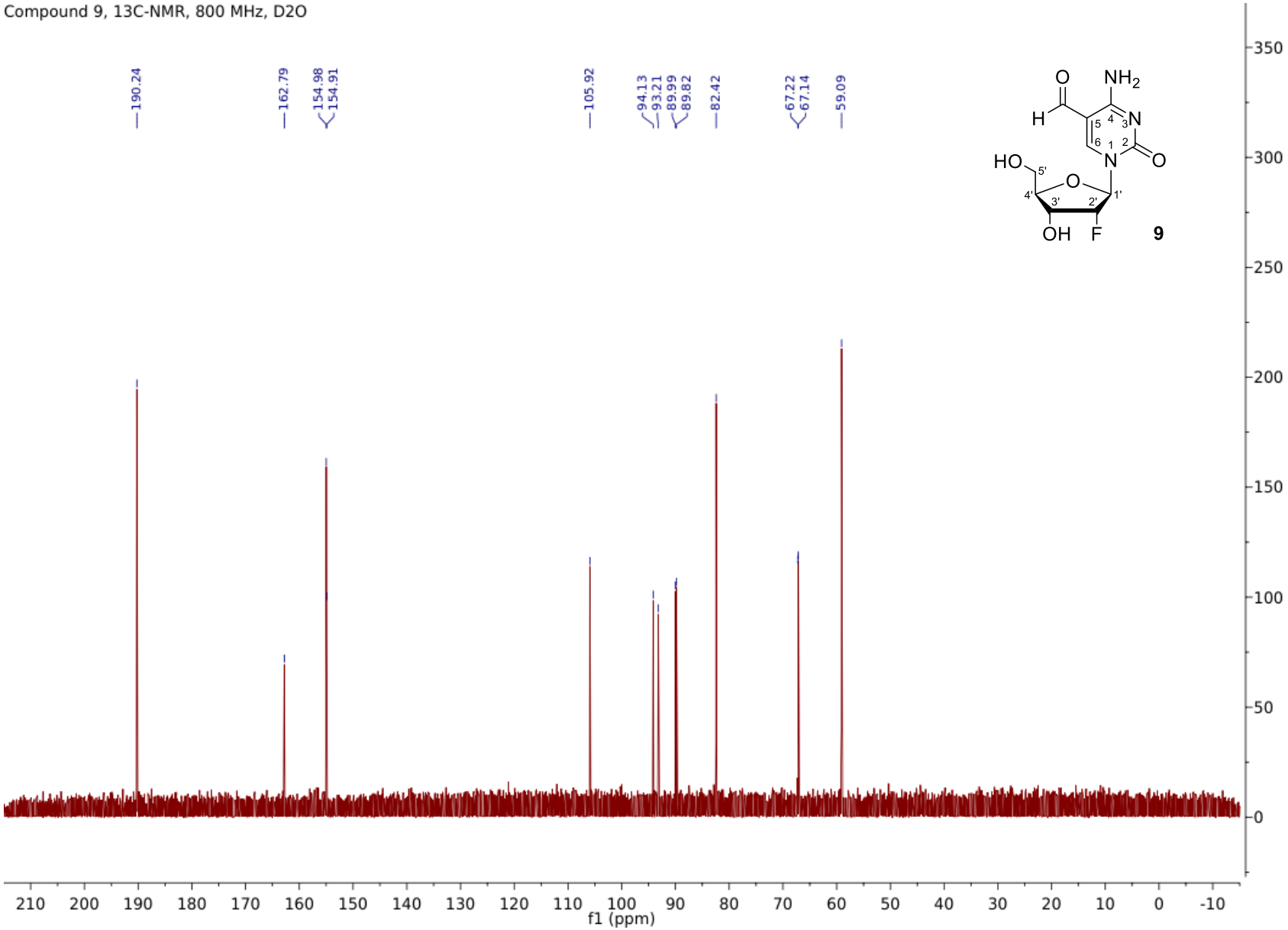


Compound 9, 1H-NMR, 400 MHz, D2O

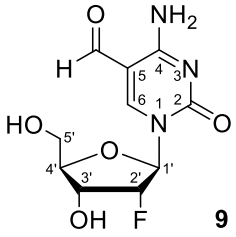



**9**

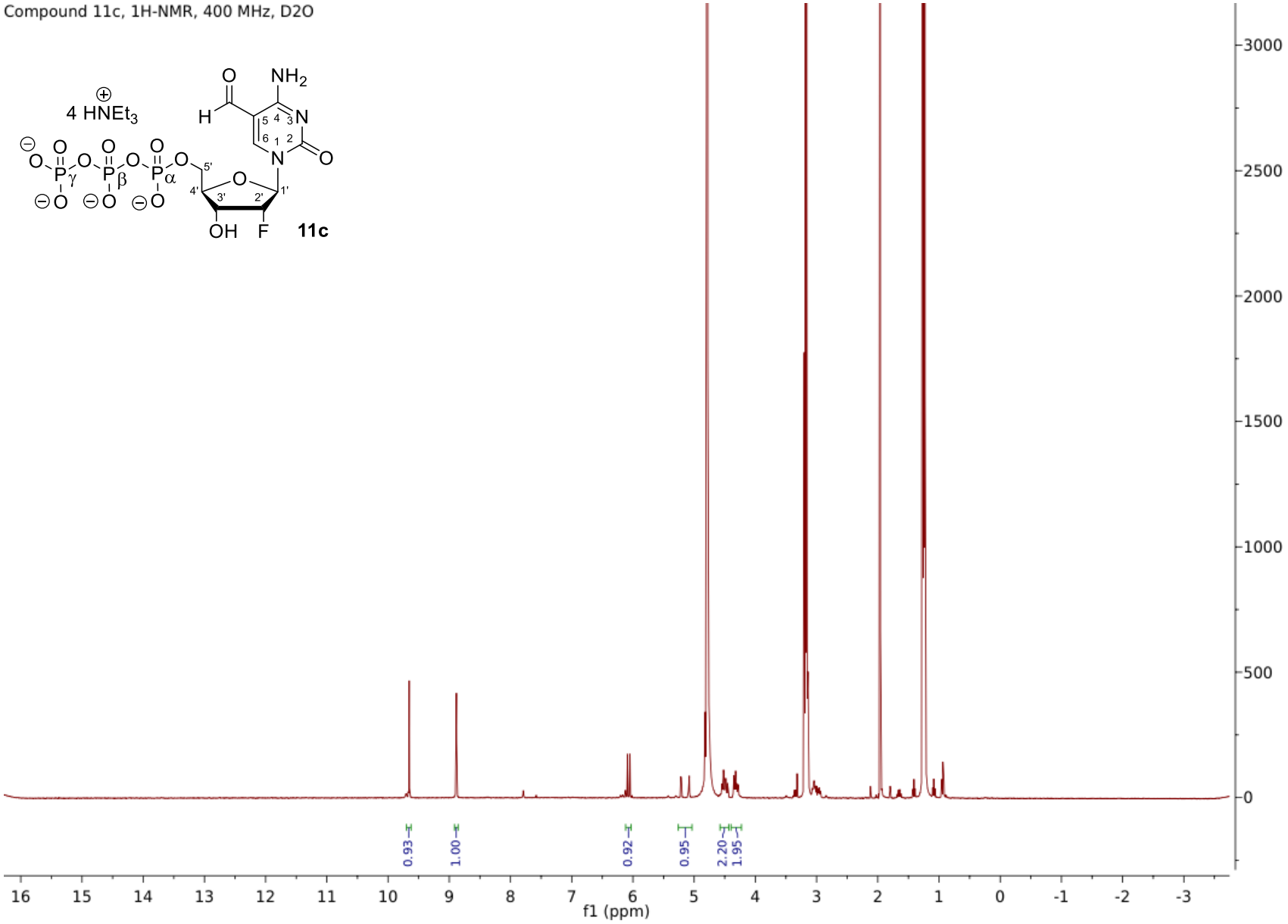
Compound 9, 13C-NMR, 800 MHz, D2O



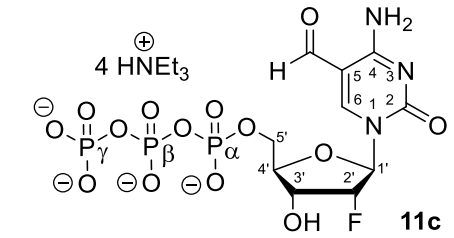
Compound 9, 19F-NMR, 400 MHz, D2O



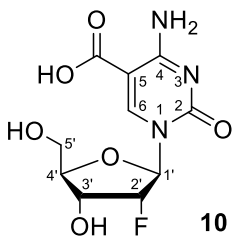

**9**


Compound 11c, 1H-NMR, 400 MHz, D2O
4 HNEt3
11c
0.93
1.00
0.92
0.95
2.20
1.95
f1 (ppm)

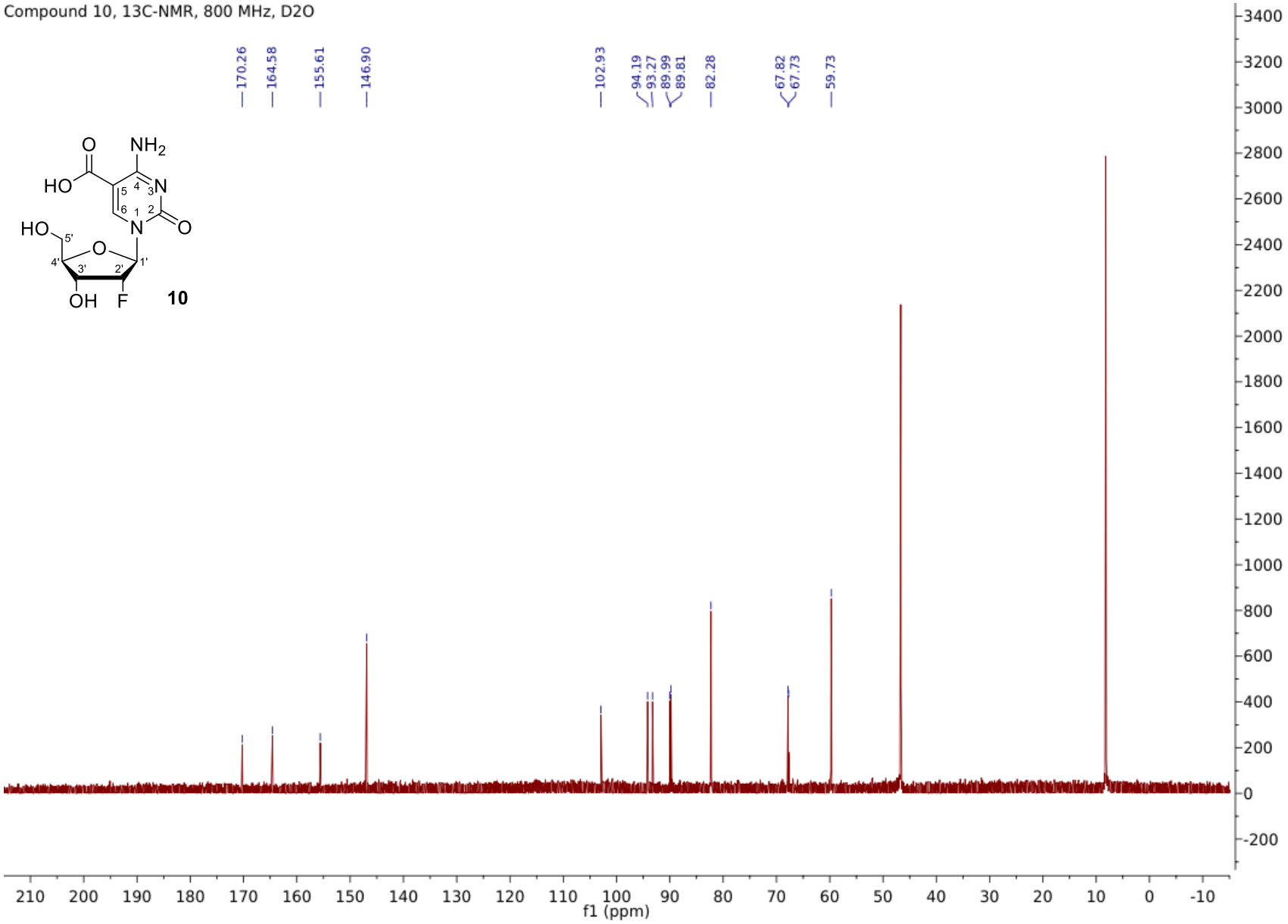
Compound 11c, 31P-NMR, 400 MHz, D2O



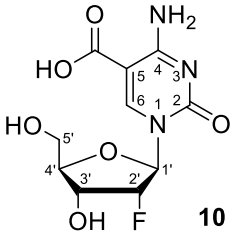
Compound 10, 1H-NMR, 400 MHz, D2O



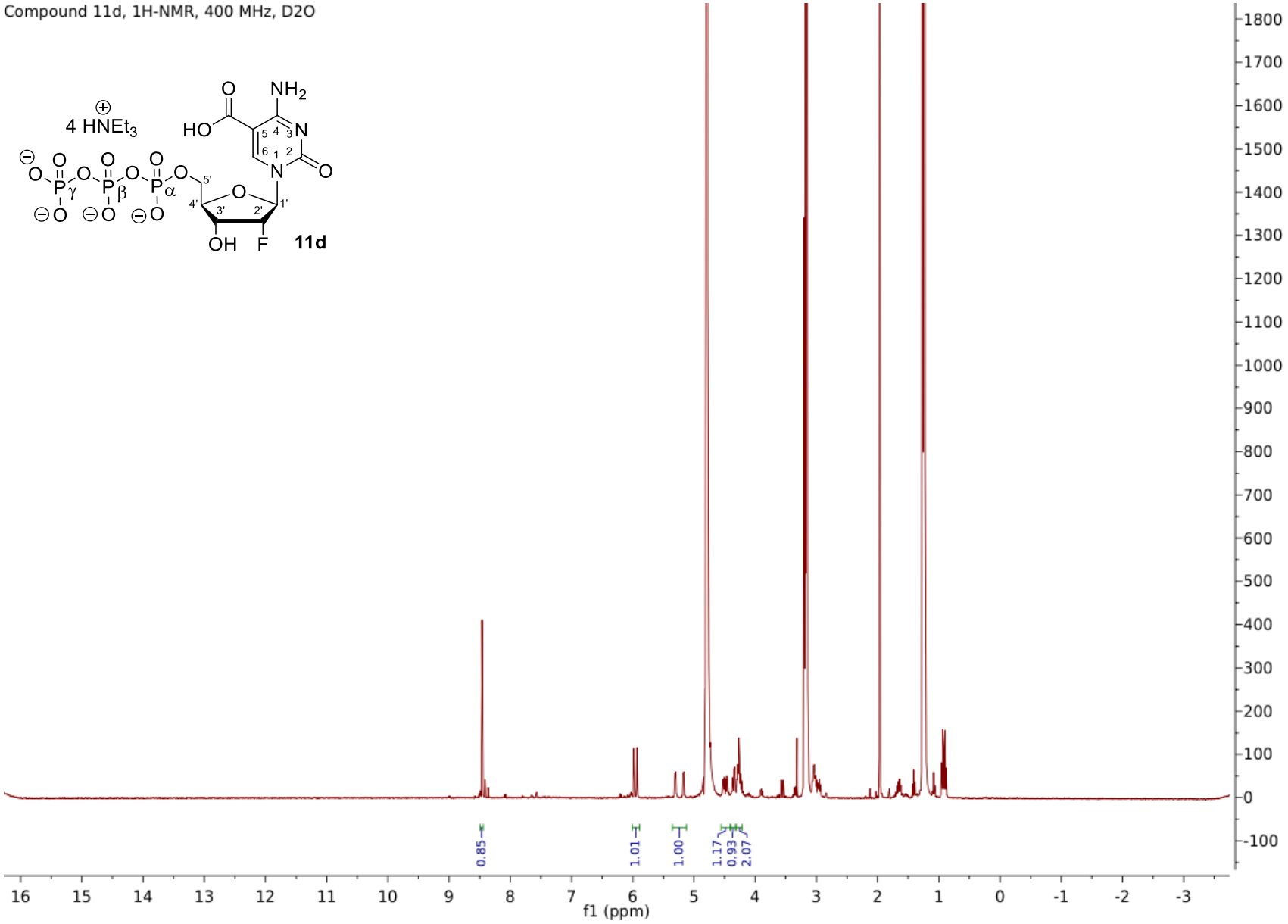

**10**


Compound 10, 13C-NMR, 800 MHz, D2O
170.26
164.58
155.61
146.90
102.93
94.19
93.27
89.99
89.81
82.28
67.82
67.73
59.73
10
f1 (ppm)

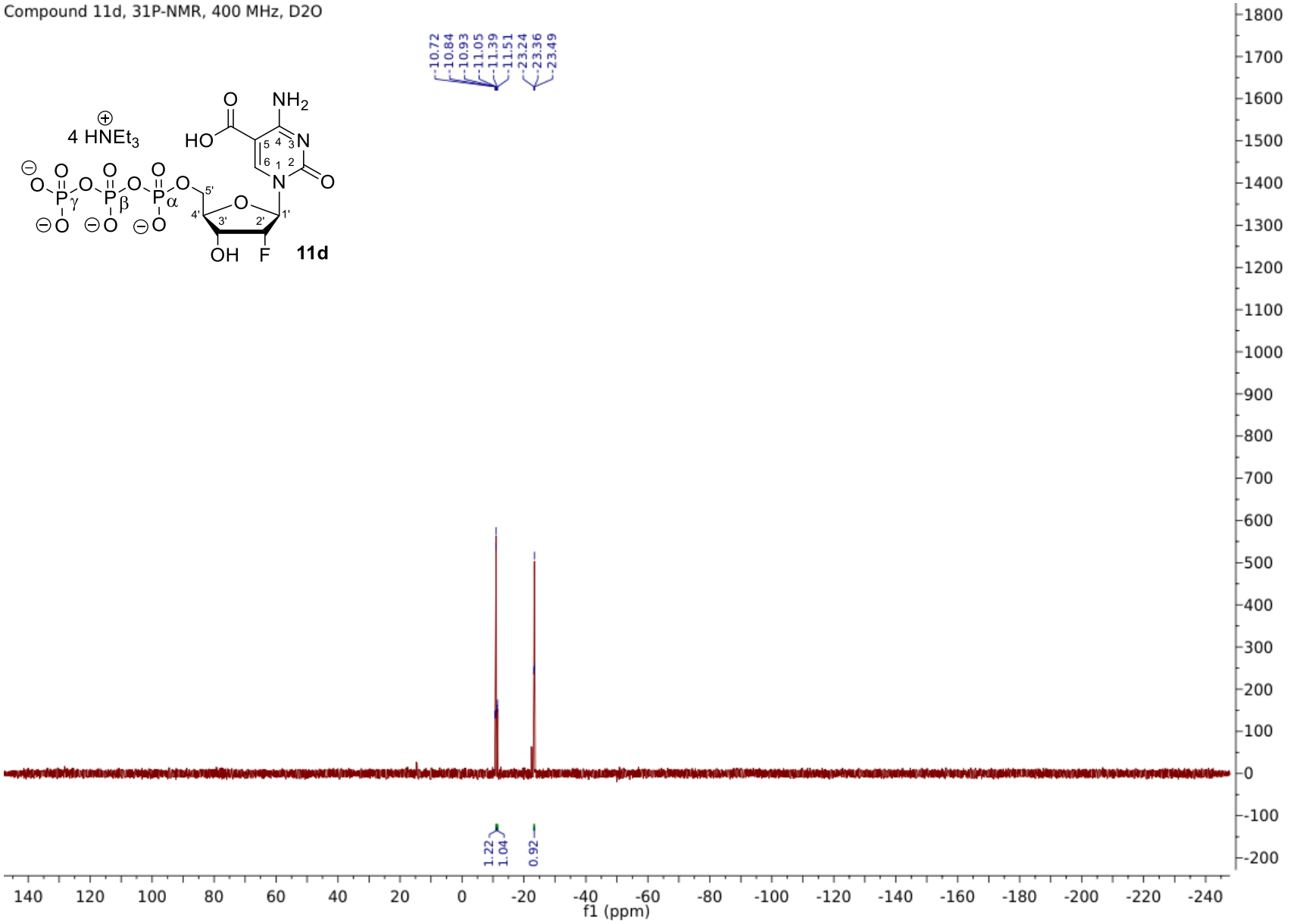
Compound 10, 19F-NMR, 400 MHz, D2O




**10**


Compound 11d, 1H-NMR, 400 MHz, D2O
4 HNEt3
11d
0.85
1.01
1.00
1.17
0.93
2.07
f1 (ppm)

Compound 11d, 31P-NMR, 400 MHz, D2O



# 9. Literature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