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Supporting Information

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Aminophenyl- and Nitrophenyl-Labeled Nucleoside Triphosphates. Synthesis, Enzymatic Incorporation and Electrochemical Detection

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SUPPORTING INFORMATION

Contents:

Experimental section

Synthesis and characterization of modified dNTPs

General

NMR spectra were measured on a Bruker Avance 600 (600 MHz for ^{1}H and 151 MHz for ^{13}C nuclei) and a Bruker 500 (500 MHz for ^{1}H, 125.7 MHz for ^{13}C and 202.3 for ^{31}P) in D_2O (referenced to dioxane as internal standard, δ_H = 3.75 ppm, δ_C = 69.3 ppm, standard for ^{31} P NMR was external H_3PO_4). Chemical shifts are given in ppm (δ-scale), coupling constants (J) in Hz. Complete assignment of all NMR signals was performed using a combination of H,H-COSY, H,C-HSQC and H,C-HMBC experiments. Mass spectra were measured on LCQ classic (Thermo-Finnigan) spectrometer using ESI or Q-Tof Micro (Waters, ESI source, internal calibration with lockspray). Preparative HPLC separations were performed on a column packed with 10 μm C18 reversed phase (Phenomenex, Luna C18(2)).

Synthesis of halogenated triphosphates

Synthesis and characterization data for 9-(2-Deoxy-*β*-D-erythro-pentofuranosyl)-7-iodo-7-deazaadenine 5'-*O*-triphosphate[1] and 1-(2-Deoxy-*β*-D-erythro-pentofuranosyl)-5-iodouracil 5'-*O*-triphosphate[2] were reported previously.

Synthesis of 2'-Deoxy-5-iodocytidine 5'-*O*-triphosphate

5-Iodo-2'-deoxycitidine (106 mg, 0.3 mmol) was suspended in trimethyl phosphate (0.75 ml) at 0°C and $POCl_3$ (35 μl, 0.36 mmol) was added. The mixture was then stirred at 0°C for 120 min, an ice-cooled solution of $(NHBu_3)_2H_2P_2O_7$ (820 mg, 1.5 mmol) and Bu_3N (0.3 ml, 1.25 mmol) in dry DMF (3 ml) was added and the mixture was stirred at 0°C for another 60 min. Then the reaction was quenched by addition of 2 M aqueous TEAB (2 ml) and the solvents were evaporated in vacuo and the residue was co-distilled with water three times. The product was isolated on DEAE Sephadex column (150 ml) eluting with a gradient 0 to 1.2 M TEAB, evaporated, co-distilled with water (3 times) and lyophilized. Title 5-I-dCTP (166 mg, 54%) was obtained as white powder.

MS(ESI^+): 695 (100, M+$HNEt_3$+2H), HRMS: for $C_{15}H_{31}N_4O_{13}P_3I$ calculated 695.014530 found 695.01697. ^{1}H NMR (500 MHz, D_2O, $ref_{dioxane}$ = 3.75 ppm): 1.27 (t, 36H, J_{vic} = 7.3, C**H**$_3$CH_2N); 2.30 (ddd, 1H, J_{gem} = 14.1, $J_{2'b,1'}$ = 7.1, $J_{2'b,3'}$ = 6.3, H-2´b); 2.42 (ddd, 1H, J_{gem} =

14.1, $J_{2'a,1'}$ = 6.1, $J_{2'a,3'}$ = 3.5, H-2′a); 3.19 (q, 36H, J_{vic} = 7.3, $CH_3C\mathbf{H_2}N$); 4.17-4.23 (m, 3H, H-4′ and H-5′); 4.61 (m, 1H, H-3′); 6.25 (dd, 1H, $J_{1'2'}$ = 7.1, 6.1, H-1′); 8.23 (s, 1H, H-6). ^{13}C NMR (125.7 MHz, D_2O, $ref_{dioxane}$ = 69.3 ppm): 10.96 ($\mathbf{C}H_3CH_2N$); 42.21 (CH_2-2′); 49.40 ($CH_3\mathbf{C}H_2N$); 61.55 (C-5); 68.06 (d, $J_{C,P}$ = 6, CH_2-5′); 73.40 (CH-3′); 88.39 (d, $J_{C,P}$ = 9, CH-4′); 88.97 (CH-1′); 150.32 (CH-6); 159.38 (C-2); 167.38 (C-4). ^{31}P (^{1}H dec.) NMR (202.3 MHz, D_2O, ref_{H3PO4} = 0 ppm): -22.64 (dd, J = 20.3, 20.0, P_β); -11.05 (d, J = 20.0, P_α); -10.15 (d, J = 20.3, P_γ).

The Suzuki-Miyaura cross-coupling reactions of halogenated dNTPs with 3-aminophenylboronic and 3-nitrophenylboronic acid

General procedure

Water-acetonitrile mixture (2:1, 0.5 ml) was added through septum to an argon purged vial containing halogenated dNTP (0.05 mmol), 3-aminoboronic acid (17.3 mg, 0.1 mmol) or 3-nitroboronic acid (16.7 mg, 0.1 mmol), Cs_2CO_3 (81 mg, 0.25 mmol). After dissolving of the solids, a solution of $Pd(OAc)_2$ (1.12 mg, 0.005 mmol) and TPPTS (14.2 mg, 0.025 mmol) in water-acetonitrile (2:1, 0.3 ml) was added and the mixture was stirred and heated up to 120 °C for 30 minutes. Products were isolated from crude reaction mixture by HPLC on C18 column with the use of linear gradient of 0.1 M TEAB (triethylammonium bicarbonate) in H_2O to 0.1 M TEAB in H_2O/MeOH (1:1) as eluent. Several co-distillations with water and conversion to sodium salt form (Dowex 50WX8 in Na^+ cycle) followed by freeze-drying from water gave white solid products.

7-(3-Aminophenyl)-2'-deoxy-7-deazaadenosine 5'-*O*-triphosphate (dA^{NH2}TP)

Yield 40%. MS(ESI$^-$): 580 (100, M-1), 500 (M – PO_3H_2 – 1), HRMS: for $C_{17}H_{21}N_5O_{12}P_3$ calculated 580.0400 found 580.0391. NMR spectra for 4xNa^+ salt at pH 7: ^{1}H NMR (500 MHz, D_2O, $ref_{dioxane}$ = 3.75 ppm, pH = 7.1): 2.45 (ddd, 1H, J_{gem} = 14.2, $J_{2'b,1'}$ = 6.3, $J_{2'b,3'}$ = 3.4, H-2′b); 2.71 (ddd, 1H, J_{gem} = 14.2, $J_{2'a,1'}$ = 7.8, $J_{2'a,3'}$ = 6.4, H-2′a); 4.09-4.20 (m, 2H, H-5′); 4.24 (m, 1H, H-4′); 4.75 (bm, 1H, H-3′); 6.65 (dd, 1H, $J_{1'2'}$ = 7.8, 6.3, H-1′); 6.87 (ddd, 1H, $J_{4,5}$ = 8.0, $J_{4,2}$ = 2.2, $J_{4,6}$ = 0.8, H-4-$C_6H_4NH_2$); 6.94 (bd, 1H, $J_{6,5}$ = 7.5, H-6-$C_6H_4NH_2$); 6.95 (bs, 1H, H-2-$C_6H_4NH_2$); 7.32 (dd, 1H, $J_{5,4}$ = 8.0, $J_{5,6}$ = 7.5, H-5-$C_6H_4NH_2$); 7.50 (s, 1H, H-8); 8.15 (bs, 1H, H-2). ^{13}C NMR (125.7 MHz, D_2O, $ref_{dioxane}$ = 69.3 ppm): 46.62 (CH_2-2′); 68.19 (d, $J_{C,P}$ = 6, CH_2-5′); 73.50 (CH-3′); 86.57 (CH-1′); 88.24 (d, $J_{C,P}$ = 9,

CH-4′); 101.87 (C-5); 121.20 (C-7); 124.11 (CH-4-$C_6H_4NH_2$); 124.78 (CH-2-$C_6H_4NH_2$); 126.21 (CH-8); 129.87 (CH-6-$C_6H_4NH_2$); 133.58 (CH-5-$C_6H_4NH_2$); 136.19 (C-1-$C_6H_4NH_2$); 136.39 (C-3-$C_6H_4NH_2$); 145.92 (CH-2); 150.10 (C-4); 153.65 (C-6). ^{31}P (^{1}H dec.) NMR (202.3 MHz, D_2O, ref_{H3PO4} = 0 ppm, pH = 7.1): -23.07 (bdd, J = 19.1, 16.7, P_β); -12.04 (d, J = 19.1, P_α); -9.15 (d, J = 16.7, P_γ).

5-(3-Aminophenyl)-2'-deoxyuridine 5'-*O*-triphosphate (dU^{NH2}TP)

Yield 43%. MS(ESI$^+$): 626 (100, M+3Na+2H), 524 (35, M – PO_3H_2+2Na), HRMS: for $C_{15}H_{18}N_3O_{14}Na_3P_3$ calculated 625.9695 found 625.9713. NMR spectra for 4xNa^+ salt at pH 7: ^{1}H NMR (500 MHz, D_2O, $ref_{dioxane}$ = 3.75 ppm, pH = 7.1): 2.41 (ddd, 1H, J_{gem} = 14.2, $J_{2'b,1'}$ = 6.6, $J_{2'b,3'}$ = 4.2, H-2′b); 2.46 (dt, 1H, J_{gem} = 14.2, $J_{2'a,1'}$ = $J_{2'a,3'}$ = 6.5, H-2′a); 4.16-4.26 (m, 3H, H-4′,5′); 4.67 (ddd, 1H, $J_{3',2'}$ = 6.5, 4.2, $J_{3',4'}$ = 3.4, H-3′); 6.37 (dd, 1H, $J_{1'2'}$ = 6.6, 6.5, H-1′); 6.91 (ddd, 1H, $J_{4,5}$ = 8.0, $J_{4,2}$ = 2.3, $J_{4,6}$ = 0.9, H-4-$C_6H_4NH_2$); 7.04 (dd, 1H, $J_{2,4}$ = 2.3, $J_{2,6}$ = 1.5, H-2-$C_6H_4NH_2$); 7.10 (ddd, 1H, $J_{6,5}$ = 7.7, $J_{6,2}$ = 1.5, $J_{6,4}$ = 0.9, H-6-$C_6H_4NH_2$); 7.31 (dd, 1H, $J_{5,4}$ = 8.0, $J_{5,6}$ = 7.7, H-5-$C_6H_4NH_2$); 7.90 (s, 1H, H-6). ^{13}C NMR (125.7 MHz, D_2O, $ref_{dioxane}$ = 69.3 ppm): 42.17 (CH_2-2′); 67.92 (d, $J_{C,P}$ = 6, CH_2-5′); 72.92 (CH-3′); 87.99 (CH-1′); 88.45 (d, $J_{C,P}$ = 9, CH-4′); 117.02 (C-5); 125.19 (CH-4-$C_6H_4NH_2$); 125.49 (CH-2-$C_6H_4NH_2$); 131.72 (CH-6-$C_6H_4NH_2$); 132.54 (CH-5-$C_6H_4NH_2$); 133.51 (C-3-$C_6H_4NH_2$); 136.54 (C-1-$C_6H_4NH_2$); 142.15 (CH-6); 153.87 (C-2); 167.00 (C-4). ^{31}P (^{1}H dec.) NMR (202.3 MHz, D_2O, ref_{H3PO4} = 0 ppm, pH = 7.1): -23.27 (t, J = 19.3, P_β); -12.41 (d, J = 19.3, P_α); -9.02 (d, J = 19.3, P_γ).

5-(3-Aminophenyl)-2'-deoxycytidine 5'-*O*-triphosphate (dC^{NH2}TP)

Yield 43%. MS(ESI$^-$): 557 (100, M-1), HRMS: for $C_{15}H_{20}N_4O_{13}P_3$ calculated 557.0240 found 557.0255. NMR spectra for 4xNa^+ salt at pH 7: ^{1}H NMR (500 MHz, D_2O, $ref_{dioxane}$ = 3.75 ppm, pH = 7.1): 2.36 (dt, 1H, J_{gem} = 14.1, $J_{2'b,1'}$ = $J_{2'b,3'}$ = 6.7, H-2′b); 2.46 (ddd, 1H, J_{gem} = 14.1, $J_{2'a,1'}$ = 6.2, $J_{2'a,3'}$ = 3.6, H-2′a); 4.15-4.20 (m, 2H, H-5′); 4.22 (m, 1H, H-4′); 4.62 (dt, 1H, $J_{3',2'}$ = 6.7, 3.6, $J_{3',4'}$ = 3.6, H-3′); 6.34 (dd, 1H, $J_{1'2'}$ = 6.7, 6.2, H-1′); 6.98-7.06 (m, 3H, H-2,4,6-$C_6H_4NH_2$); 7.38 (t, 1H, $J_{5,4}$ = $J_{5,6}$ = 7.8, H-5-$C_6H_4NH_2$); 7.80 (s, 1H, H-6). ^{13}C NMR (151 MHz, D_2O, $ref_{dioxane}$ = 69.3 ppm): 42.61 (CH_2-2′); 67.64 (d, $J_{C,P}$ = 5, CH_2-5′); 72.65 (CH-3′); 88.93 (d, $J_{C,P}$ = 9, CH-4′); 89.42 (CH-1′); 110.61 (C-5); 126.59 (CH-4-$C_6H_4NH_2$); 127.40 (CH-2-$C_6H_4NH_2$); 131.71 (CH-6-$C_6H_4NH_2$); 133.43 (CH-5-$C_6H_4NH_2$); 134.30 (C-1-

$C_6H_4NH_2$); 134.67 (C-3-$C_6H_4NH_2$); 145.64 (CH-6); 151.81 (C-2); 161.57 (C-4). ^{31}P (^1H dec.) NMR (202.3 MHz, D_2O, ref_{H3PO4} = 0 ppm, pH = 7.1): -22.98 (t, J = 19.3, P_β); -11.67 (d, J = 19.3, P_α); -10.14 (d, J = 19.3, P_γ).

2'-Deoxy-5-(3-nitrophenyl)-7-deazaadenosine 5'-*O*-triphosphate (dA^{NO2}TP)

Yield 28%. MS(ESI$^-$): 610 (100, M-1), 530 (60, M-PO_3H_2-1), HRMS: for $C_{17}H_{19}N_5O_{14}P_3$ calculated 610.0141 found 610.0125. NMR spectra for 4xEt_3NH^+ salt: ^1H NMR (500 MHz, D_2O, $ref_{dioxane}$ = 3.75 ppm): 1.26 (t, 32H, J_{vic} = 7.3, C**H$_3$**CH_2N); 2.51 (ddd, 1H, J_{gem} = 14.2, $J_{2'b,1'}$ = 6.2, $J_{2'b,3'}$ = 3.0, H-2′b); 2.76 (ddd, 1H, J_{gem} = 14.2, $J_{2'a,1'}$ = 8.3, $J_{2'a,3'}$ = 6.3, H-2′a); 3.18 (q, 24H, J_{vic} = 7.3, CH_3C**H$_2$**N); 4.11 (ddd, 1H, J_{gem} = 11.1, $J_{5'b,P}$ = 5.0, $J_{5'b,4'}$ = 4.3, H-5′b); 4.18 (ddd, 1H, J_{gem} = 11.1, $J_{5'a,P}$ = 6.2, $J_{5'a,4'}$ = 4.1, H-5′a); 4.25 (m, 1H, H-4′); 4.77 (dt, 1H, $J_{3',2'}$ = 6.3, 3.0, $J_{3',4'}$ = 3.0, H-3′); 6.68 (dd, 1H, $J_{1'2'}$ = 8.3, 6.2, H-1′); 7.67 (s, 1H, H-8); 7.70 (dd, 1H, $J_{5,4}$ = 8.2, $J_{5,6}$ = 7.6, H-5-$C_6H_4NO_2$); 7.94 (bd, 1H, $J_{6,5}$ = 7.6, H-6-$C_6H_4NO_2$); 8.20 (bs, 1H, H-2); 8.23 (ddd, 1H, $J_{4,5}$ = 8.2, $J_{4,2}$ = 2.3, $J_{4,6}$ = 0.8, H-4-$C_6H_4NO_2$); 8.29 (dd, 1H, $J_{2,4}$ = 2.3, $J_{2,6}$ = 1.7, H-2-$C_6H_4NO_2$). ^{13}C NMR (151 MHz, D_2O, $ref_{dioxane}$ = 69.3 ppm): 10.94 (**C**H_3CH_2N); 41.22 (CH_2-2′); 49.36 (CH_3**C**H_2N); 65.37 (d, $J_{C,P}$ = 6, CH_2-5′); 74.12 (CH-3′); 85.73 (CH-1′); 87.96 (d, $J_{C,P}$ = 9, CH-4′); 103.34 (C-5); 119.13 (C-7); 124.26 (CH-8); 124.96 (CH-4-$C_6H_4NO_2$); 125.94 (CH-2-$C_6H_4NO_2$); 132.99 (CH-5-$C_6H_4NO_2$); 137.63 (C-1-$C_6H_4NO_2$); 137.98 (CH-6-$C_6H_4NO_2$); 150.77 (C-3-$C_6H_4NH_2$); 152.68 (C-4); 153.54 (CH-2); 159.35 (C-6). ^{31}P (^1H dec.) NMR (202.3 MHz, D_2O, ref_{H3PO4} = 0 ppm): -22.51 (dd, J = 19.7, 18.7, P_β); -10.71 (d, J = 19.7, P_α); -9.75 (d, J = 18.7, P_γ).

2'-Deoxy-5-(3-nitrophenyl)uridine 5'-*O*-triphosphate (dU^{NO2}TP)

Yield 26%. MS(ESI$^-$): 588 (100, M-1), HRMS: for $C_{15}H_{17}N_3O_{16}P_3$ calculated 587.9822 found 587.9840. NMR spectra for 4xEt_3NH^+ salt: ^1H NMR (500 MHz, D_2O, $ref_{dioxane}$ = 3.75 ppm): 1.27 (t, 32H, J_{vic} = 7.3, C**H$_3$**CH_2N); 2.43 (ddd, 1H, J_{gem} = 14.2, $J_{2'b,1'}$ = 6.7, $J_{2'b,3'}$ = 3.6, H-2′b); 2.48 (ddd, 1H, J_{gem} = 14.2, $J_{2'a,1'}$ = 7.6, $J_{2'a,3'}$ = 6.0, H-2′a); 3.19 (q, 24H, J_{vic} = 7.3, CH_3C**H$_2$**N); 4.12-4.36 (m, 3H, H-4′,5′); 4.67 (ddd, 1H, $J_{3',2'}$ = 6.3, 3.6, $J_{3',4'}$ = 3.1, H-3′); 6.37 (dd, 1H, $J_{1'2'}$ = 7.6, 6.7, H-1′); 7.71 (dd, 1H, $J_{5,4}$ = 8.3, $J_{5,6}$ = 7.8, H-5-$C_6H_4NO_2$); 7.91 (ddd, 1H, $J_{6,5}$ = 7.8, $J_{6,2}$ = 1.7, $J_{6,4}$ = 1.0, H-6-$C_6H_4NO_2$); 8.07 (s, 1H, H-6); 8.25 (ddd, 1H, $J_{4,5}$ = 8.3, $J_{4,2}$ = 2.4, $J_{4,6}$ = 1.0, H-4-$C_6H_4NO_2$); 8.43 (dd, 1H, $J_{2,4}$ = 2.4, $J_{2,6}$ = 1.7, H-2-$C_6H_4NO_2$). ^{13}C NMR (125.7 MHz, D_2O, $ref_{dioxane}$ = 69.3 ppm): 10.95 (**C**H_3CH_2N); 41.49 (CH_2-2′); 49.37

(CH_3**C**H_2N); 68.16 (d, $J_{C,P}$ = 6, CH_2-5′); 73.58 (CH-3′); 88.51 (CH-1′); 88.54 (d, $J_{C,P}$ = 7, CH-4′); 116.65 (C-5); 125.79 (CH-4-$C_6H_4NO_2$); 126.40 (CH-2-$C_6H_4NO_2$); 132.87 (CH-5-$C_6H_4NO_2$); 136.07 (C-1-$C_6H_4NO_2$); 138.01 (CH-6-$C_6H_4NO_2$); 142.50 (CH-6); 150.45 (C-3-$C_6H_4NO_2$); 153.94 (C-2); 167.12 (C-4). ^{31}P (^{1}H dec.) NMR (202.3 MHz, D_2O, ref_{H3PO4} = 0 ppm): -22.61 (dd, J = 20.3, 19.2, P_β); -11.10 (d, J = 20.3, P_α); -9.51 (d, J = 19.2, P_γ).

2'-Deoxy-5-(3-nitrophenyl)cytidine 5'-*O*-triphosphate (dC^{NO2}TP)

Yield 26%. MS (ESI$^+$): 677 (100, M+4Na), HRMS: for $C_{15}H_{16}N_4O_{15}Na_4P_3$ calculated 676.9416 found 676.9416. NMR spectra for 4xNa^+ salt at pH 7: ^{1}H NMR (500 MHz, D_2O, $ref_{dioxane}$ = 3.75 ppm, pH = 7.1): 2.37 (dt, 1H, J_{gem} = 14.0, $J_{2'b,1'} = J_{2'b,3'}$ = 6.8, H-2′b); 2.55 (ddd, 1H, J_{gem} = 14.0, $J_{2'a,1'}$ = 6.1, $J_{2'a,3'}$ = 3.5, H-2′a); 4.11-4.20 (m, 2H, H-5′); 4.22 (m, 1H, H-4′); 4.61 (dt, 1H, $J_{3',2'}$ = 6.8, 3.5, $J_{3',4'}$ = 3.5, H-3′); 6.35 (dd, 1H, $J_{1'2'}$ = 6.8, 6.1, H-1′); 7.75 (dd, 1H, $J_{5,4}$ = 9.1, $J_{5,6}$ = 7.7, H-5-$C_6H_4NO_2$); 7.85 (ddd, 1H, $J_{6,5}$ = 7.7, $J_{6,2}$ = 1.6, $J_{6,4}$ = 1.0, H-6-$C_6H_4NO_2$); 7.88 (s, 1H, H-6); 8.31 (ddd, 1H, $J_{4,5}$ = 9.1, $J_{4,2}$ = 2.3, $J_{4,6}$ = 1.0, H-4-$C_6H_4NO_2$); 8.32 (dd, 1H, $J_{2,4}$ = 2.3, $J_{2,6}$ = 1.6, H-2-$C_6H_4NO_2$). ^{13}C NMR (125.7 MHz, D_2O, $ref_{dioxane}$ = 69.3 ppm): 42.31 (CH_2-2′); 68.01 (d, $J_{C,P}$ = 6, CH_2-5′); 72.69 (CH-3′); 89.27 (d, $J_{C,P}$ = 9, CH-4′); 90.19 (CH-1′); 109.89 (C-5); 127.43 (CH-4-$C_6H_4NO_2$); 127.54 (CH-2-$C_6H_4NO_2$); 133.41 (C-1-$C_6H_4NO_2$); 133.84 (CH-5-$C_6H_4NO_2$); 139.70 (CH-6-$C_6H_4NO_2$); 146.36 (CH-6); 150.91 (C-3-$C_6H_4NO_2$); 151.00 (C-2); 161.34 (C-4). ^{31}P (^{1}H dec.) NMR (202.3 MHz, D_2O, ref_{H3PO4} = 0 ppm, pH = 7.1): -23.52 (bdd, J = 19.1, 18.7, P_β); -12.94 (bd, J = 19.1, P_α); -8.81 (bd, J = 18.7, P_γ).

Primer extension incorporation of the nitrophenyl- and/or aminophenyl-modified nucleotides into oligonucleotides

Materials. Synthetic ONs (summarized in Table 1-SI for sequences) were purchased from VBC genomics (Austria). Dynabeads® M-270 Streptavidin (DB_{Stv}) were obtained from Dynal A.S. (Norway), Klenow Fragment (3′ → 5′ exo-) and T4 polynucleotide kinase from New England Biolabs (Great Britain), DyNAzyme™ II DNA Polymerase from Finnzymes (Finland), unmodified nucleoside triphosphates (dATP, dTTP, dCTP and dGTP) from Sigma and γ-^{32}P-ATP from MP Empowered Discovery (USA). Other chemicals were of analytical grade.

Table 1-SI: Synthetic oligonucleotides used in the work

primer	5‘-CATGGGCGGCATGGG-3‘
temp^{AT}	5‘-**TATATATATAT**<u>CCCATGCCGCCCATG</u>-3‘
temp^{AC}	5‘-**TGTGTGTGTGT**<u>CCCATGCCGCCCATG</u>-3‘
temp^{TC}	5‘-**AGAGAGAGAGA**<u>CCCATGCCGCCCATG</u>-3‘
temp^{C}	5‘-**CCCG**<u>CCCATGCCGCCCATG</u>-3‘
temp^{U}	5‘-**CCCA**<u>CCCATGCCGCCCATG</u>-3‘
temp^{A}	5‘-**CCCT**<u>CCCATGCCGCCCATG</u>-3‘
temp^{ATnr2}	5‘-**TTATATTTAAT**<u>CCCATGCCGCCCATG</u>-3‘
temp^{rnd16}	5‘-**CTAGCATGAGCTCAGT**<u>CCCATGCCGCCCATG</u>-3‘

Notes:

- ✓ in the template (temp) ONs the segments forming duplex with the primer are underlined, the replicated segments are in bold
- ✓ templates used in experiments involving the DB_{Stv} magnetoseparation procedure were biotinylated at their 5’-ends
- ✓ acronyms used in this work for primer extension (PEX) products are analogous to those introduced for the templates (e.g., a PEX product pex^{AT} was synthesized on temp^{AT} template, etc.)

Primer extension: The primer (0.7 μM) was mixed with a template ON (0.7 μM), dNTPs (125 μM each) and a DNA polymerase (1.25 or 1.0 U per sample for Klenow or DyNAzyme

polymerases, respectively). For polyacrylamide gel electrophoresis (PAGE) experiments, the primer was ^{32}P-prelabeled at its 5'-end and non-biotinylated templates were used. For electrochemical analysis, unlabeled primer and biotinylated templates allowing magnetic separation of the extended primer (see below) were used. Reaction mixtures were incubated for 5 minutes at 37 ºC (with Klenow) or for 45 min at 60 °C (with DyNAzyme).

Polyacrylamide gel electrophoresis: Products of primer extension reaction were mixed with loading buffer (80% formamide, 10 mM EDTA, 1 mg ml^{-1} xylene cyanol, 1 mg ml^{-1} bromphenol blue) and electrophoresed in 15 % denaturing polyacrylamide gel containing 1xTBE buffer (pH=8) and 7 M urea at 25 W for 50 minutes. Gels were dried, autoradiographed and visualized using Phosphorimager Storm.

Magnetoseparation procedure and electrochemical analysis

Isolation of the labeled PEX product strands by a DB_{Stv} magnetoseparation procedure (Scheme 1-SI)**:** The PEX products were captured at DB_{Stv} via biotin tags tethered to the 5'-ends of the template strands. 50-μl aliquots of the PEX reaction mixtures were added to the DB_{Stv} [25 μl of the stock suspension washed twice by 100 μl of 0.3 M NaCl, 10 mM Tris-HCl, pH 7.4 (buffer H)]. The mixtures were incubated on a shaker for 30 min at 20 °C. Then the beads were subsequently washed thrice by 100 μl of PBS (0.14 M NaCl, 3 mM KCl, 4 mM sodium phosphate pH 7.4) with 0.01 % Tween 20, thrice by 100 μl of the buffer H and resuspended in 50 μl of deionized water. The extended primer strands were released by heating at 75 °C for 2 min. Each medium exchange was performed using a magnetoseparator (Dynal, Norway). Prior to the *ex situ* electrochemical measurements, NaCl was added into the samples (final concentration 0.2 M).

Similar techniques have recently been proposed to improve electrochemical detection of DNA hybridization or DNA protein interactions,[3-6] as well as to prepare ferrocene-labeled PEX products for the electrochemical analysis.[7]

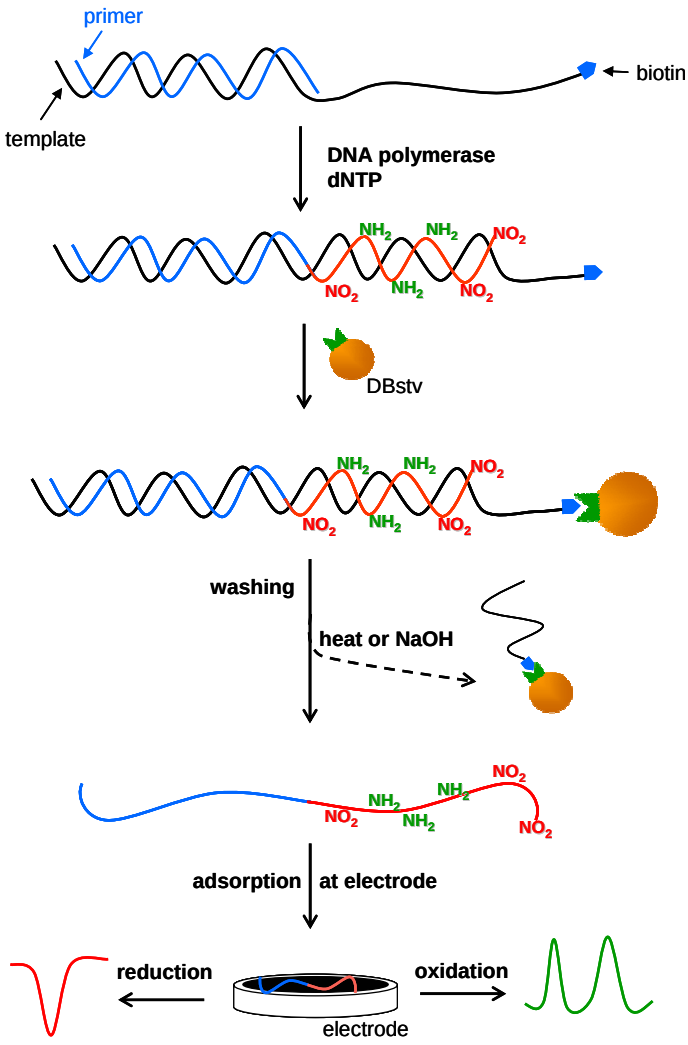
Voltammetric analysis: The PEX oligonucleotide products were analyzed using *ex-situ* (adsorptive transfer stripping) voltammetric procedure that proved particularly suitable for analysis of small amounts of nucleic acids, proteins or other biomolecules strongly adsorbing at electrode surfaces.[8-10] The technique takes the advantages of a firm adsorptive accumulation of DNA at the electrode surface from a small sample volume, followed by transfer of the DNA-modified electrode into blank background electrolyte in an ordinary electrochemical cell.

The PEX products were accumulated at the basal-plane pyrolytic graphite electrode (PGE; prepared and pretreated as described[3, 11]) surface from 5-μl aliquots containing 0.2 M NaCl for 60 s. Then the electrode was rinsed by deionized water and placed into the electrochemical cell.

Electrochemical responses of $\mathbf{dN^{NO2}TP}$ and $\mathbf{dN^{NH2}TP}$ (Fig. 2 in the Communication) were measured in a conventional *in situ* mode (with the analyte dissolved in background electrolyte).

Square-wave voltammetry (SWV;[12] and references therein) was chosen as a technique yielding well-defined signals of the nitrophenyl and aminophenyl tags as well as

intrinsic DNA responses due to guanine oxidation[9, 10] at the carbon electrodes. *SWV parameters*: **oxidation of amino-labels** (and guanine residues): initial potential 0 V, final potential +1.5 V, pulse amplitude 25 mV, frequency 200 Hz, potential step 5 mV; **reduction of nitro-labels**: initial potential 0 V, final potential -1.5 V, other parameters the same. The measurements were performed at ambient temperature in 0.1 M Tris, 0.2 M NaCl, pH 7.3 using an Autolab analyzer (EcoChemie, The Netherlands) in a three-electrode setup (with the PGE as working, Ag/AgCl/3M KCl as reference and platinum wire as counter electrode). The voltammograms were baseline-corrected by means of a moving average algorithm (GPES 4 software, EcoChemie).



Scheme 1-SI

Magnetic separation and ex-situ voltammetric analysis of the nitro and/or amino-labeled PEX products.

Supplementary results

Influence of aminophenyl and/or nitrophenyl tags on the PEX nucleotide incorporation

PEX incorporation of $\mathbf{A}^{\mathbf{NH2}}$, $\mathbf{A}^{\mathbf{NO2}}$, $\mathbf{U}^{\mathbf{NH2}}$, $\mathbf{U}^{\mathbf{NO2}}$, $\mathbf{C}^{\mathbf{NH2}}$ or $\mathbf{C}^{\mathbf{NO2}}$ was tested using different template nucleotide sequences (see Table 1 in Communication). Synthesis of DNA stretches accommodating the labeled nucleobases at isolated positions was practically as feasible as synthesis of the same stretches with unlabeled dNTPs. The same was true for doublet repeat sequences in which a labeled nucleotide alternated with another unlabeled. Incorporation of $\mathbf{A}^{\mathbf{NH2}}$, $\mathbf{A}^{\mathbf{NO2}}$, $\mathbf{U}^{\mathbf{NH2}}$ and/or $\mathbf{U}^{\mathbf{NO2}}$ at mutually adjacent position was also easy, in contrast to ferrocene-labeled U or A incorporation into homonucleotide blocks (that exhibited early termination at sites of clustered incorporation of the labeled bases[7]).

On the contrary, incorporation of $\mathbf{C}^{\mathbf{NH2}}$ or $\mathbf{C}^{\mathbf{NO2}}$ next to any other labeled base was less feasible (resulting in early termination of PEX), especially when DyNAzyme was used and the PEX reaction was performed at 60 °C (see Fig. 1-SI for examples). With Klenow (exo-) polymerase (PEX temperature 37 °C) lacking the proofreading nuclease activity, carefully controlled reaction conditions were required to avoid erroneous nucleotide incorporation. High frequency of (both modified and unmodified) nucleobase misincorporation was observed especially when higher enzyme concentrations or longer incubation times were used.

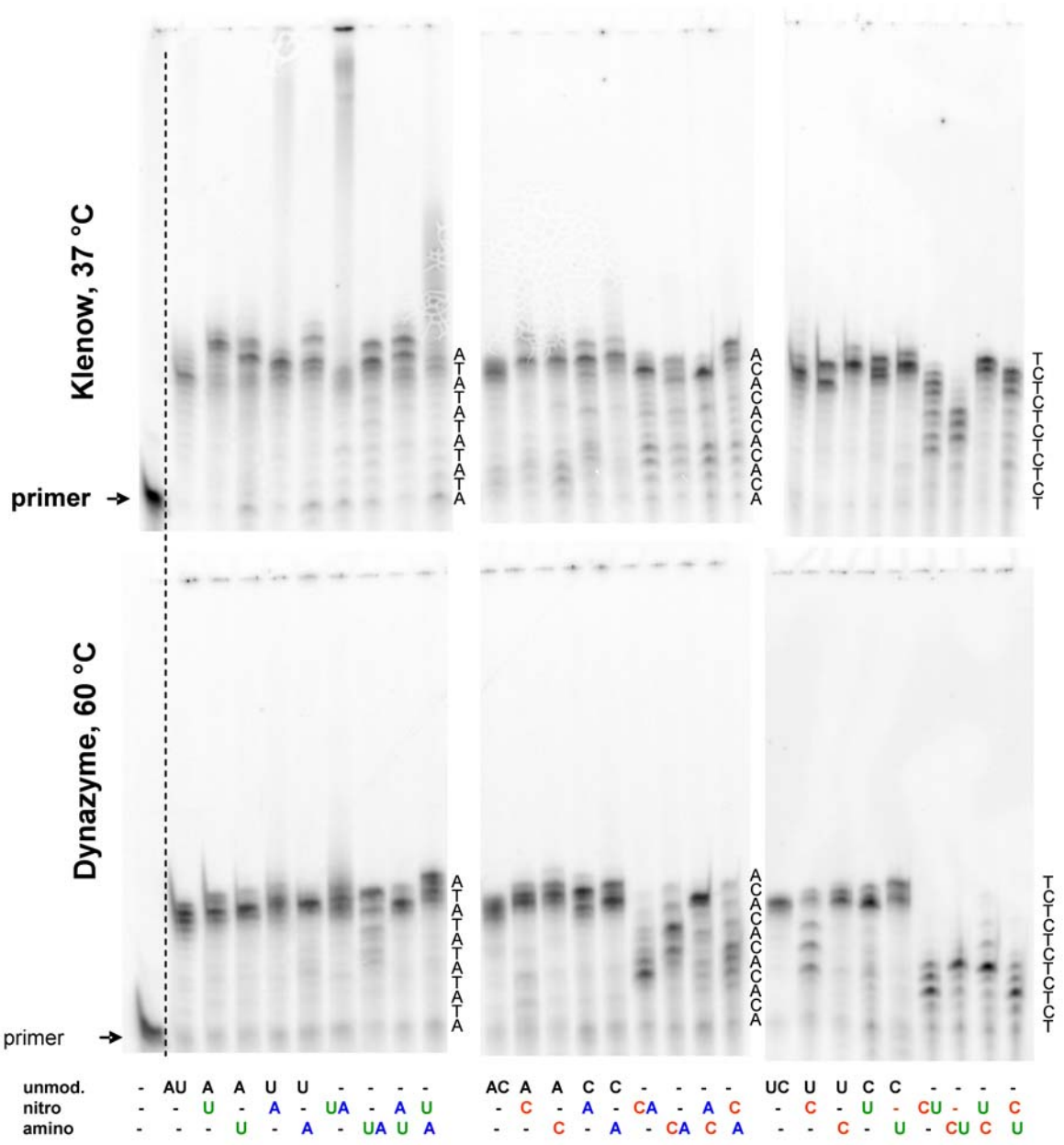


Figure 1-SI

Denaturing PAGE analysis of PEX products synthesized on templates (from left to right) temp^{AT}, temp^{AC} or temp^{TC} using the nitro- and/or amino-labeled nucleobases. Nucleotide sequences of the synthesized stretches are shown along the panel left edges, composition of the dNTP mixes on the bottom. PEX reactions in the top panels were performed with Klenow (exo-) polymerase at 37 °C, those in the bottom panels with a thermostable DyNAzyme polymerase at 60 °C.

Electrochemical analysis of DNA bearing both nitro and amino tags.

Electrochemical signals of the DNA nitro-tags arise from irreversible reduction of the nitro group close to -700 mV while irreversible oxidation of the amino-labels at the PGE takes place around +750 to +800 mV (Figs. 3 in Communication and 2-SI). This makes it possible not only to discriminate perfectly between the two labels, but also to detect successively (one-by-one) both types of markers in the same DNA (ON) molecule after a single accumulation of the analyte at the electrode surface. When the initial potential is set less negative than potential of the nitro group reduction but less positive than potential of the amino group oxidation, it is in principle possible to measure signal of one of the tags (e.g., nitro via negative potential scan) followed by measurement of the other tag (e.g., amino via positive potential scan, or *vice versa*). Fig. 2A-SI shows that both NO_2 and NH_2 peaks could be measured in this way. Partial decrease of the markers signals (relative to intensities detected in first potential scans with newly adsorbed ON) was probably due to a change of the adsorbed DNA properties upon charging the electrode to rather positive or negative potentials during the first scans; these effect can easily be eliminated by a proper calibration. The possibility of simultaneous detection of the amino group signal and the guanine oxidation peak[9, 10] is also advantageous since it offers an internal control of the magnetoseparation procedure efficacy (intensity of the guanine peak can be used for normalization of the tag signal intensities i.e., for counting number of tags incorporated per guanine residue.[7] In all PEX products, 8 guanine residues are present in the primer stretch).

For sequential detection of the nitro and amino labels in the same DNA molecule, it is critical that one tag is not electrochemically converted into the other (otherwise products of the first electrode reaction would contribute to signal intensity of the label measured as the second). Arylamines are primarily oxidized at carbon electrodes by one or two electrons to cation radicals and/or biradicals that can oligomerize or polymerize ([13-16] and references therein). On the other hand, nitro compounds can be reduced, depending on conditions, either by four electrons to hydroxylamines, or by another two electrons to amines.[17, 18] Fig. 2B-SI shows that after reduction of a PEX product labeled only by a nitrophenyl-base conjugate, no signal due to the amino group was detected in the following positively going potential scan. Thus, the nitro tags are most likely reduced in a four-electron process to the hydroxylamine derivatives and products of this reaction do not interfere with the subsequent detection of the amino tags.

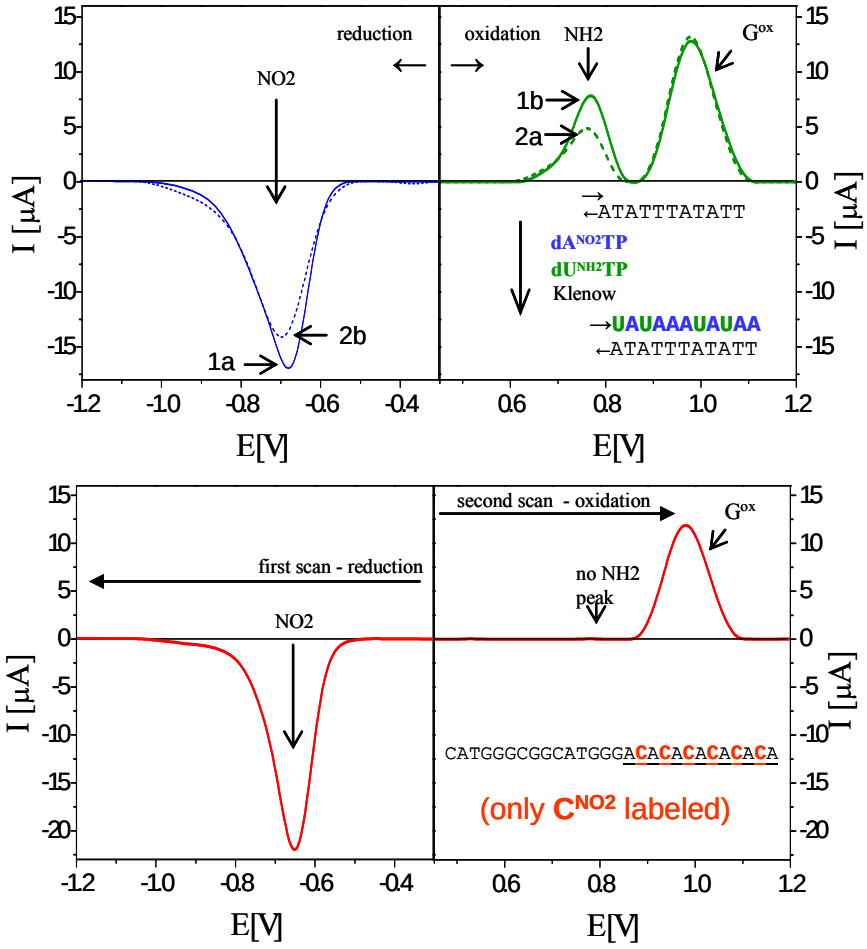


Figure 2-SI

(**A**) Detection of nitro- and amino-labels in the same PEX product (pex^{AT} synthesized using a mix of **dA^{NO2}TP** and **dU^{NH2}TP**, see inset). Solid curves (1a and 1b) are first scans obtained with PEX products newly adsorbed at the PGE. Dashed curves 2a and 2b were measured as second scans after recording curves 1a or 1b, respectively (i.e., both label types were detected successively after a single adsorption step). (**B**) Electrochemical reduction followed by oxidation of pex^{AC} bearing only **C^{NO2}** labeled nucleotides. No signal due to the amino group was detected suggesting that the nitro group was reduced via a four electron process to a hydroxylamine derivative.

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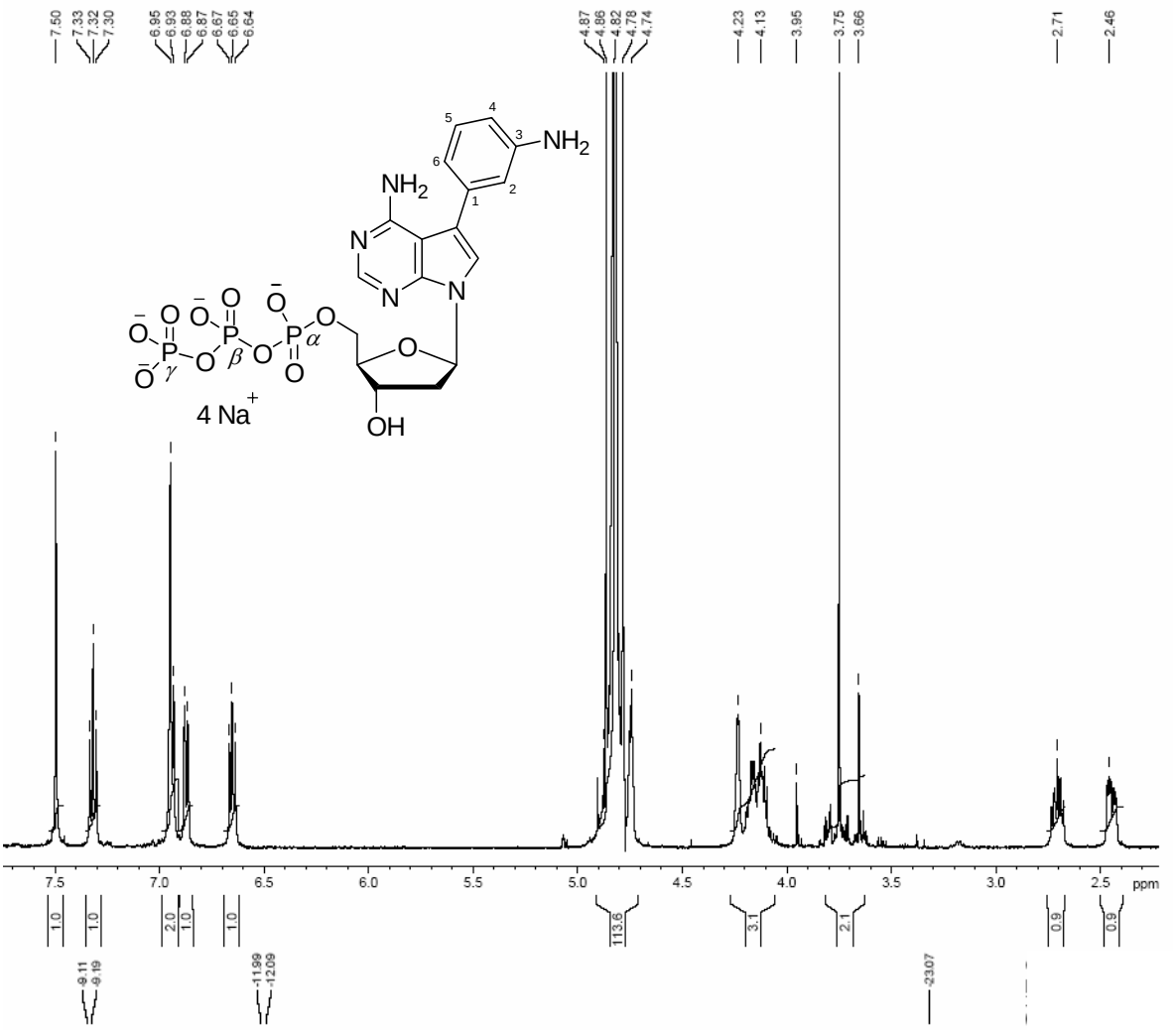
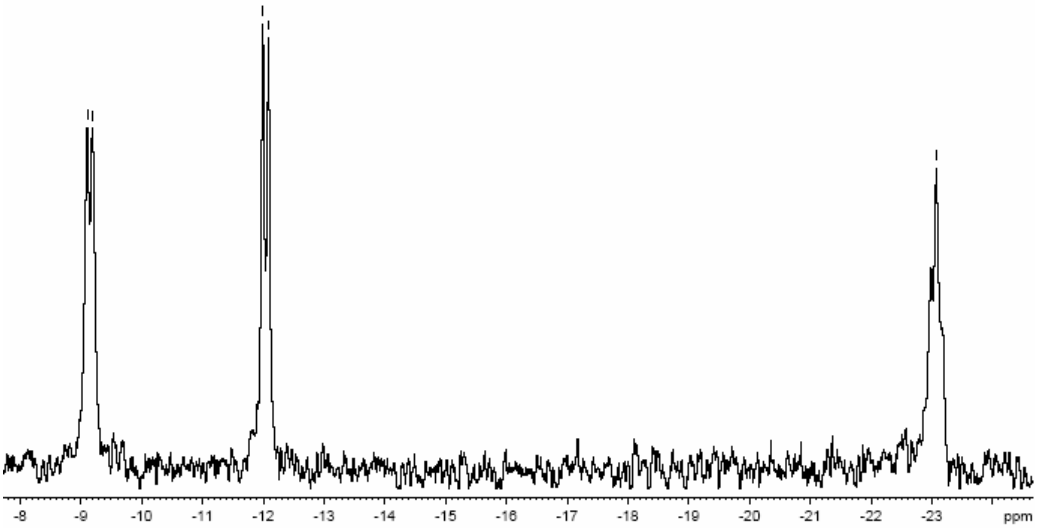
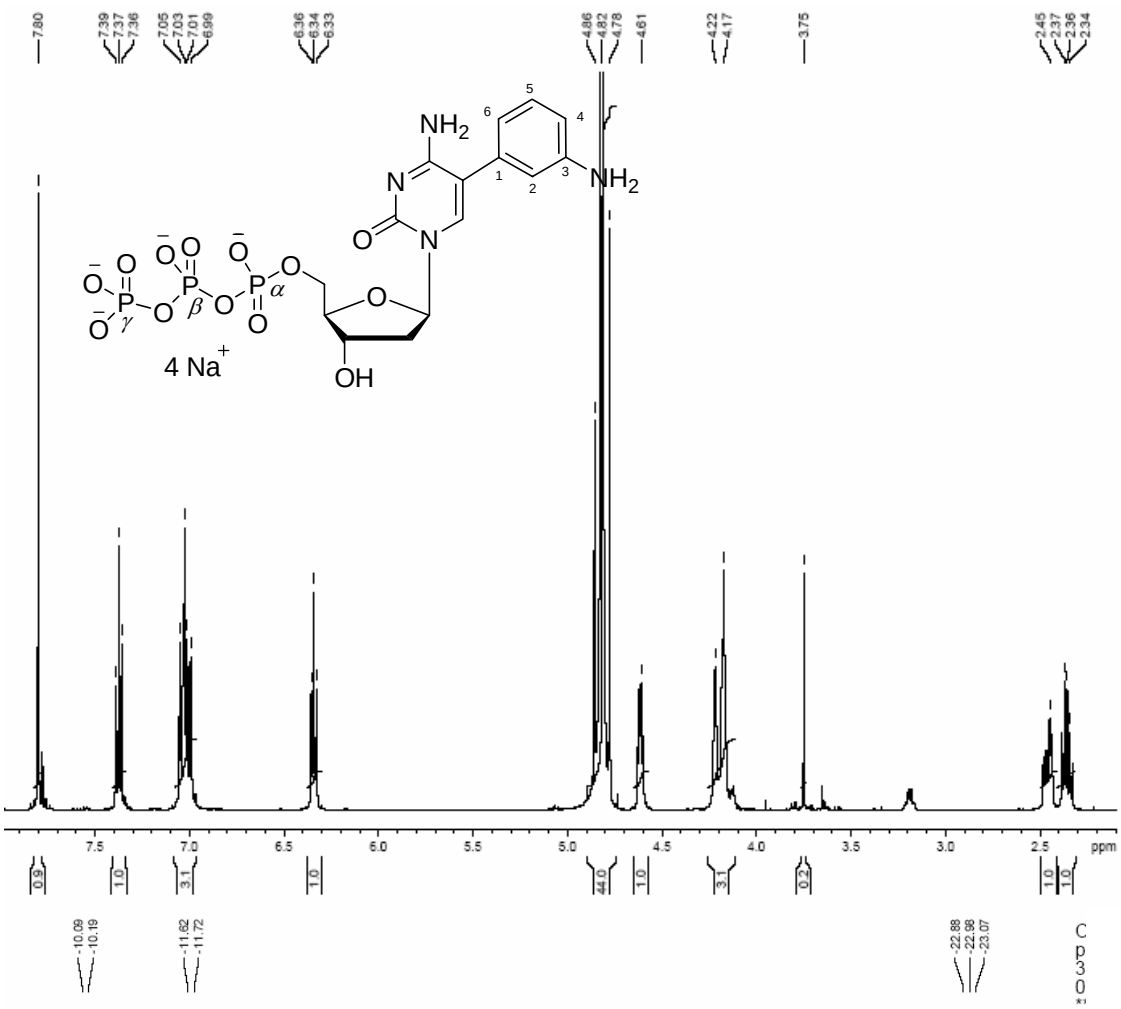
Selected copies of NMR spectra



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4.17
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2.36
2.34
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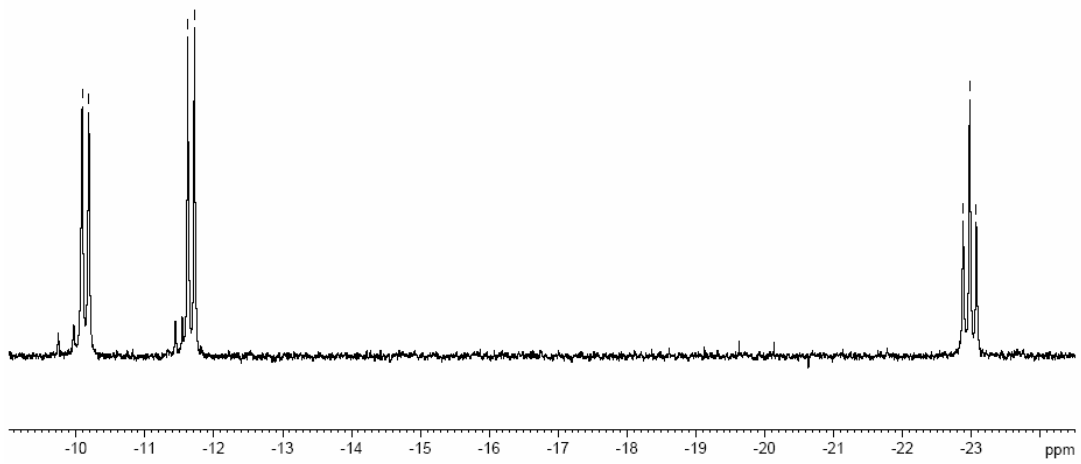
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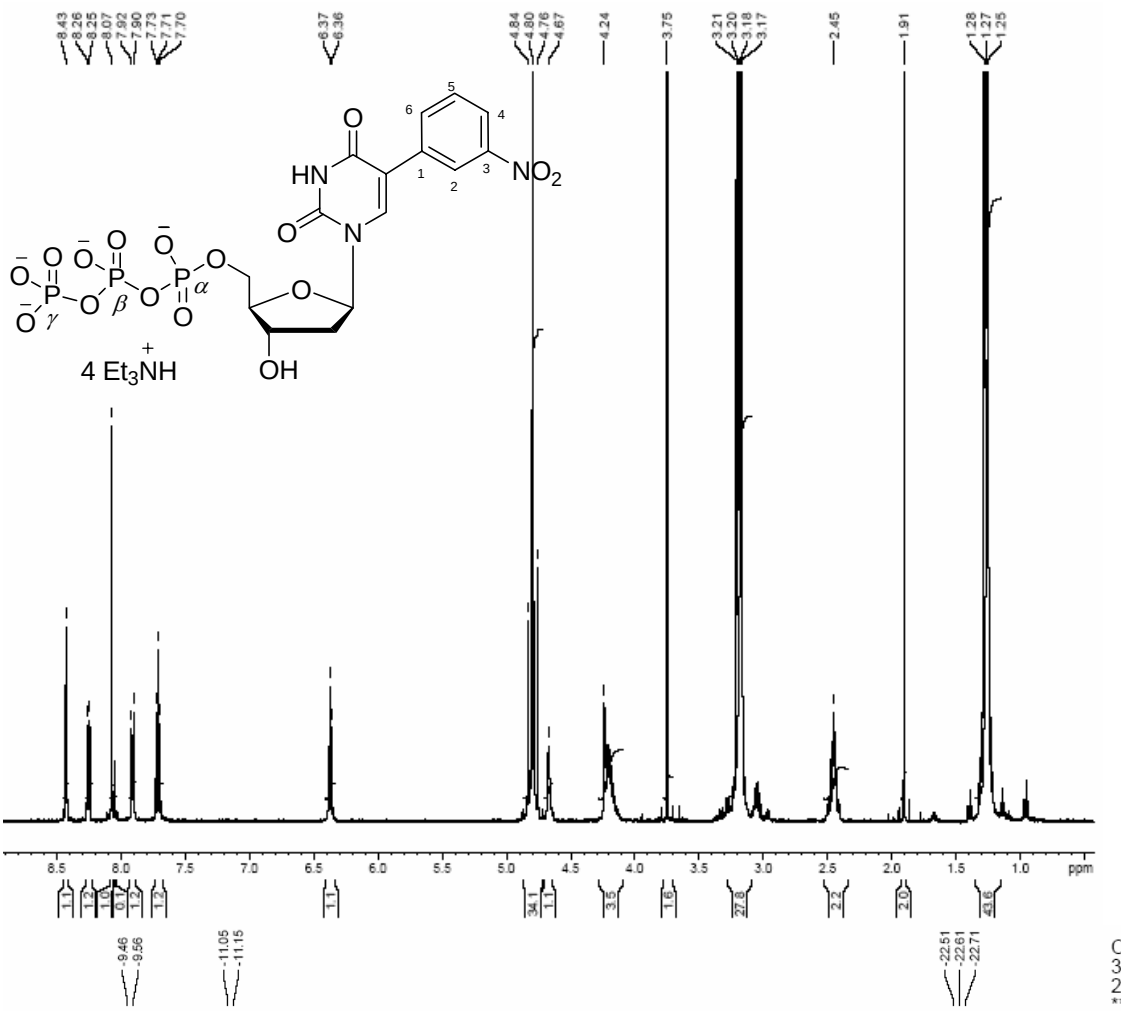
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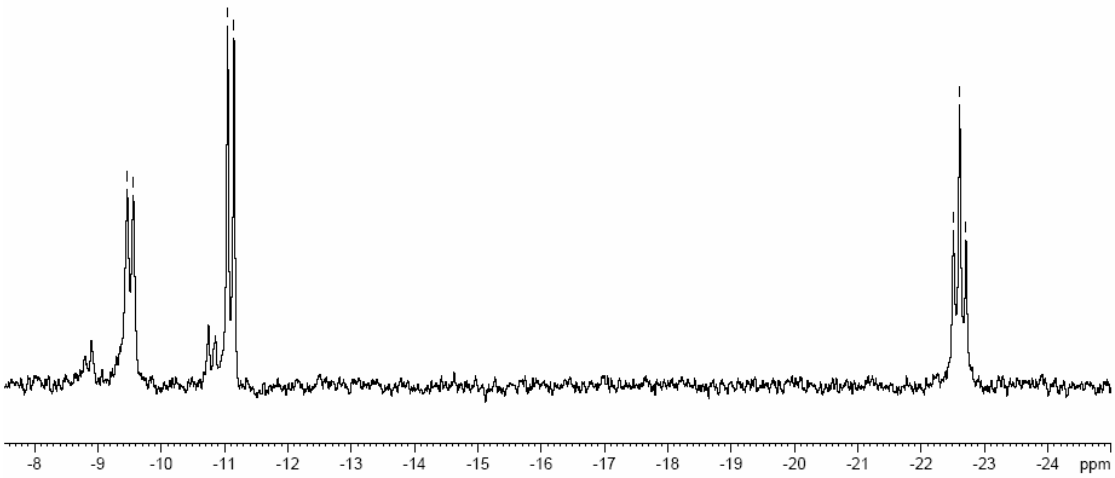
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