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New insights for the preparation of cytidine containing nucleotides using a soluble ether-linked polyethylene glycol support

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Text S1: Experimental Details.

Materials.

Polyethylene glycol-4000 was purchased from Aldrich. Yields of supported reactions were calculated considering an average molecular weight for PEG-OH_{4000} = 4000 $g.mol^{-1}$. Anhydrous DMF and Et_2O were commercially available, whereas CH_3OH and CH_2Cl_2 were distilled from CaH_2, triethylphosphate was distilled over BaO under reduced pressure. Solids were dried over KOH pellets or P_2O_5 under reduced pressure at rt. Moisture sensitive reactions were performed under argon atmosphere using oven-dried glassware.

Tri-*n*-butylammonium phosphate and bis(tri-*n*-butylammonium) pyrophosphate were obtained following previously published procedures.[1]

Methods.

Thin layer chromatography (TLC) was performed on pre-coated aluminium sheets of silica gel 60 F254, visualization of products being accomplished by UV absorbance followed by charring with 5% ethanolic sulphuric acid, or using $KMnO_4$ dip, and then heating.

NMR experiments were accomplished on Bruker Avance spectrometers at 20°C. ^{1}H-NMR spectra were recorded at 300 MHz, 400 or 500 MHz, ^{13}C-NMR spectra at 126, 101 or 75 MHz with proton decoupling. ^{31}P NMR spectra were recorded at 121 MHz with proton decoupling. Chemical shifts are given in δ values referenced to the solvent peak and relative to tetramethylsilan (TMS), or to an external reference H_3PO_4 for ^{31}P NMR. ^{1}H–^{1}H COSY and ^{1}H–^{13}C heteronuclear experiments were performed in order to confirm assignments. Coupling constants, *J*, are reported in Hertz (Hz).

HPLC-UV analysis was performed using a Waters HPLC system (2695 separation module 996 photodiode array detector 2996). LC-MS/MS system consisted of an ACQUITY™ Ultra Performance Liquid Chromatography integrated system from Waters (Milford, MA, USA), coupled to a triple quadrupole mass spectrometer TSQ Quantum Ultra™ (Thermo Fisher Scientific Inc., Waltham, MA, USA) operating in full scan mode. The chromatographic separation was achieved on a hypercarb column 50 mm × 2.1 mm with 5 μm particles size (Thermo Fisher Scientific). The mobile phase

[1] B. H. A. Knoblauch, C. E. Muller, L. Jarlebark, G. Lawoko, T. Kottke, M. A. Wikstrom and E. Heilbronn, *Eur. J. Med. Chem.*, 1999, **34**, 809-824.
A. El-Tayeb, A. D. Qi and C. E. Muller, *J. Med. Chem.*, 2006, **49**, 7076-7087.

consisted of: (A) a mixture of hexylamine (5 mM) and DEA (0.4%, v/v), the pH was adjusted to 10.5 with acetic acid, and (B) a mixture of acetonitrile and A eluent (60/40, v/v) applied at a flow-rate of 0.3 $mL.min^{-1}$ in a gradient mode. A linear 15 min gradient from 0% to 30% B, followed by 10 min of isocratic 30% B, and then a linear 10 min gradient from 30% to 100% B was used to elute the nucleotides. The analytical column was thermostated at 30°C. The auto sampler temperature was set at 10°C throughout the analysis. Injection of the sample (10µL) was performed in full loop mode.

Analyses of each supported intermediates were performed by nuclear magnetic resonance spectra (^{1}H, ^{13}C, and ^{31}P NMR, if applicable).

Preparation of PEG bis(carboxylethoxy) (1). The PEG_{4000} (10 g, 2.5 mmol) was dissolved in dry toluene (25 mL) and the solution was heated to 40°C, under argon. After complete dissolution, potassium *tert*-butoxyde (28 mg, 0.25 mmol) was added and the solution was stirred at 40°C for 30 minutes. Then, *tert*-butyl acrylate (2.2 mL, 15 mmol) was added and the reaction mixture was stirred for 6 h at 40°C. The solution was concentrated under reduced pressure and the residue was dissolved in a minimum of CH_2Cl_2 (50 mL) before to be poured on a large excess of cold diethyl ether (500 mL). The resulting precipitate was filtered, affording the intermediate **2** which was dried under vacuum on KOH pellets (9.89 g). The latter was dissolved in anhydrous CH_2Cl_2 (10 mL), TFA (10 mL) was added and the reaction mixture was stirred overnight under argon at rt. The volatiles were removed under reduced pressure and the residue was dissolved in a minimum of CH_2Cl_2 (80 mL). The resulting solution was slowly poured onto a large excess of cold diethyl ether (800 mL). After filtration, the precipitate was dried under vacuum on KOH pellets affording compound **1** as a white powder (9.12 g, 88% yield).

^{1}H NMR (D_2O, 500 MHz): δ 3.80 (t, J = 6.0 Hz, 2H, $CH_2\beta$), 3.76 – 3.62 (m, 188H, $CH_{2(PEG)}$), 2.67 (t, J = 6.0 Hz, 2H, $CH_2\alpha$). ^{13}C NMR (D_2O, 126 MHz): δ 176.0 (CO_2H), 69.6 ($CH_{2(PEG)}$), 66.1 ($CH_2\beta$), 34.3 ($CH_2\alpha$).

Preparation of PEG-supported cytidine (3). To a solution of PEG bis(carboxylethoxy) **1** (3 g, 0.72 mmol) in anhydrous CH_2Cl_2 (50 mL) was added a solution of cytidine (352 mg, 1.45 mmol) in dry DMF (21 mL) under argon atmosphere. Then, DCC (597 mg, 2.90 mmol) and HOBt (195 mg, 1.45 mmol) were added and the resulting reaction mixture was stirred at 60°C overnight. The solvents were evaporated under reduced pressure. The residue was then

dissolved in CH_2Cl_2 (2 mL) and left overnight at 4°C. The dicyclohexylurea was filtered off, and the filtrate was concentrated under reduced pressure and dissolved in CH_2Cl_2 (10 mL). The resulting solution was slowly added to an excess volume of cold diethyl ether (150 mL) and the precipitate was filtered, then washed with cold diethyl ether. The final product was recrystallized from isopropanol (200 mL) and dried under vacuum over KOH pellets to afford PEG-bis(cytidine) **3** as a white powder (2.55 g, 77% yield).

^{1}H NMR (D_2O, 500 MHz): δ 8.40 (d, J = 7.5 Hz, 1H, H_6), 7.41 (d, J = 7.5 Hz, 1H, H_5), 5.93 (d, J = 2.7 Hz, 1H, $H_{1'}$), 4.33 – 4.20 (m, 2H, $H_{2'}$, $H_{3'}$), 4.01 – 3.63 (m, $H_{4'}$, $H_{5'}$, $CH_{2(PEG)}$), 2.83 (t, J = 5.9 Hz, 2H, $CH_2\alpha$). ^{13}C NMR (D_2O, 126 MHz): δ 174.0 (C=O), 162.6 (C_4), 156.8 (C_2), 145.7 (C_6), 98.0 (C_5), 91.4 ($C_{1'}$), 83.8 ($C_{4'}$), 74.5 ($C_{2'}$), 69.6 (CPEG), 68.7 ($C_{3'}$), 65.9 ($CH_2\beta$), 60.2 ($C_{5'}$), 37.2 ($CH_2\alpha$).

PEG-supported cytidine 5'-monophosphate bis(triethylammonium) salt (6). PEG-cytidine **3** (800 mg, 0.17 mmol) was dissolved in distilled triethylphosphate (4 mL) at 40°C under argon atmosphere. After complete dissolution, phosphorus oxychloride (478 µL, 5.22 mmol) was slowly added and the mixture was stirred for 1 h at 40°C. the conversion rate was complete, as shown by ^{1}H NMR (Fig. S3). Excess of reagent was hydrolysed by addition of TEAB 1 M, pH = 7.5 (≈ 24 mL) until the pH of the solution reached 7. The resulting mixture was concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2 and the organic layer was washed with water to remove the inorganic phosphate salts. The aqueous layer was extracted several times by CH_2Cl_2, the organic layers were combined and evaporated under reduced pressure. The residue was dissolved in CH_2Cl_2 (20 mL) and the resulting solution was slowly added to an excess volume of cold diethyl ether (200 mL). The precipitate was filtered, washed with cold diethyl ether, and recrystallized from isopropanol (150 mL). The final product was dried under vacuum over KOH pellets to afford PEG-cytidine 5'-monophosphate as a bis(triethylammonium) salt and as a white powder (767 mg, 85% yield).

^{1}H NMR (D_2O, 400 MHz): δ 8.50 (d, J = 7.6 Hz, 1H, H_6), 7.42 (d, J = 7.4 Hz, 1H, H_5), 6.01 (d, J = 2.4 Hz, 1H, $H_{1'}$), 4.31 – 4.04 (m, 5H, $H_{2'}$, $H_{3'}$, $H_{4'}$, $H_{5'}$), 3.9 – 3.7 (m, $CH_{2(PEG)}$), 3.23 (q, J = 7.3 Hz, 12H, $N(CH_2CH_3)_3$), 2.70 (t, J = 6.0 Hz, 2H, $CH_2\alpha$), 1.31 (t, J = 7.3 Hz, 18H, $N(CH_2CH_3)_3$). ^{13}C NMR (D_2O, 126 MHz): δ 173.9 (CO), 162.4 (C_4), 156.6 (C_2), 145.8 (C_6), 98.2 (C_5), 90.7 ($C_{1'}$), 82.8 ($C_{4'}$), 74.7 ($C_{2'}$), 69.6 (C_{PEG}), 68.6 ($C_{3'}$), 66.2 ($CH_2\beta$), 63.3 ($C_{5'}$), 46.6 ($N(\underline{C}H_2CH_3)_3$), 34.3 ($CH_2\alpha$), 8.2 ($N(CH_2\underline{C}H_3)_3$). ^{31}P NMR ($D_2O$, 162 MHz): δ 0.24.

General procedures for mono- and di-phosphorylation of PEG supported cytidine 5'-monophosphate and LC/MS analysis. PEG-supported cytidine 5'-monophosphate **6** (200 mg, 0.019 mmol) was suspended in tributylamine (930 µL, 1.93 mmol) under argon atmosphere and the solution was stirred for 10 min at r.t. Cold diethyl ether was added and the precipitate was filtered, washed with cold diethyl ether, and dried overnight under vacuum over KOH pellets. Then, the resulting supported nucleoside 5'-monophosphate was dissolved in anhydrous DMF (380 µL) and CDI (38 mg, 0.116 mmol) was added. The reaction mixture was stirred for 2 h at rt and then treated with anhydrous CH_3OH (38 µL, 0.47 mmol) to hydrolyse the excess of CDI. After 15 min, a solution of tributylammonium phosphate (165 mg, 0.58 mmol) or bis(tributylammonium)-pyrophosphate (319 mg, 0.58 mmol) in DMF (0.58 mL, 1 M) was added and the reaction mixture was stirred for 24 h at rt. The mixture was finally treated with dry CH_3OH (v/v, ≈ 1 mL) and then concentrated under reduced pressure. The residue was precipitated with cold diethyl ether (15 mL) and the precipitate was filtered and washed with diethyl ether.

An aliquot of the crude supported compound (≈ 20 mg) was treated with aqueous triethylamine (1 mL, pH > 8) at rt for 3 h. The solution was then concentrated under reduced pressure and the residue was treated with concentrated NH_4OH (0.5 mL) for 30 minutes. After concentration under reduced pressure, the residue was dissolved in water (≈ 1 mL). The aqueous solution was then extracted several times with CH_2Cl_2. Aqueous layer was diluted three times and analysed by LC-UV and LC-MS.

PEG-supported cytidine 5'-diphosphate (11). Retention time (CDP, after cleavage, LC/MS) = 9.89 min.

PEG-supported cytidine 5'-triphosphate (12). Retention time (CTP, after cleavage, LC/MS) = 12.84 min.

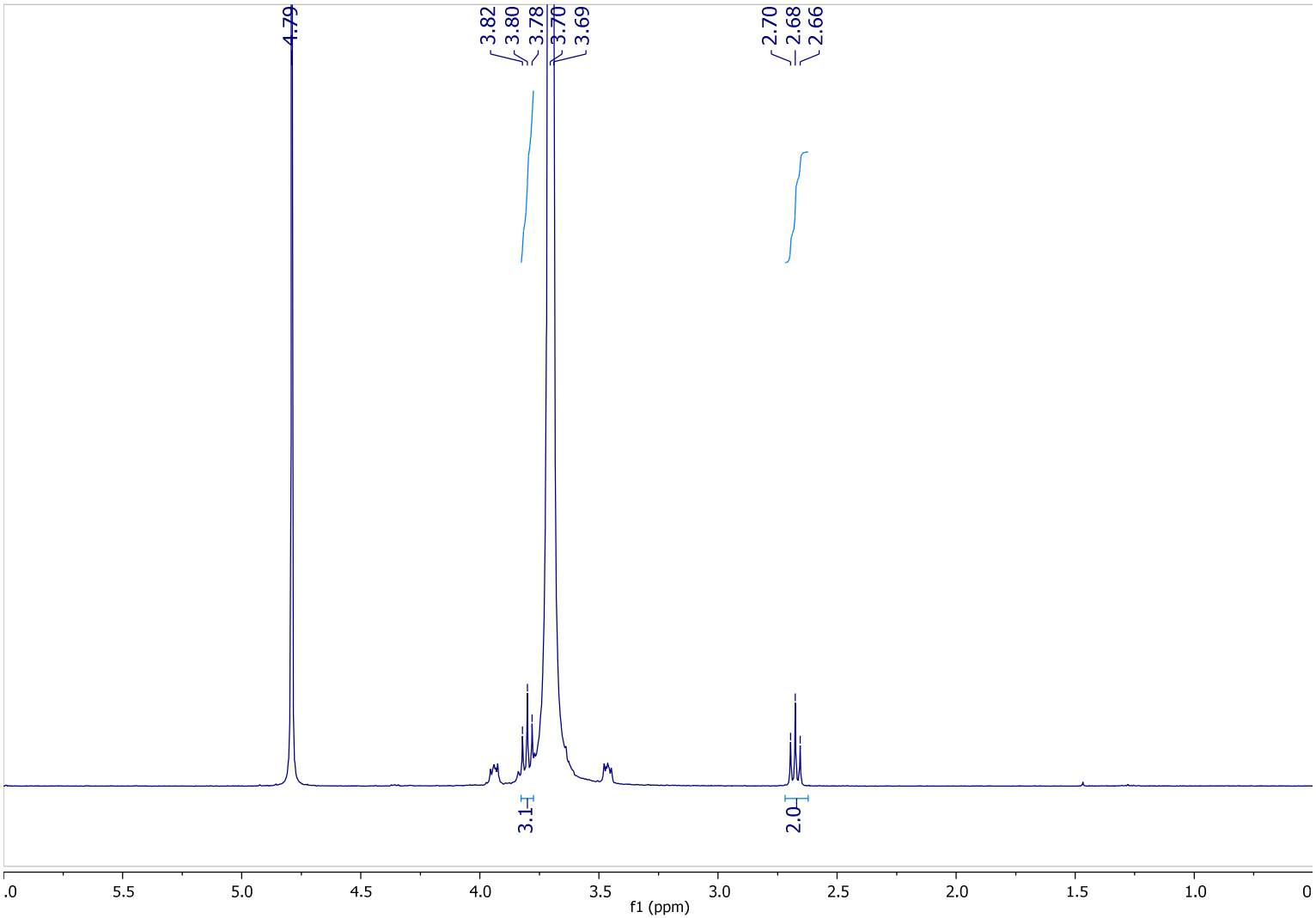

Fig. S1. ^{1}H NMR spectrum of PEG bis(carboxylethoxy) **1**.

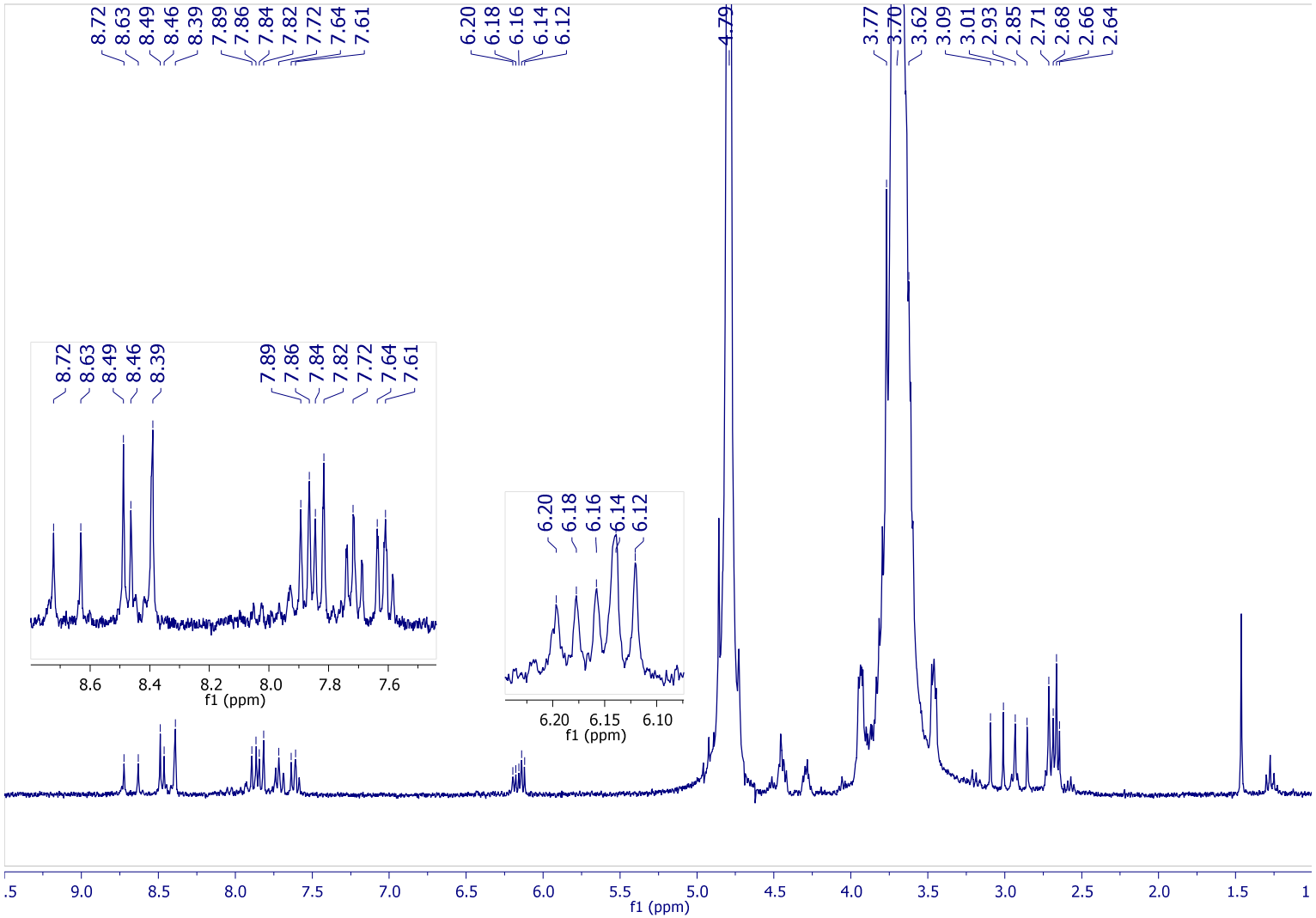

Fig S2. ^{1}H NMR spectrum after precipitation of the crude reaction mixture obtained by reacting adenosine with PEG **1** under the standard conditions (HOBt/DCC in DMF/CH_2Cl_2 at 60°C for 6h).

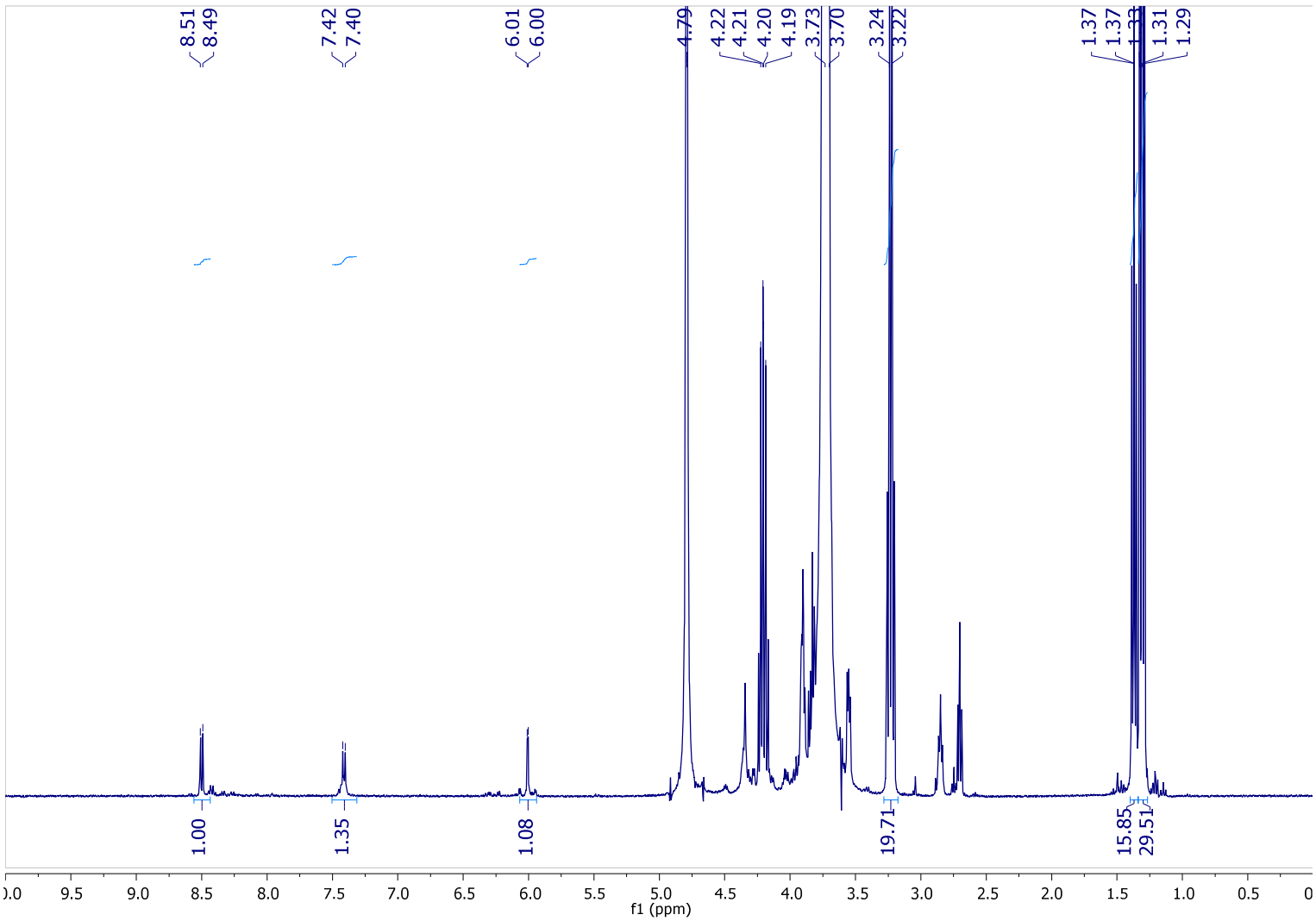

Fig S3. ^{1}H NMR spectrum after precipitation of the crude reaction mixture obtained by reacting PEG-bis(cytidine) **3** with $POCl_3$ in triethylphosphate for 1h at 40°C.

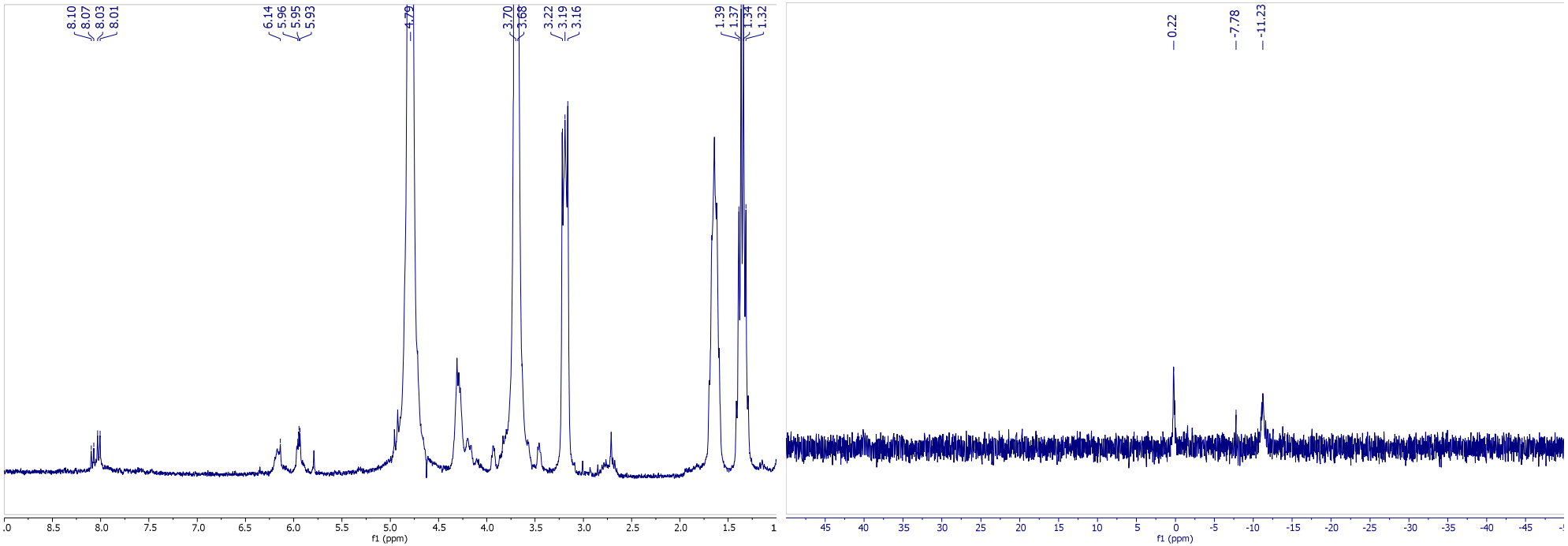

Fig S4. ^{1}H and ^{31}P NMR spectra of the crude reaction mixture obtained by reacting cytidine 5'-monophosphate with PEG **1** under the standard conditions (HOBt/DCC in DMF/CH_2Cl_2 at 60°C for 6h).

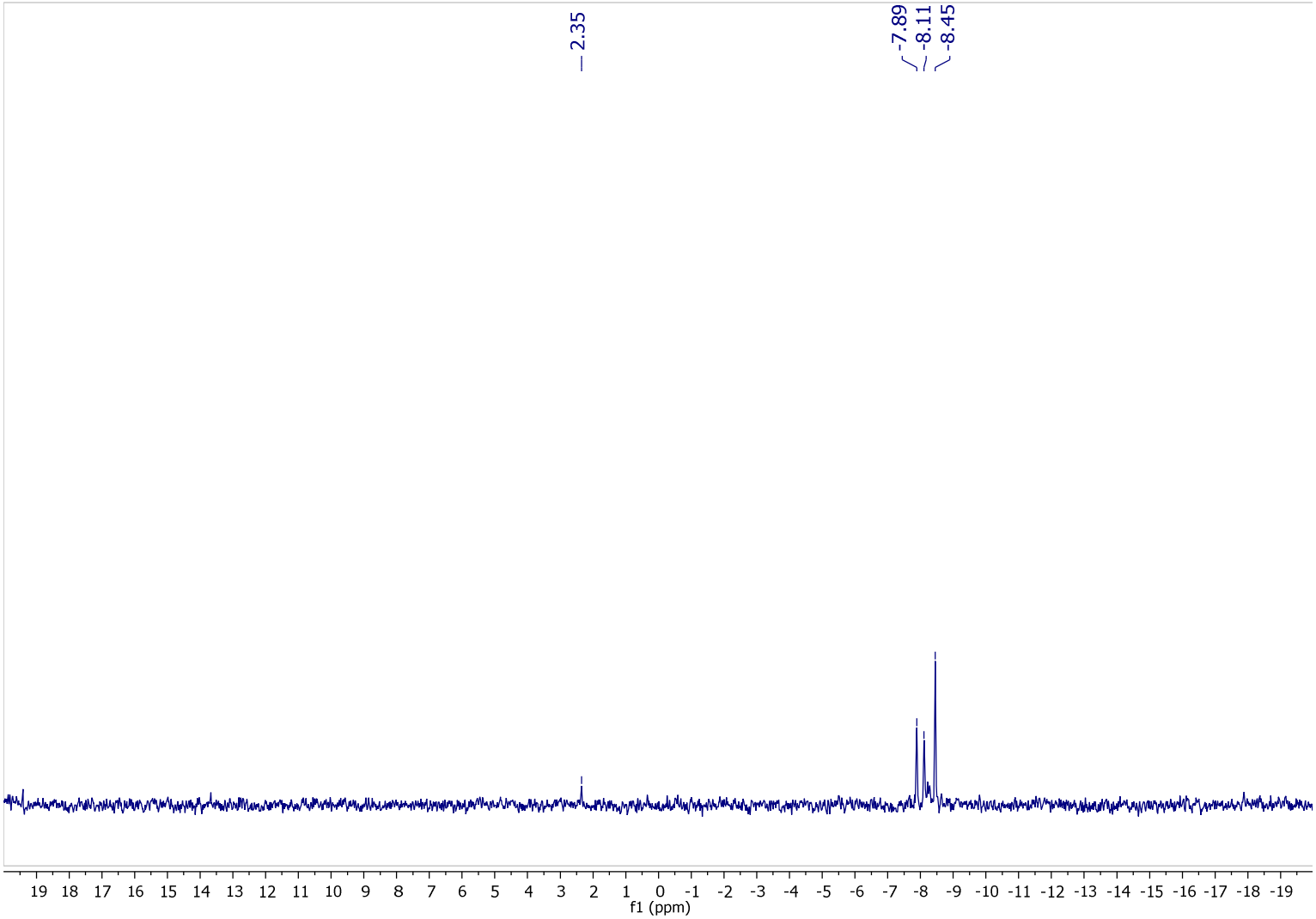

Fig S5. ^{31}P NMR spectrum after precipitation of a sample of the crude reaction mixture obtained after 2h of activation of PEG-bis(CMP) **6** by CDI.

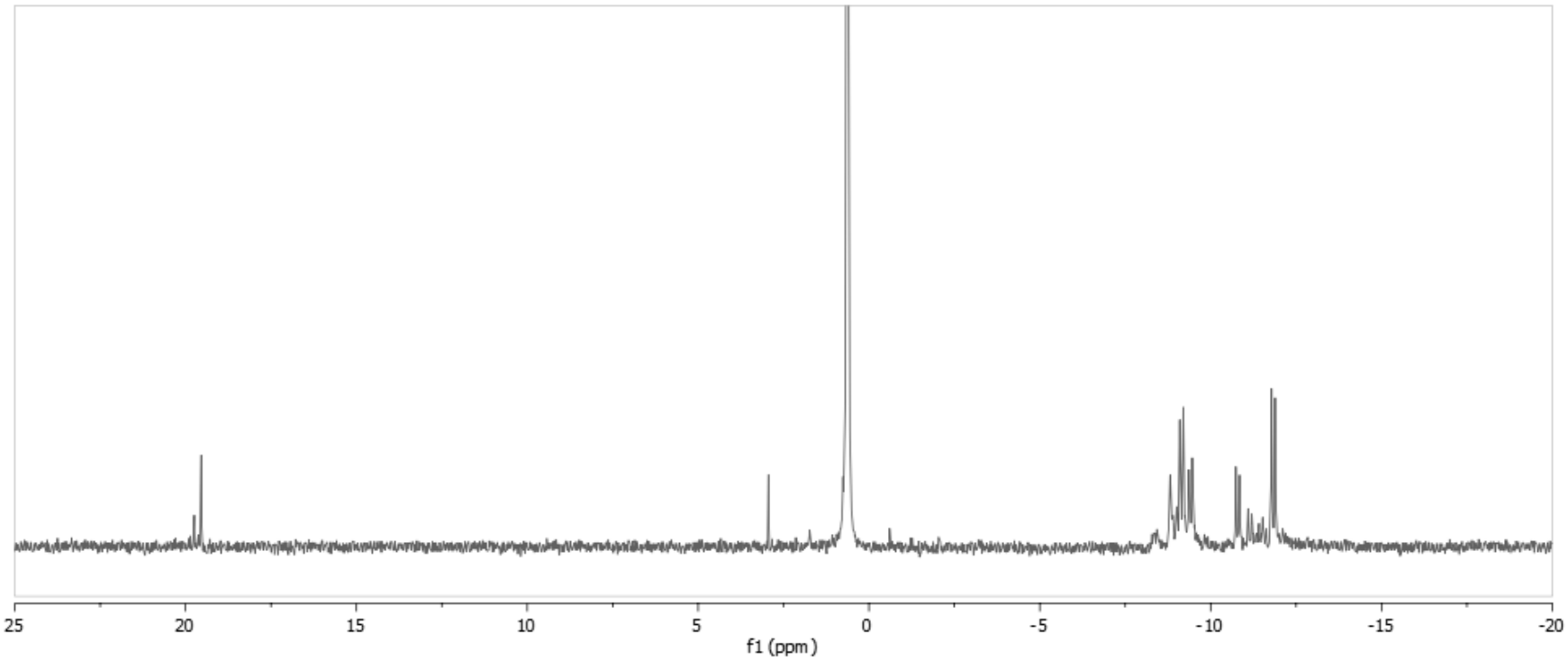

Fig S6. ^{31}P NMR spectrum of the crude reaction mixture obtained after treatment of activated PEG-bis(CMP) with tri(n-butyl)ammonium phosphate.