Electronic Supporting Information

Protected 2'-deoxyribonucleoside triphosphate building blocks for photocaging of epigenetic 5-(hydroxymethyl)cytosine in DNA

Soňa Boháčová,[a] Zuzana Vaníková,[a,b] Lenka Poštová Slavětínská,[a] and Michal Hocek[a,b*]

[a] *Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences, Flemingovo namesti 2, CZ-16610 Prague 6, Czech Republic*

[b] *Department of Organic Chemistry, Faculty of Science, Charles University in Prague, Hlavova 8, Prague 2, CZ-12843, Czech Republic*

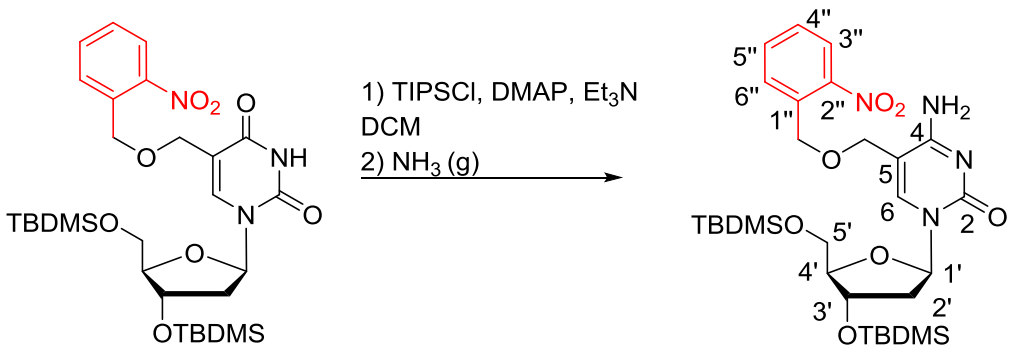
Contents

1. SYNTHESIS

Materials and methods

NMR spectra were recorded on 500 MHz (500.0 MHz for ^{1}H, 202.3 MHz for ^{31}P, 125.7 MHz for ^{13}C) spectrometer from sample solutions in D_2O or DMSO-d_6. Chemical shifts (in ppm, δ scale) were referenced as follows: D_2O (referenced to dioxane as internal standard: 3.75 ppm for ^{1}H NMR and 69.30 ppm ^{13}C NMR) and DMSO-d_6 (referenced to solvent signal: 2.50 ppm for ^{1}H NMR and 39.70 ppm for ^{13}C NMR). Coupling constants (J) are given in Hz. Complete assignment of all NMR signals was achieved by using a combination of H,H−COSY, H,C-HSQC, and H,C-HMBC experiments. High resolution mass spectra were measured on a LTQ Orbitrap XL spectrometer (Thermo Fisher Scientific). Mass spectra of functionalized DNA were measured by MALDI-TOF, Reflex IV (Bruker) with nitrogen laser. The reactions were monitored by thin-layer chromatography using Merck silica gel 60 F254 plates and visualized at 254 nm. Column chromatography was performed using silica gel (40‑63 μm). Semi-preparative separation of nucleoside triphosphates was performed using HPLC on a column packed with 10 µm C18 reversed phase (Phenomenex, Luna C18 (2) 100 Å). Sample elution was monitored by its absorbance at 264 nm. Absorption spectra were measured on a Cary 100 UV-Vis spectrometer (Agilent Technologies). UV LEDs which were used for uncaging of modified PEX products XSL-355-E (5 mm; 353-360 nm; 0.8-1.2 mW; 1.06 mW/cm2), XRL400-5O (5 mm; 400-410 nm; 21.0-29.4 mW), VL425-5-15 (5 mm; 420-430 nm; 10-16 mW), were purchased from Roithner LaserTechnik. Synthetic oligonucleotides (primers, templates and biotinylated templates) were purchased from Generi Biotech. Natural nucleoside triphosphate (dATP, dCTP, dGTP, dTTP) were purchased from New England Biolabs. DNA polymerases were purchased from Merck (KOD XL) and from New England Biolabs (Vent exo-). Streptavidine magnetic particles were purchased from Roche. PCR products were purified using magnetic particles Agencourt AMPure XP. The PAGE gels were visualized by a fluorescence scanner (Typhoon FLA 9500, GE Healthcare).

3′, 5′-Bis-*O*-(*tert*-butyldimethylsilyl)-5-(2-nitrobenzyloxy)methyl-2′-deoxycytidine (2a)

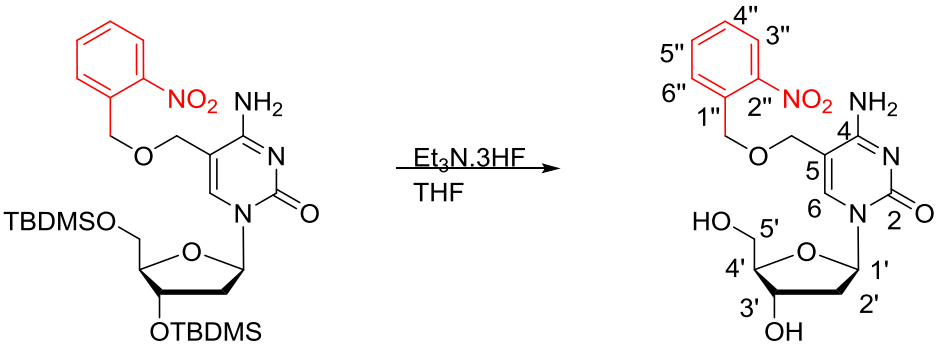


Compound **1a** (150 mg; 0.24 mmol), DMAP (35 mg; 0.29 mmol) and Et_3N (350 µL; 2.5 mmol) were dissolved in anhydrous DCM (9 mL) and mixture was stirred for 10 minutes at room temperature. 2,4,6- Triisopropyl benzenesulfonyl chloride (350 mg, 1.16 mmol) was added and the reaction mixture was stirred at room temperature overnight under argon atmosphere. The

solvent was removed under reduced pressure and the residue was dissolved in anhydrous dioxane. The flask with solution was evacuated three times and flushed with gaseous ammonia. The reaction mixture was stirred for 3 hours under gaseous ammonia at room temperature. After concentration in vacuum, the residue was purified by gradient column chromatography (gradient from 100% DCM to 25:1 DCM:MeOH) to yield protected 5-(2-nitrobenzyloxy)methyl-2´-deoxycytidine (120 mg; 80% over two steps).

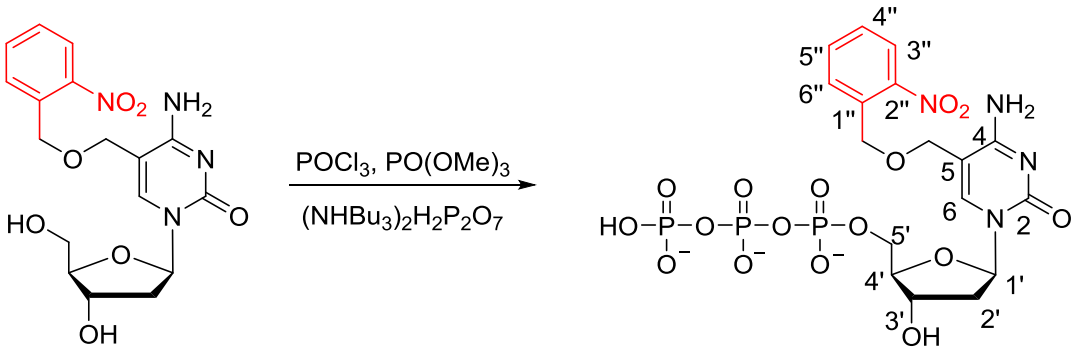
The spectral data were in accordance with literature[1,2].

5-(2-Nitrobenzyloxy)methyl-2´-deoxycytidine (dC^{NB})



Compound **2a** (120 mg; 0.19 mmol) was dissolved in dry THF (9 mL), trimethylamine trihydrofluoride (100 µL; 0.61 mmol) was added and the reaction mixture was stirred overnight at room temperature. Then, another portion of trimethylamine trihydrofluoride (200 µL; 1.22 mmol) was added, and the stirring was continued overnight. The reaction mixture was concentrated under reduced pressure and the residue was purified by gradient column chromatography (gradient from 100% DCM to 10:1 DCM:MeOH). The obtained compound was re-purified by HPLC (gradient H_2O →MeOH) to yield 5-(2-nitrobenzyloxy)methyl-2´-deoxycytidine (31 mg; 41%).
The spectral data were in accordance with literature[1, 2].

5-(2-Nitrobenzyloxy)methyl-2´-deoxycytidine triphosphate (dC^{NB}TP)



Nucleoside **dC^{NB}** (28 mg; 0.07 mmol) was dried for 15 minutes at 80 °C under vacuum. Subsequently, the vial with starting material was cooled to room temperature and proton sponge (15 mg; 0.07 mmol) was added. The content was evacuated again and suspended in trimethylphosphate (0.28 mL) at 0 °C under argon atmosphere. $POCl_3$ (8 µL; 0.09 mmol) was added and the reaction mixture was then stirred at 0 °C for 1 hour. An ice-cooled solution of tri-*n*-butylammonium pyrophosphate (198 mg; 0.36 mmol) in anhydrous DMF (0.7 mL) was added to the reaction mixture. After 1 hour of stirring, the solution was quenched by addition of aqueous TEAB (2M, 2 mL). The solvents were evaporated in vacuum and the residue was co-distilled several times with water. The product was isolated on HPLC (linear gradient: 0.1M TEAB 100% H_2O → 0.1M TEAB 100% MeOH → 100% MeOH), evaporated, co-distilled with water; converted into a sodium salt form (Dowex 50 in Na+ cycle) and lyophilized to yield **dC^{NB}TP** as a white powder (10 mg; 21%).

^{1}H NMR (500.0 MHz, D_2O): 2.33 (dt, 1H, J_{gem} = 13.8 Hz, $J_{2'b,1'}$ = $J_{2'b,3'}$ = 6.5 Hz, H-2′); 2.45 (ddd, 1H, J_{gem} = 13.8 Hz, $J_{2'a,1'}$ = 6.5 Hz, $J_{2'a,3'}$ = 4.6 Hz, H-2′); 4.23 (m, 1H, H-4′); 4.27 – 4.35 (m, 2H, H-5′); 4.63 and 4.66 (2 × d, 2 × 1H, J_{gem} = 13.1 Hz, OCH_2-5); 4.70 (ddd, 1H, $J_{3',2'}$ = 6.5, 4.6 Hz, $J_{3',4'}$ = 4.1 Hz, H-3′); 4.98 and 5.04 (2 × d, 2 × 1H, J_{gem} = 13.0 Hz, OCH_2-1 ″); 6.28 (t, 1H, $J_{1',2'}$ = 6.5 Hz, H-1′); 7.58 (ddd, 1H, $J_{4'',3''}$ = 8.2 Hz, $J_{4'',5''}$ = 7.8 Hz, $J_{4'',6''}$ = 1.5 Hz, H-4-$C_6H_4NO_2$); 7.65 (dd, 1H, $J_{6'',5''}$ = 7.8 Hz, $J_{6'',4''}$ = 1.5 Hz, H-6-$C_6H_4NO_2$); 7.73 (td, 1H, $J_{5'',4''}$ = $J_{5'',6''}$ = 7.8 Hz, $J_{5'',3''}$ = 1.3 Hz, H-5-$C_6H_4NO_2$); 7.99 (s, 1H, H-6); 8.06 (dd, 1H, $J_{3'',4''}$ = 8.2 Hz, $J_{3'',5''}$ = 1.3 Hz, H-3-$C_6H_4NO_2$).

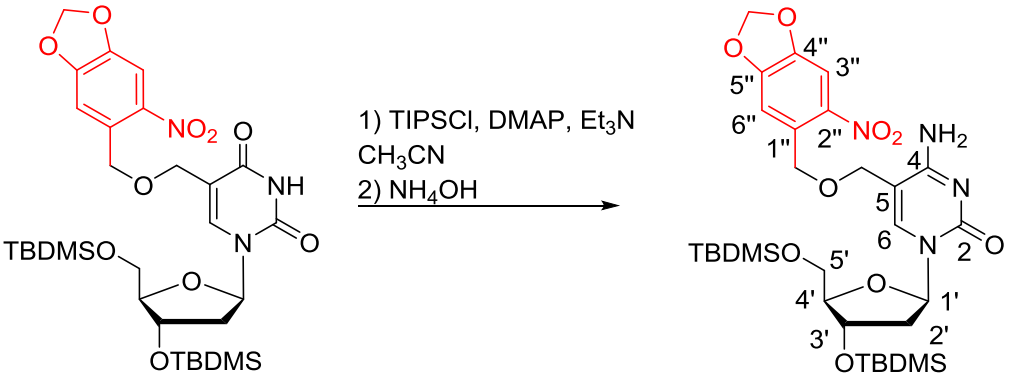
^{13}C NMR (125.7 MHz, D_2O): 42.15 (CH_2-2′); 67.6.7 (d, $J_{C,P}$ = 4.2 Hz, CH_2-5′); 69.90 (OCH_2-5); 72.11 (OCH_2-1″); 72.62 (CH-3′); 88.32 (d, $J_{C,P}$ = 9.3 Hz, CH-4′); 88.51 (CH-1′); 107.04 (C-5); 127.81 (CH-3″ -$C_6H_4NO_2$); 132.04 (CH-4″ -$C_6H_4NO_2$); 134.32 (CH-6″ -$C_6H_4NO_2$); 135.54 (C-1″ -$C_6H_4NO_2$); 136.77 (CH-5″ -$C_6H_4NO_2$); 144.46 (CH-6); 150.73 (C-2″ -$C_6H_4NO_2$); 159.88 (C-2); 167.64 (C-4).

^{31}P NMR (202.3 MHz, D_2O): -22.03 (dd, 1P, $J_{\beta,\chi}$ = 20.2 Hz, $J_{\beta,\alpha}$ = 19.8 Hz, P_β); -11.41 (d, 1P, $J_{\alpha,\beta}$ = 19.8, P_α); -6.12 (d, 1P, $J_{\chi,\beta}$ = 20.2 Hz, P_γ).

MS (ESI): m/z (%): 471.1 (51) $[M\text{-}2PO_3]^-$, 551.1 (100) $[M\text{-}PO_3\text{+}H]^-$, 573.0 (30) $[M\text{-}PO_3\text{+}Na]^-$, 653.0 (19) $[M\text{+}H\text{+}Na]^-$, 675.0 (24) $[M\text{+}2Na]^-$.

HRMS (ESI): m/z calcd for $C_{17}H_{20}O_{16}N_4Na_2P_3$: 674.9888 $[M+2Na]^-$, found: 674.9885; m/z calcd for $C_{17}H_{21}O_{16}N_4NaP_3$: 653.00686 $[M+H+Na]^-$; found 653.00662.

3′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-5-(6-nitropiperonyloxy)methyl-2′-deoxycytidine (2b)



Compound **1b** (125 mg, 0.19 mmol), DMAP (88 mg, 0.58 mmol) and 2,4,6-triisopropylbenzenesulfonyl chloride (217 mg, 0.53 mmol) were dissolved in dry CH_3CN. Triethylamine (105 µL, 0.59 mmol) was added and the reaction was stirred overnight at rt. Aqueous ammonia was added and stirring was continued for 2 h. The solvent was removed under reduced pressure. The crude product was extracted with EtOAc followed by chromatography (DCM:MeOH 10:1) to give **2b** as yellow powder (118 mg, 95 %).

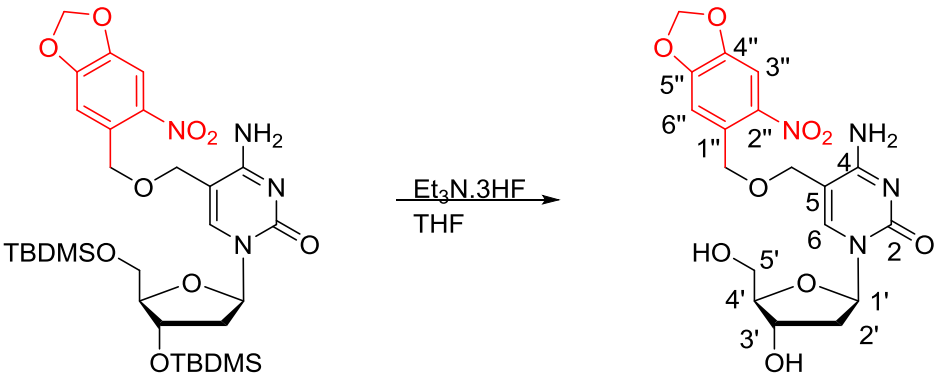
^{1}H NMR (500 MHz, DMSO-d_6): 0.037, 0.042, 0.078, and 0.081 (4x s, 4x 3H, CH_3Si); 0.84 and 0.87 (2x s, 2 x 9H, $(CH_3)_3C$); 2.05 (ddd, 1H, J_{gem}= 13.3 Hz, $J_{2'a,1'}$= 7.9 Hz, $J_{2'a,3'}$= 5.8 Hz, H-2′a); 2.13 (ddd, 1H, J_{gem}= 13.3 Hz, $J_{2'b,1'}$= 6.0 Hz, $J_{2'b,3'}$= 2.9 Hz, H-2′b); 3.71 (m, 2H, H-5′a, H-5′b); 3.81 (td, 1H, $J_{4',5'a}$= $J_{4',5'b}$= 4.2 Hz, $J_{4',3'}$= 2.6 Hz, H-4′); 4.30 (s, 2H, B-CH_2O); 4.33 (dt, $J_{3',2'b}$= 5.6 Hz, $J_{3',4'}$= $J_{3',2'a}$= 2.8 Hz, H-3′); 4.75 (s, 2H, Ph- CH_2O); 6.15 (dd, 1H, $J_{1',2'a}$= 7.9 Hz, $J_{1',2'b}$= 5.9 Hz, H-1′); 6.23 (s, 2H, OCH_2O); 6.83 (bs, 1H, NH_2-a); 7.20 (s, 1H, H-6′′); 7.47 (bs, 1H, NH_2-b); 7.62 (s, 1H, H-6); 7.68 (s, 1H, H-3′′)

^{13}C NMR (125.7 MHz, DMSO-d_6): -5.34, -4.73 and -4.58 (CH_3Si); 17.91 and 18.14 ($(CH_3)_3$**C**); 25.86 and 25.92 ((**C**$H_3)_3$C); 39.80 (CH_2-2′); 63.03 (CH_2-5′); 66.51 (B-CH_2O); 67.92 (Ph-CH_2O); 72.55 (CH-3′); 84.96 (CH-1′); 87.03 (CH-4′); 102.35 (C-5); 103.62 (OCH_2O); 105.28 (CH-3′′); 107.53 (CH-6′′); 132.04 (C-1′′); 140.78 (CH-6); 140.99 (C-2′′); 146.97 (C-4′′); 152.32 (C-5′′); 154.88 (C-2); 164.58 (C-4)

MS (ESI): m/z (%): 665 (100) $[M+H]^+$, 687 (64) $[M+Na]^+$.

HRMS (ESI): m/z calcd for $C_{30}H_{49}N_4O_9Si_2$: 665.30326 $[M+H]^+$; found: 665.30336.

5-(6-Nitropiperonyloxy)methyl-2´-deoxycytidine (dC^{NP})



Compound **2b** (118 mg, 0.18 mmol) was dissolved in dry THF, triethylamine trihydrofluoride (205 µL, 1.24 mmol) was added and the reaction was stirred overnight at rt in a plastic bottle. The solvent was removed under reduced pressure and the mixture was separated by chromatography (DCM:MeOH 7:1) to give **dC^{NP}** as a yellow powder (30 mg, 39 %).

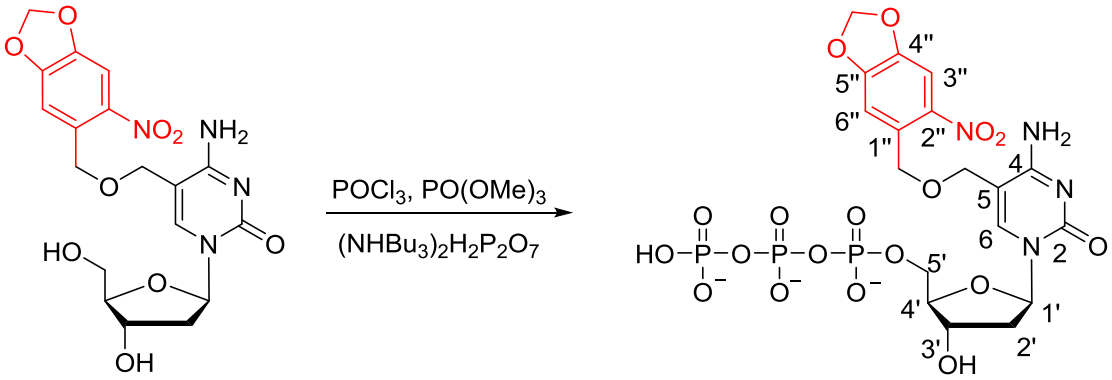
^{1}H NMR (500 MHz, DMSO-d$_6$): 1.97 (ddd, 1H, J_{gem}= 13.4 Hz, $J_{2´a,1´}$= 7.4 Hz, $J_{2´a,3´}$= 5.9 Hz, H-2´a); 2.11 (ddd, 1H, J_{gem}= 13.4 Hz, $J_{2´b,1´}$= 6.0 Hz, $J_{2´b,3´}$= 3.2 Hz, H-2´b); 3.56 (m, 2H, H-5´a, H-5´b); 3.77 (m, 1H, H-4´); 4.21 (m, 1H, H-3´); 4.32 (m, 2H, B-CH$_2$O); 4.75 (d, 2H, $J_{CH2,6´}$= 0.7 Hz, Ph-CH$_2$O); 4.97 (t, 1H, $J_{OH,5´}$= 5.4 Hz, HO-5´); 5.20 (d, 1H, $J_{OH,3´}$= 4.2 Hz, HO-3´); 6.14 (dd, 1H, $J_{1´,2´a}$= 7.4 Hz, $J_{1´,2´b}$= 6.0 Hz, H-1´); 6.23 (s, 2H, OCH$_2$O); 6.76 (bs, 1H, NH$_2$-a); 7.22 (s, 1H, H-6´´); 7.41 (bs, 1H, NH$_2$-b); 7.68 (s, 1H, H-3´´); 7.90 (s, 1H, H-6).

^{13}C NMR (125.7 MHz, DMSO-d$_6$): 40.54 (CH$_2$-2´); 61.52 (CH$_2$-5´); 66.57 (B-CH$_2$O); 67.93 (Ph-CH$_2$O); 70.57 (CH-3´); 85.10 (CH-1´); 87.47 (CH-4´); 102.13 (C-5); 103.60 (OCH$_2$O); 105.25 (CH-3´´); 107.54 (CH-6´´); 132.34 (C-1´´); 140.89 (C-2´´); 141.43 (CH-6); 146.93 (C-4´´); 152.39 (C-5´´); 155.02 (C-2); 164.59 (C-4).

MS (ESI): m/z (%): 459 (100) [M+Na]$^+$, 437 (72) [M+H]$^+$.

HRMS (ESI): m/z calcd for C$_{18}$H$_{21}$N$_4$O$_9$: 437.13030 [M+H]$^+$; found: 437.13054.

5-(6-Nitropiperonyloxy)methyl-2´-deoxycytidine triphosphate (dC^{NP}TP)



Nucleoside **dC^{NP}** (12 mg, 0.03 mmol) and proton sponge (12 mg, 0.06 mmol) were suspended in trimethyl phosphate (80 µL) in an argon purged flask and the suspension was cooled to 0 °C. Then, redistilled $POCl_3$ (5 µL, 0.06 mmol) was added. The reaction mixture was stirred at 0 °C for 40 min and then ice-cold solution of $(NHBu_3)_2H_2P_2O_7$ (77 mg, 0.11 mmol) and tributyl amine (28 µL, 0.12 mmol) in anhydrous DMF (2 mL) was added. The reaction mixture was stirred at 0 °C for another 1 h. Then aqueous solution of TEAB (2M, 2 mL, 4 mmol) was added and the mixture was evaporated under reduced pressure. The residue was co-evaporated several times with water. The product was purified by HPLC chromatography (0.1 M aq. TEAB 0→50 % methanol), co-evaporated several times with water and converted to sodium salt on Dowex. Triphosphate (4.2 mg, 21 %) was obtained as a yellowish lyophilizate (water).

^{1}H NMR (500 MHz, D_2O): 2.28 (dt, 1H, J_{gem} = 14.0 Hz, $J_{2'a,1'}$ = $J_{2'a,3'}$ = 6.5 Hz, H-2´a); 2.41 (ddd, 1H, J_{gem} = 14.0 Hz, $J_{2'b,1'}$ = 6.3 Hz, $J_{2'b,3'}$ = 4.2 Hz, H-2´b); 4.19 (m, 1H, H-4´); 4.25 (m, 2H, H-5´); 4.57 (d, 1H, J_{gem} = 13.1 Hz, B-CH_2O-a); 4.62 (d, 1H, J_{gem} = 13.1 Hz, B-CH_2O-b); 4.63 (dt, 1H, 1H, $J_{3',2'a}$ = 6.3 Hz, $J_{3',2'b}$ = $J_{3',4'}$ = 4.0 Hz, H-3´); 4.85 (d, 1H, J_{gem} = 13.2 Hz, OCH_2-Ph-a); 4.94 (d, 1H, J_{gem} = 13.2 Hz, OCH_2-Ph-b); 6.15 (s, 2H, OCH_2O); 6.24 (t, 1H, $J_{1',2'a}$ = $J_{1',2'b}$ = 6.5 Hz, H-1'); 7.04 (s, 1H, H-6´´); 7.57 (s, 1H, H-3´´); 7.95 (s, 1H, H-6).

^{13}C NMR (125.7 MHz, D_2O): 40.14 (CH_2-2´); 65.67 (d, $J_{C,P}$ = 5.6 Hz, CH_2-5´); 67.69 (B-CH_2O); 70.22 (OCH_2-Ph); 70.82 (CH-3'); 86.11 (d, $J_{C,P}$ = 9.1 Hz, CH-4'); 86.51 (CH-1'); 104.15 (OCH_2O); 105.08 (C-5); 106.40 (CH-3´´); 110.67 (CH-6´´); 131.45 (C-1´´); 142.11 (CH-6); 142.27 (C-2´´); 148.10 (C-4´´); 150.79 (C-5´´); 157.65 (C-2); 165.43 (C-4).

^{31}P NMR (202.3 MHz, D_2O): -22.02 (m, 1P, P_β); -10.84 (d, 1P, $J_{\alpha,\beta}$ = 19.8 Hz, P_α); -8.76 (bs, 1P, P_γ).

MS (ESI): m/z (%): 595 (100) $[M\text{-}HPO_3+2H]^-$.

HRMS (ESI): m/z calcd for $C_{18}H_{22}N_4O_{18}P_3$: 675.01474 $[M+2H]^-$; found: 675.01355.

2. ABSORPTION SPECTRA AND MOLAR ABSORPTION COEFFICIENTS

Molar absorption coefficients were determined for modified cytosine triphosphates in water at four different wavelengths in the range of 355-425 nm. Graphical evaluations are shown for 365 nm, for comparison with modified deoxyuridines[3].

2.1 dC^{NB}TP

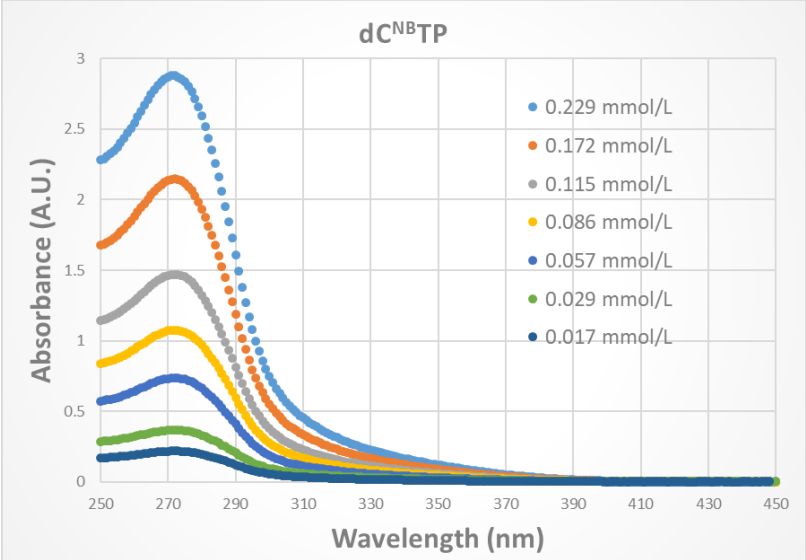


Figure S1: Absorption spectra of **dC^{NB}TP** in the range of 250-450 nm.

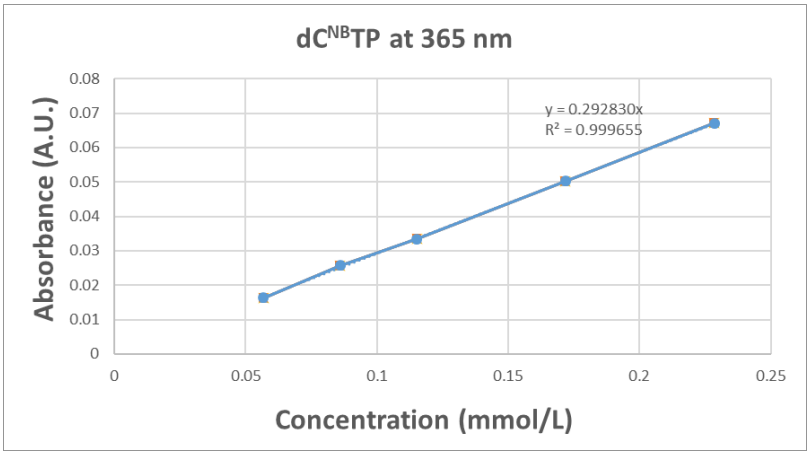


Figure S2: Calculation of the molar absorption coefficient of **dC^{NB}TP** using linear regression of concentrations and absorbances at 365 nm.

2.2 dC^{NP}TP

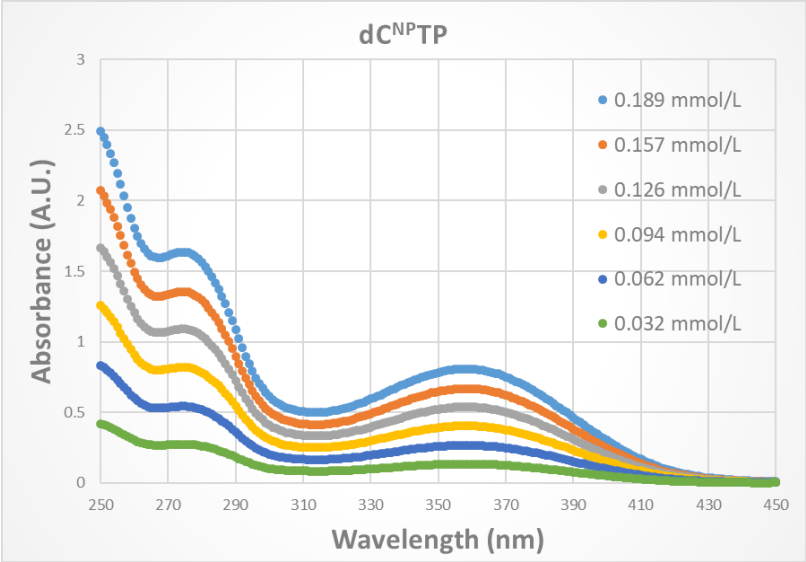


Figure S3: Absorption spectra of **dC^{NP}TP** in the range of 250-450 nm.

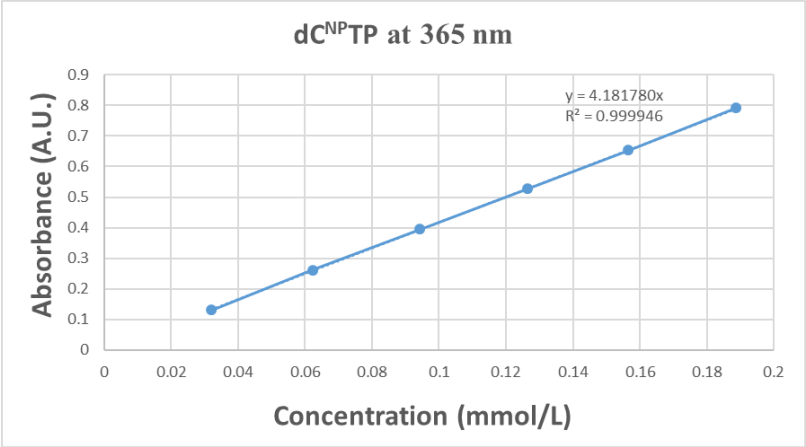


Figure S4: Calculation of the molar absorption coefficient of **dC^{NP}TP** using linear regression of concentrations and absorbances at 365 nm.

Table S1: Molar absorption coefficients in the range of 355-425 nm.

Compound	**dC^{NB}TP**	**dC^{NP}TP**
ε (355 nm)	443	4258
ε (365 nm)	293	4182
ε (400 nm)	35	1598
ε (425 nm)	14	290

3. BIOCHEMISTRY

Table S2: List of oligonucleotides

Name	*Sequence 5'→3'* [a,b]	*Length*
Primer 248sh	CATGGGCGGCATGGG	15-mer
Oligo1C	CCCGCCCATGCCGCCCATG	19-mer
Prb4basII	CTAGCATGAGCTCAGTCCCATGCCGCCCATG	31-mer
AflC	AACTACTAC**CTTAAG**CCCATGCCGCCCATG	30-mer
EcC	AACTACTAC**GAATTC**CCCATGCCGCCCATG	30-mer
PvC	AACTACTAC**CAGCTG**CCCATGCCGCCCATG	30-mer
ScC	AACTACTAC**AGTACT**CCCATGCCGCCCATG	30-mer
RsC	AACTACTACT**GTAC**ACCCATGCCGCCCATG	30-mer
KpC	AACTACTAC**GGTACC**CCCATGCCGCCCATG	30-mer
Primer L20	GACATCATGAGAGACATCGC	20-mer
Primer LT25TH	CAAGGACAAAATACCTGTATTCCTT	25-mer
FVLA	GACATCATGAGAGACATCGCCTCTGGGCTAATA GGACTACTTCTAATCTGTAAGAGCAGATCCCTG GACAGGCAAGGAATACAGGTATTTTGTCCTTG	98-mer
Primer FOR-ins100bp	TTCAGCCATATATCCTCTGGCTAATAGG	28-mer
Primer P-REV-P/P_2	GGAGAGCGTTCACCGACAAACAACAG	26-mer
Pveg-modif-2	TTCAGCCATATATCCTCTGGCTAATAGGACTACT TCTAATCTGTAAGAGCAGATCCATACGCCTGGA CAGGCAATCAGGCTAGAGGAATTCTATTTGACA AAAATGGGCTCGTGTTGTACAATAAATGTGTCT AAGCTTGGGTCCCACCTGACCCCATGCCGAACT CAGAAGTGAAACGCCGTAGCGCCGATGGTAGTG TGGGGTCTCCCCATGCGAGAGTAGGGAACTGCC AGGCATCAAATAAAACGAAAGGCTCAGTCGAA AGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCG GTGAACGCTCTCC	311-mer

[a] position of modified nucleobases in product strand is underlined

[b] specific sequence for restriction endonuclease is bold

3.1 A single modification incorporation by PEX

3.1.1 KOD XL DNA polymerase

The reaction mixture (20 µL) contained KOD XL DNA polymerase (2.5 U/µL, 0.2 µL), dNTPs (1 mM, 0.6 µL or 0.8 µL for **dC^{NB}TP** and **dC^{NP}TP**), primer 248sh (3 µM, 1 µL), 19-mer template oligo1C (3 µM, 1.5 µL) and 10x buffer for KOD XL DNA polymerase (2 µL) supplied by the manufacturer. Reaction mixtures were incubated for 30 min at 60 °C in a thermomixer. After reaction samples were denatured by the addition of the stop solution (20 µL, 80% [v/v] formamide, 20 mM EDTA, 0.025%, [w/v] bromophenol blue, 0.025 % [w/v] xylene cyanol, PCR water) followed by heating for 5 min at 95 °C. Reaction mixtures were separated using 12.5 % denaturing PAGE.

3.1.2 Vent exo- DNA polymerase

The reaction mixture (20 µL) contained Vent exo- polymerase (2 U/µL, 0.08 µL), dNTPs (1mM, 0.6 µL or 0.8 µL for **dC^{NB}TP** and **dC^{NP}TP**), primer 248sh (3 µM, 1 µL), 19-mer template oligo1C (3 µM, 1.5 µL) and 10x Thermopol buffer (2 µL) supplied by the manufacturer. Reaction mixtures were incubated for 30 min at 60 °C in a thermomixer. After reaction, samples were denatured by addition of stop solution (20 µL, 80% [v/v] formamide, 20 mM EDTA, 0.025%, [w/v] bromophenol blue, 0.025 % [w/v] xylene cyanol, PCR water) followed by heating for 5 min at 95 °C. Reaction mixtures were separated using 12.5 % denaturing PAGE.

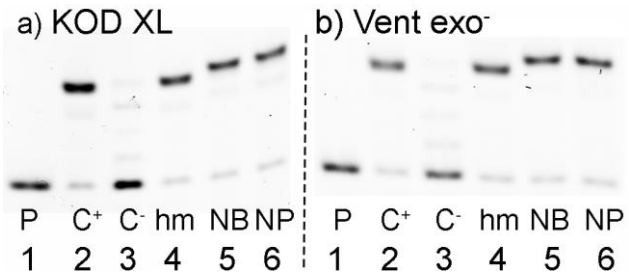


Figure S5: Denaturing PAGE analysis of PEX with oligo1C template using a) KOD XL DNA polymerase b) Vent exo- polymerase: Lane 1, P: primer; lane 2, C$^+$: product of PEX with natural dNTPs; lane 3, C$^-$: products of PEX in the absence of dCTP; lane 4, hm: product of PEX with modified **dC^{hm}TP** and three natural dNTPs; lane 5, NB: product of PEX with modified **dC^{NB}TP** and three natural dNTPs; lane 6, NP: product of PEX with modified **dC^{NP}TP** and three natural dNTPs.

3.1.3 MALDI analysis of PEX products

The reaction mixture (100 µL) contained KOD XL DNA polymerase (2.5 U/µL, 0.2 µL), GTP (1 mM, 21 µL), dCTP or modified dCTP (1 mM, 25 µL), primer 248sh (10 µM, 3.2 µL), 19-mer biotinylated template oligo1C (100 µM, 3.2 µL) and 10x buffer for KOD XL DNA polymerase (10 µL) supplied by the manufacturer. Reaction mixtures were incubated for 40 min at 60 °C in a thermomixer. After reaction, samples were purified on magnetic beads and MALDI was measured.

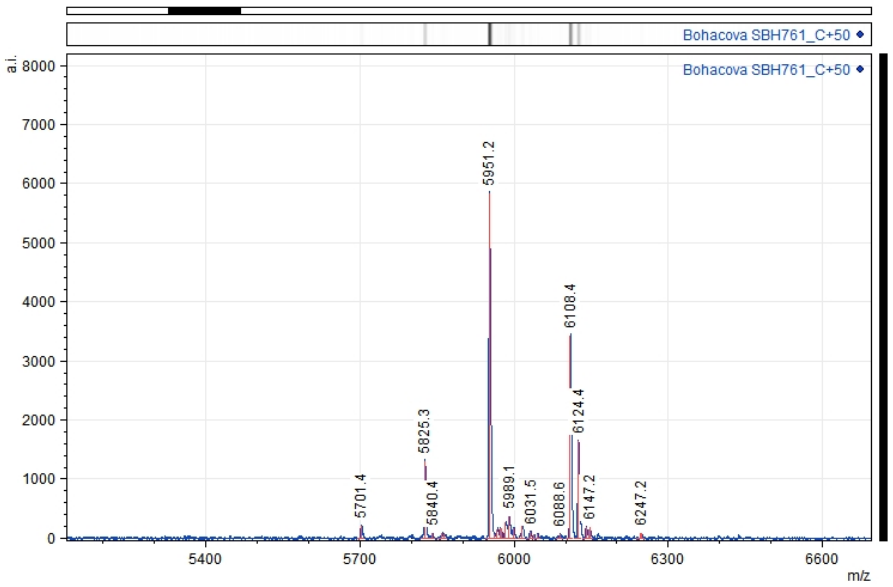


Figure S6: MALDI of positive control: M(calc) 5951; M(found) 5951 [M], 6108 [Temp+3].

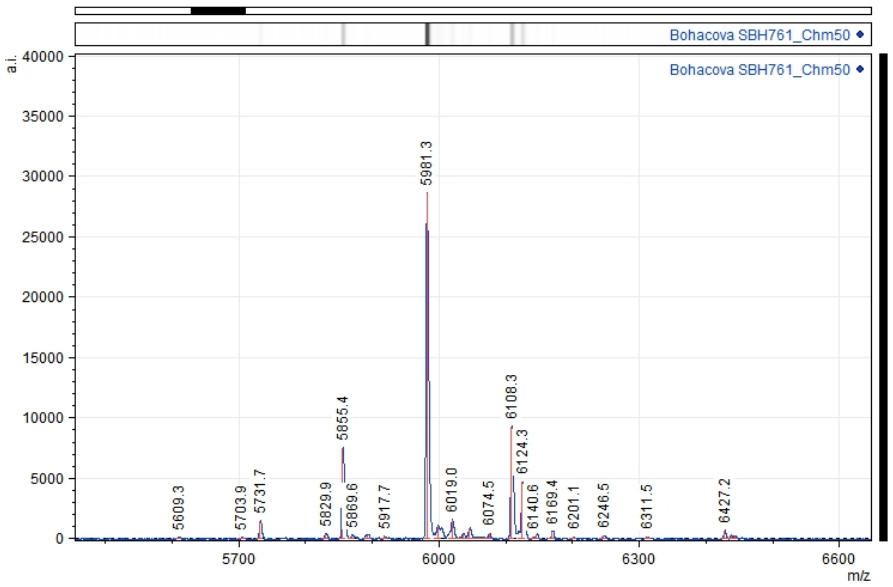


Figure S7: MALDI of hm modified PEX product: M(calc) 5981; M(found) 5981 [M], 6108 [Temp+3].

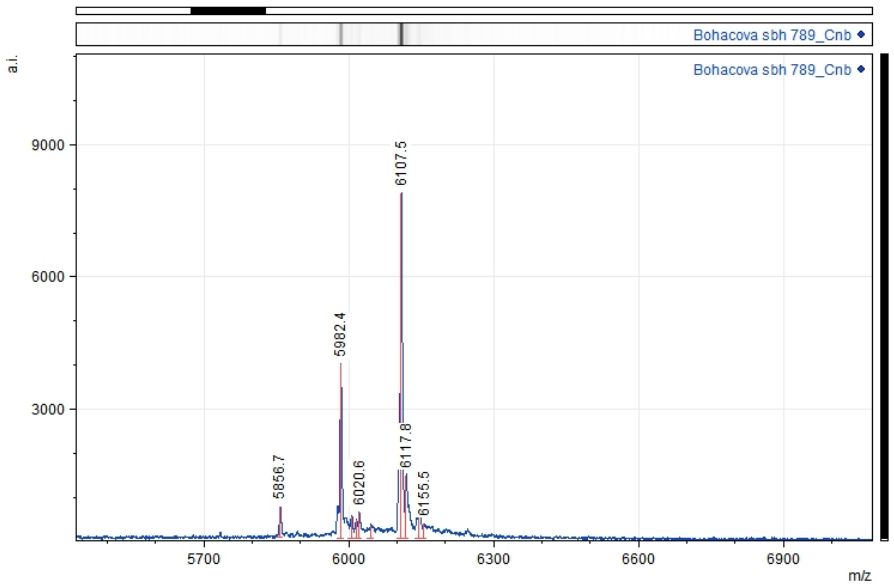


Figure S8: MALDI of NB modified PEX product: M(calc) 6116; M(found) 5982.4 [M-C^{NB}+C^{hm}], 6107.6 [Temp+2], 6117.6 [M+2].

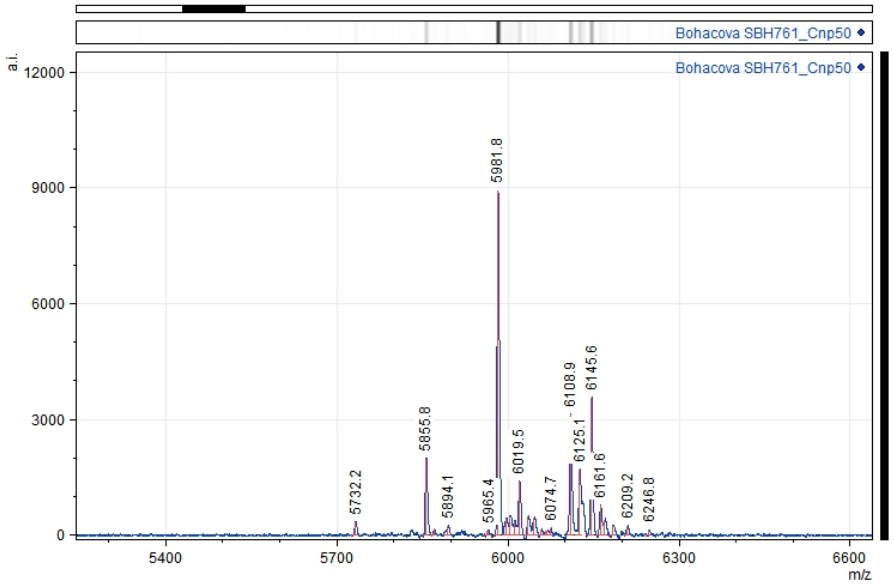


Figure S9: MALDI of NP modified PEX product: M(calc) 6160; M(found) 5982 [M-C^{NP}+C^{hm}].

3.2 Kinetics of single modification incorporation by PEX

3.2.1 KOD XL DNA polymerase

The reaction mixture (20 µL) contained KOD XL DNA polymerase (2.5 U/µL, 0.02 µL), dCTP or modified dCTP (1 mM, 0.8 µL), 6-FAM labelled primer 248sh (3 µM, 1 µL), 19-mer oligo1C template (3 µM, 1.5 µL) and 10x buffer for KOD XL DNA polymerase (2 µL) supplied by the manufacturer. Reaction mixtures were incubated for five different times (0.1, 0.5, 1, 2, 5 min)

at 60 °C in a thermomixer. After reaction, samples were denatured by the addition of the stop solution (20 µL, 80% [v/v] formamide, 20 mM EDTA, 0.025%, [w/v] bromophenol blue, 0.025 % [w/v] xylene cyanol, PCR water) followed by heating for 5 min at 95 °C. Reaction mixtures were separated using 12.5 % denaturing PAGE.

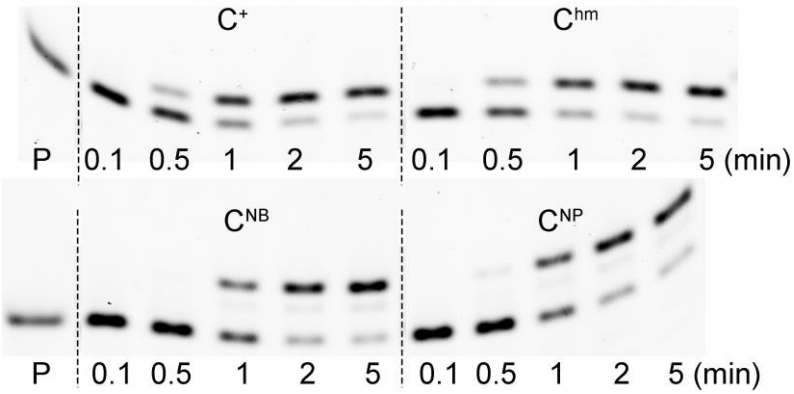


Figure S10: Kinetics of single modification incorporation by PEX using KOD XL polymerase

3.2.2 Vent exo- DNA polymerase

The reaction mixture (20 µL) contained Vent exo- DNA polymerase (2.5 U/µL, 0.08 µL), dCTP or modified dCTP (1 mM, 0.8 µL), 6-FAM labelled primer 248sh (3 µM, 1 µL) and 19-mer oligo1C template (3 µM, 1.5 µL) and 10x buffer for KOD XL DNA polymerase (2 µL) supplied by the manufacturer. Reaction mixtures were incubated for five different times (0.1, 0.5, 1, 2, 5 min) at 60 °C in a thermomixer. After reaction samples were denatured by the addition of the stop solution (20 µL, 80% [v/v] formamide, 20 mM EDTA, 0.025%, [w/v] bromophenol blue, 0.025 % [w/v] xylene cyanol, PCR water) and heating for 5 min at 95 °C. Reaction mixtures were separated using 12.5 % denaturing PAGE.

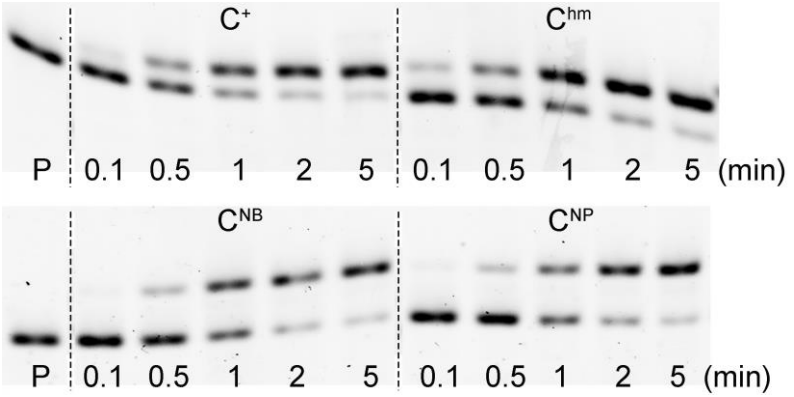


Figure S11: Kinetics of single modification incorporation by PEX using Vent exo- polymerase

3.3 Multiple modification incorporation by PEX

The reaction mixture (20 µL) contained KOD XL DNA polymerase (2.5 U/µL, 0.02 µL), dNTPs (1mM, 1.5 µL or 3 µL for **$C^{NB}TP$** and **$C^{NP}TP$**), primer 248sh (3 µM, 1 µL), 31-mer template prb4basII (3 µM, 1.5 µL) and 10x buffer for KOD XL DNA polymerase (2 µL) supplied by the manufacturer. Reaction mixtures were incubated for 40 min at 60 °C in a thermomixer. After reaction samples were denatured by addition of stop solution (20 µL, 80% [v/v] formamide, 20 mM EDTA, 0.025%, [w/v] bromophenol blue, 0.025 % [w/v] xylene cyanol, PCR water) followed by heating for 5 min at 95 °C. Reaction mixtures were separated using 12.5 % denaturing PAGE.

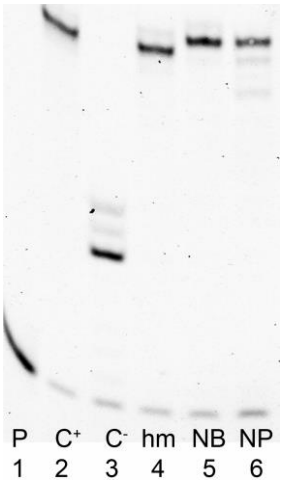


Figure S12: Denaturing PAGE analysis of PEX with prb4basII 31-mer template: Lane 1, P: primer; lane 2, C^+: product of PEX with natural dNTPs; lane 3, C^-: products of PEX in the absence of dCTP; lane 4, hm: product of PEX with modified **$dC^{hm}TP$** and three natural dNTPs; lane 5, NB: product of PEX with modified **$dC^{NB}TP$** and three natural dNTPs, lane 6, NP: product of PEX with modified **$dC^{NP}TP$** and three natural dNTPs.

3.3.1 MALDI analysis of PEX products

The reaction mixture (100 µL) contained KOD XL DNA polymerase (2.5 U/µL, 0.2 µL), dNTPs (1mM, 21 µL or 25 µL for modified dCTP), primer 248sh (10µM, 3.2 µL) and biotinylated 31-mer template prb4basII (100µM, 3.2 µL) and 10x buffer for KOD XL DNA polymerase (10 µL) supplied by the manufacturer. Reaction mixtures were incubated for 40 min at 60 °C in a thermomixer. After reaction samples were purified on magnetic beads and MALDI was measured.

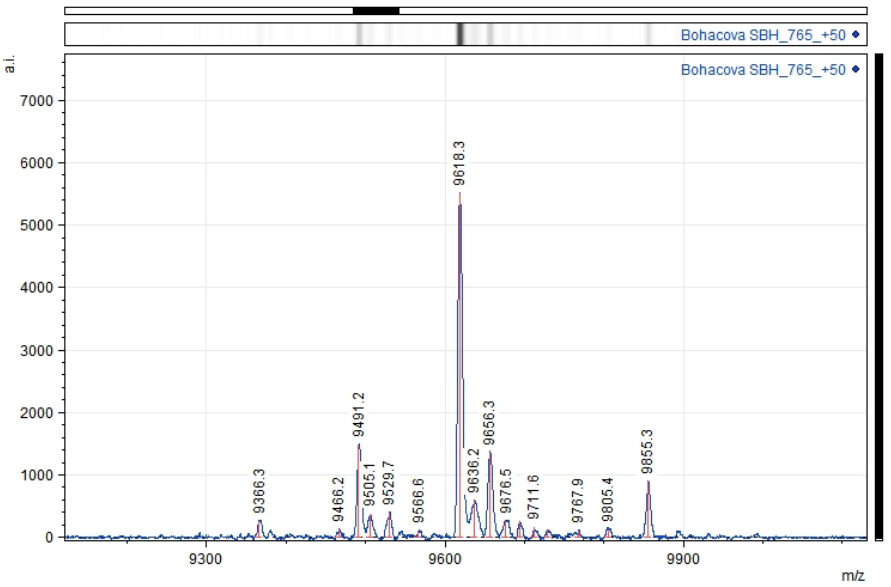


Figure S13: MALDI of positive control: M(calc) 9617; M(found) 9618 [M+1].

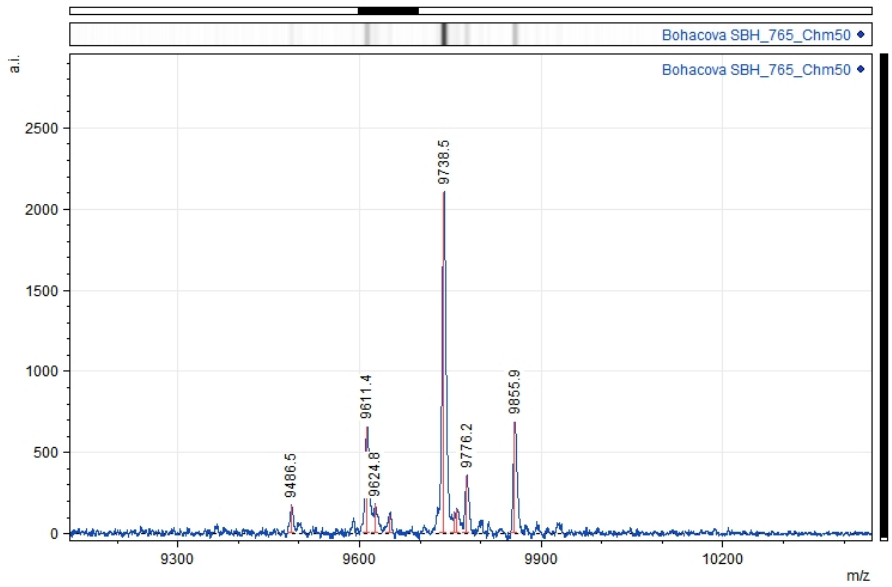


Figure S14: MALDI of hm modified PEX product: M(calc) 9737; M(found) 9738 [M+1].

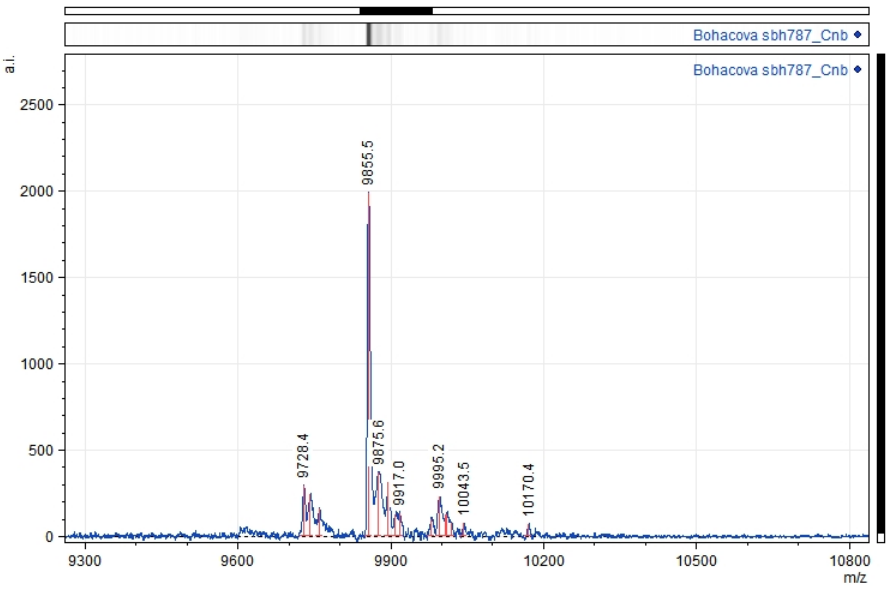


Figure S15: MALDI of NB modified PEX product: M(calc) 10277.5; M(found) 9855.5 [Temp+3], 9875.6 [M-3C^{NB}+3C^{hm}+3H].

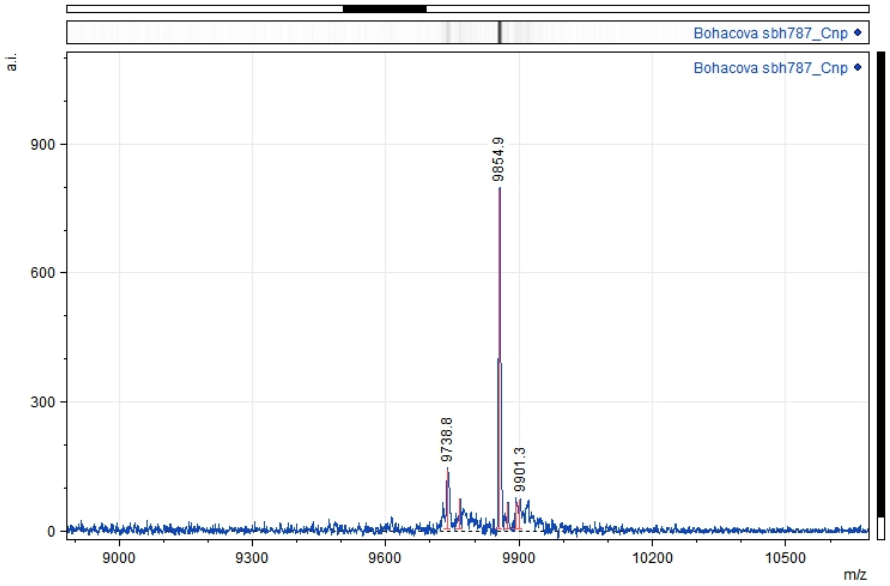


Figure S16: MALDI of NP modified PEX product: M(calc) 10453.5; M(found) 9854.9 [Temp+3], 9738.8 [M-4C^{NP}+4C^{hm}+H].

3.4 PEX product uncaging

3.4.1 Searching for REs that tolerate C^{hm}

PEX procedure for templates KpC, PvC, ScC

The reaction mixtures (40 μL each) contained Pwo DNA polymerase (Peqlab, 1 U/μL, 0.4 μL), natural dNTPs (4 mM, 2 μL), functionalized-dCTP (4 mM, 2 μL), FAM-labelled primer 248sh (3μM, 2 μL), and 30-mer template **KpC, PvC** or **ScC** (3μM, 3 μL) in Pwo reaction buffer (4 μL) supplied by the manufacturer. Reaction mixtures were incubated for 30 min at 60 °C in a thermomixer. Subsequently, the each reaction mixture was divided into two portions. The first one was denatured by addition of stop solution (20 μL) and heated 5 min at 95 °C. The second portion was used in the study of cleavage by restriction endonucleases.

PEX procedure for templates AflC, EcC, RsC

The reaction mixtures (40 μL each) contained Pwo DNA polymerase (Peqlab, 1 U/μL, 0.26 μL), natural dNTPs (4 mM, 0.5 μL), functionalized-dCTP (4 mM, 1 μL), FAM- labelled primer 248sh (3 μM, 2 μL), and 30-mer template AflC, EcC or RsC (3 μM, 3 μL) in Pwo reaction buffer (4 μL) supplied by the manufacturer. Reaction mixtures were incubated for 30 min at 60 °C in a thermomixer. Subsequently, the each reaction mixture was divided into two portions. The first one was denatured by addition of stop solution (20 μL,) and heated 5 min at 95 °C. The second portion was used in the study of cleavage by restriction endonucleases.

Cleavage by restriction endonucleases-general procedure

The second portions of reaction mixtures after PEX experiment (20 μL each) were mixed with 2 μL of reaction buffer (buffer for KpnI - NEBuffer-1.1; EcoRI – EcoRIBuffer; for AflII, RsaI, PvuII and HF-ScaI CutSmart Buffer was used) supplied by the manufacturer, together with one of the restriction endonuclease (KpnI/ EcoRI/ AflII/ PvuII/ HF-ScaI - NewEngland Biolabs, 2U, 0.5 μL or 0.75 μL in the case of RsaI). Each reaction mixture was incubated at 37 °C for 60 min. The products of cleavage were denatured by addition of stop solution (20 μL) and heated for 5 min at 95 °C.

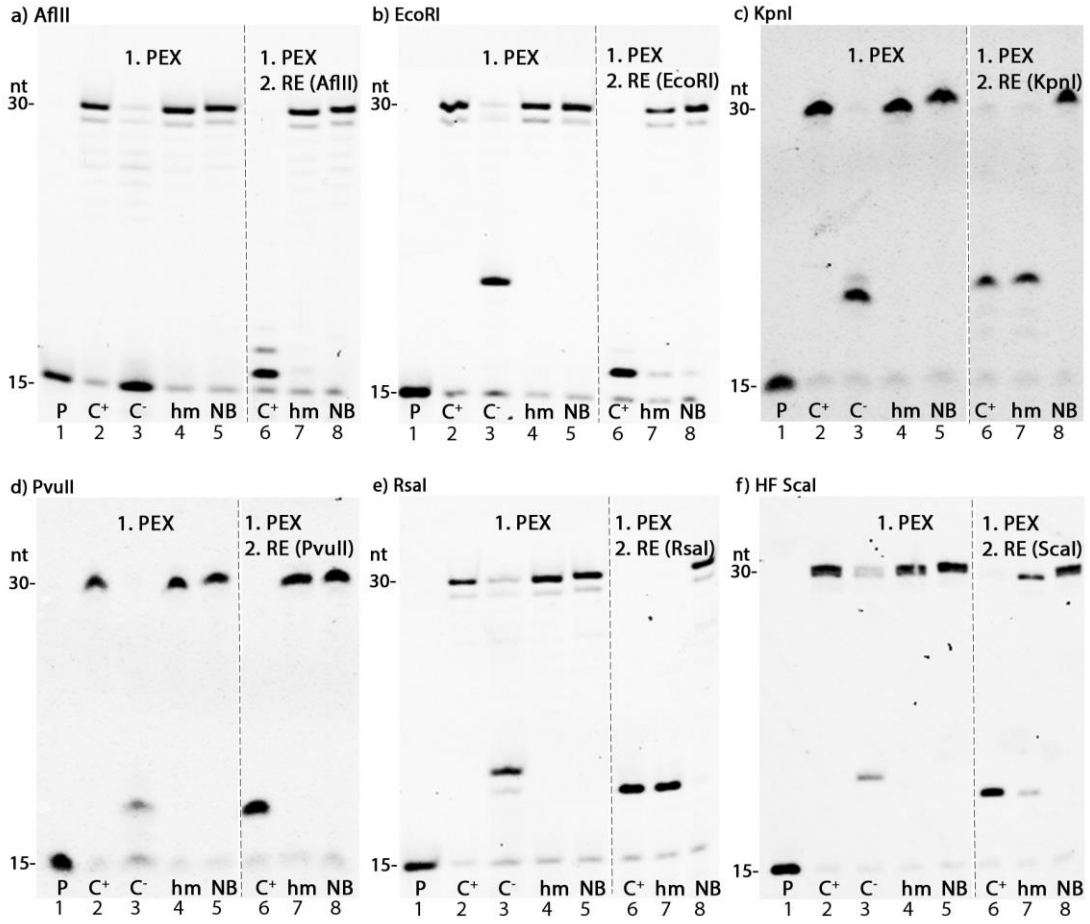


Figure S17: Primer extension experiment and study of cleavage by restriction endonucleases: lanes 1, **P**: primer; lanes 2, C^+: positive control experiments (product of PEX with natural dNTPs); lanes 3, $\mathbf{C^-}$: negative control experiments (absence of natural dCTP); lanes 4, $\mathbf{C^{hm}}$ product of PEX in the presence of $\mathbf{dC^{hm}TP}$ and 3 other natural dNTPs; lanes 5, **NB**: product of PEX with $\mathbf{dC^{NB}TP}$ and 3 other natural dNTPs; lanes 6, $\mathbf{C^+}$: product of PEX with natural dNTPs followed by cleavage with a RE (AflII/ EcoRI/ PvuII/ KpnI/ RsaI/ ScaI), lanes 7, $\mathbf{C^{hm}}$: product of PEX in the presence of $\mathbf{dC^{hm}TP}$ and 3 other natural dNTPs followed by cleavage with a RE (AflII/ EcoRI/ KpnI/ PvuII/ RsaI/ ScaI); lanes 8, **NB** product of PEX with $\mathbf{dC^{NB}TP}$ and 3 other natural dNTPs followed by cleavage with a RE (no cleavage).

3.4.2 PEX product interaction with restriction enzymes KpnI and RsaI

KpC template

The reaction mixture (20 µL) contained KOD XL DNA polymerase (2.5 U/µL, 0.02 µL), dNTPs (1mM, 0.5 µL or 1 µL for $\mathbf{dC^{NB}TP}$ and $\mathbf{dC^{NP}TP}$), primer 248sh (3 µM, 1 µL), 30-mer template KpC (3 µM, 1.5 µL) and 10x buffer for KOD XL DNA polymerase (2 µL) supplied by the manufacturer. Reaction mixtures were incubated for 30 min at 60 °C in a thermomixer.

After reaction, samples were either:

a) denatured by addition of stop solution (20 µL, 80% [v/v] formamide, 20 mM EDTA, 0.025%, [w/v] bromophenol blue, 0.025 % [w/v] xylene cyanol, PCR water) followed by heating for 5 min at 95 °C. Reaction mixtures were separated using 12.5 % denaturing PAGE.
b) Incubated with KpnI (1.6 µL) in 1.1 buffer (2.2 µL) and denatured.

RsC template

The reaction mixture (20 µL) contained KOD XL DNA polymerase (2.5 U/µL, 0.02 µL), dNTPs (1mM, 0.2 µL or 0.25 µL for **dC^{NB}TP** and **dC^{NP}TP**), primer 248sh (3 µM, 1 µL), 30-mer template RsC (3 µM, 1.5 µL) and 10x buffer for KOD XL DNA polymerase (2 µL) supplied by the manufacturer. Reaction mixtures were incubated for 30 min at 60 °C in a thermomixer.

After reaction, samples were either:

a) denatured by addition of stop solution (20 µL, 80% [v/v] formamide, 20 mM EDTA, 0.025%, [w/v] bromophenol blue, 0.025 % [w/v] xylene cyanol, PCR water) followed by heating for 5 min at 95 °C. Reaction mixtures were separated using 12.5 % denaturing PAGE.
b) Incubated with RsaI (1.6 µL) in CutSmart buffer (2.2 µL) and denatured.

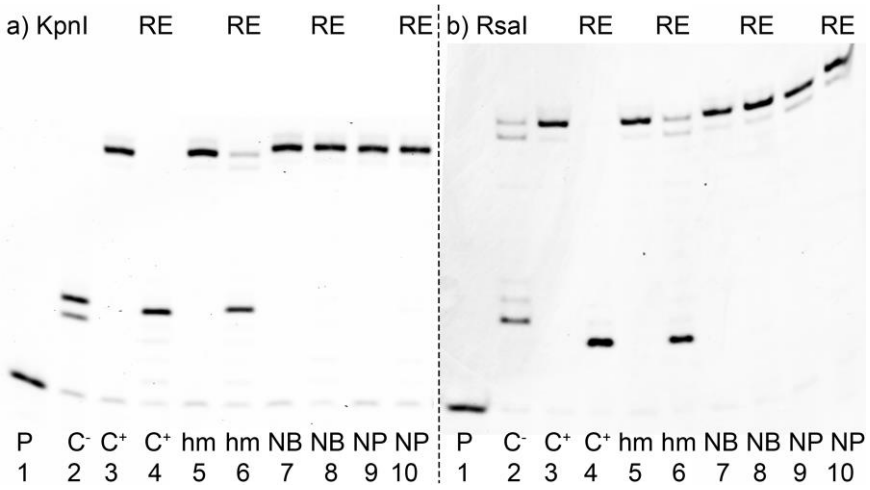


Figure S18: Denaturing PAGE analysis of PEX with a) KpC b) RsC 30-mer template: Lane 1, P: primer; lane 2, C$^-$: product of PEX in the absence of dCTP; lane 3, C$^+$: product of PEX with natural dNTPs; lane 4, C$^+$: products of PEX with natural NTPs followed by the reaction with RE; lane 5, hm: product of PEX with modified **dC^{hm}TP** and three natural dNTPs; lane 6, hm: product of PEX with modified **dC^{hm}TP** and three natural dNTPs followed by reaction with RE; lane 7, NB: product of PEX with modified **dC^{NB}TP** and three natural dNTPs; lane 8, NB: product of PEX with modified **dC^{NB}TP** and three natural dNTPs followed by the reaction with RE; lane 9, NP: product of PEX with modified **dC^{NP}TP** and three natural dNTPs; lane 10, NP: product of PEX with modified **dC^{NP}TP** and three natural dNTPs followed by the reaction with RE.

3.4.3 Uncaging

KpC template

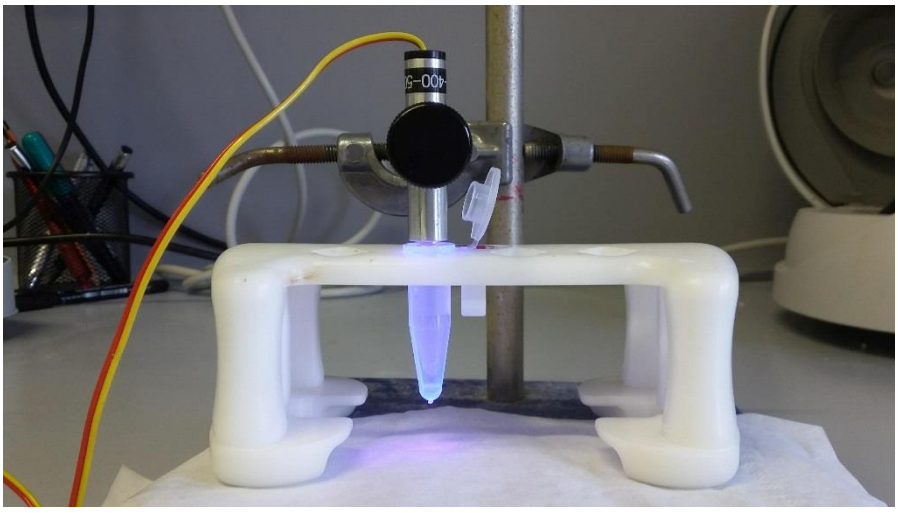
The reaction mixture (20 µL) contained KOD XL DNA polymerase (2.5 U/µL, 0.02 µL), dNTPs (1mM, 0.5 µL or 1 µL for **dC^{NB}TP** and **dC^{NP}TP**), primer 248sh (3 µM, 1 µL), 30-mer template KpC (3 µM, 1.5 µL) and 10x buffer for KOD XL DNA polymerase (2 µL) supplied by the manufacturer. Reaction mixtures were incubated for 30 min at 60 °C in a thermomixer.

After reaction, samples were either:

a) denatured by addition of stop solution (20 µL, 80% [v/v] formamide, 20 mM EDTA, 0.025%, [w/v] bromophenol blue, 0.025 % [w/v] xylene cyanol, PCR water) followed by heating for 5 min at 95 °C. Reaction mixtures were separated using 12.5 % denaturing PAGE.
b) Incubated with KpnI (1.6 µL) in 1.1 NEBuffer (2.2 µL) and denatured
c) Irradiated with UV LED followed by incubation with KpnI (1.6 µL) in 1.1 NEBuffer (2.2 µL) and denaturation.

Experimental set-up for the UV irradiation:

The solution of photocaged DNA (20 µL) after PEX reaction was irradiated with UV LED mounted directly on the top of an opened Eppendorf vial (see the photograph below). The distance between the LED and the sample solution was approximately 3.6 cm. The irradiation at 8°C was performed for the particular time interval. In the next step, the irradiated dsDNA was treated with restriction endonuclease RsaI or KpnI under conditions described in the part b) and the product was finally denatured by addition of stop solution and followed by heating for 5 min at 95 °C.



RsC template

The reaction mixture (20 µL) contained KOD XL DNA polymerase (2.5 U/µL, 0.02 µL), dNTPs (1mM, 0.2 µL or 0.25 µL for **dC^{NB}TP** and **dC^{NP}TP**), primer 248sh (3 µM, 1 µL), 30-mer template RsC (3 µM, 1.5 µL) and 10x buffer for KOD XL DNA polymerase (2 µL) supplied by the manufacturer. Reaction mixtures were incubated for 30 min at 60 °C in a thermomixer.

After reaction samples were either:

c) denatured by addition of stop solution (20 µL, 80% [v/v] formamide, 20 mM EDTA, 0.025%, [w/v] bromophenol blue, 0.025 % [w/v] xylene cyanol, PCR water) followed by heating for 5 min at 95 °C. Reaction mixtures were separated using 12.5 % denaturing PAGE.
d) Incubated with RsaI (1.6 µL) in CutSmart buffer (2.2 µL) and denatured
e) Irradiated with UV LED followed by incubation with RsaI (1.6 µL) in CutSmart buffer (2.2 µL) and denaturation.

UV LED 355 nm (0.8-1.2 mW)

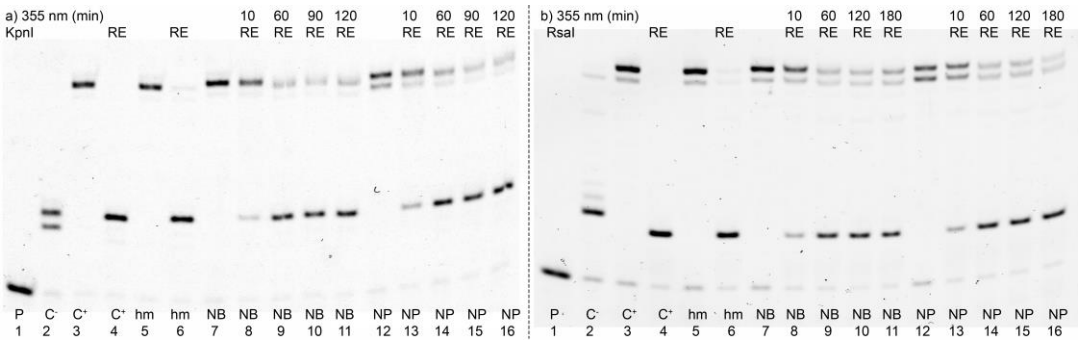


Figure S19: Denaturing PAGE analysis of PEX with 30-mer template after uncaging at 355 nm a) KpC template b) RsC template: lane 1, P: primer; lane 2, C^-: product of PEX in the absence of dCTP; lane 3, C^+: product of PEX with natural dNTPs; lane 4, C^+: product of PEX with natural dNTPs after incubation with RE; lane 5, hm: product of PEX with modified **dC^{hm}TP** and three natural dNTPs; lane 6, hm: product of PEX with modified **dC^{hm}TP** and three natural dNTPs after incubation with RE; lane 7, NB: product of PEX with modified **dC^{NB}TP** and three natural dNTPs, lanes 8-11, NB: product of PEX with modified **dC^{NB}TP** and three natural dNTPs after irradiation with UV LED followed by incubation with RE; lane 12, NP: product of PEX with modified **dC^{NP}TP** and three natural dNTPs, lanes 13-16, NP: product of PEX with modified **dC^{NP}TP** and three natural dNTPs after irradiation with UV LED followed by incubation with RE.

Table S3: DNA uncaging conversions quantification at 355 nm evaluated from gels using ImageJ software.

Temp.	Mod.	t1 (min)	Uncaged (%)	t2 (min)	Uncaged (%)	t3 (min)	Uncaged (%)	t4 (min)	Uncaged (%)
KpC	**NB**	10	16	60	67	90	72	120	66
KpC	**NP**	10	24	60	62	90	70	120	71
RsC	**NB**	10	19	60	57	120	61	180	50
RsC	**NP**	10	19	60	60	120	61	180	73

UV LED 400 nm (21-29.4 mW)

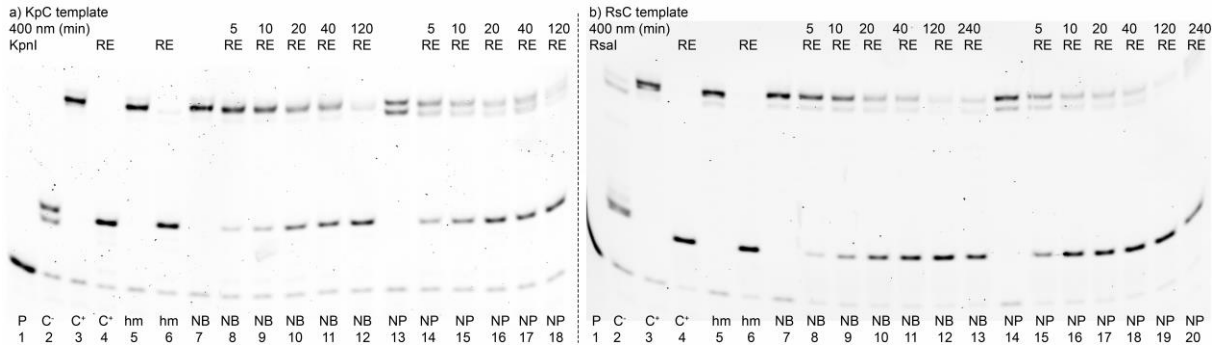


Figure S20: Denaturing PAGE analysis of PEX with 30-mer template after uncaging at 400 nm a) KpC template b) RsC template: lane 1, P: primer; lane 2, C^-: product of PEX in the absence of dCTP; lane 3, C^+: product of PEX with natural dNTPs; lane 4, C^+: product of PEX with natural dNTPs after incubation with RE; lane 5, hm: product of PEX with modified **dC^{hm}TP** and three natural dNTPs; lane 6, hm: product of PEX with modified **dC^{hm}TP** and three natural dNTPs after incubation with RE; lane 7, NB: product of PEX with modified **dC^{NB}TP** and three natural dNTPs, lanes 8-12 or 8-13, NB: product of PEX with modified **dC^{NB}TP** and three natural dNTPs after irradiation with UV LED followed by incubation with RE; lane 13 or 14, NP: product of PEX with modified **dC^{NP}TP** and three natural dNTPs, lanes 14-18 or 15-20, NP: product of PEX with modified **dC^{NP}TP** and three natural dNTPs after irradiation with UV LED followed by incubation with RE.

Table S4: DNA uncaging conversions quantification at 400 nm evaluated from gels using ImageJ software.

Temp.	Mod.	t1 (min)	Uncaged (%)	t2 (min)	Uncaged (%)	t3 (min)	Uncaged (%)
KpC	**NB**	5	9	10	17	20	43
KpC	**NP**	5	27	10	45	20	65
RsC	**NB**	5	10	10	26	20	55
RsC	**NP**	5	36	10	69	20	71

Temp.	Mod.	t4 (min)	Uncaged (%)	t5 (min)	Uncaged (%)	t6 (min)	Uncaged (%)
KpC	**NB**	40	46	120	81	-	-
KpC	**NP**	40	53	120	78	-	-
RsC	**NB**	40	64	120	85	240	73
RsC	**NP**	40	79	120	95	240	71

UV LED 425 nm (10-16 mW)

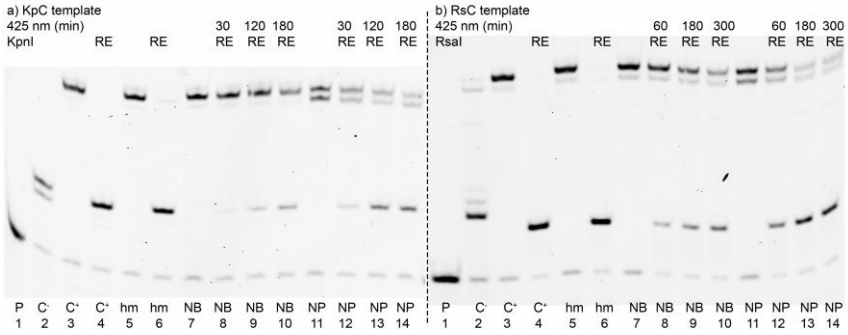


Figure S21: Denaturing PAGE analysis of PEX with 30-mer template after uncaging at 425 nm a) KpC template b) RsC template: lane 1, P: primer; lane 2, C^-: product of PEX in the absence of dCTP; lane 3, C^+: product of PEX with natural dNTPs; lane 4, C^+: product of PEX with natural dNTPs after incubation with RE; lane 5, hm: product of PEX with modified **dC^{hm}TP** and three natural dNTPs; lane 6, hm: product of PEX with modified **dC^{hm}TP** and three natural dNTPs after incubation with RE; lane 7, NB: product of PEX with modified **dC^{NB}TP** and three natural dNTPs, lanes 8-10, NB: product of PEX with modified **dC^{NB}TP** and three natural dNTPs after irradiation with UV LED followed by incubation with RE; lane 11, NP: product of PEX with modified **dC^{NP}TP** and three natural dNTPs, lanes 12-14, NP: product of PEX with modified **dC^{NP}TP** and three natural dNTPs after irradiation with UV LED followed by incubation with RE.

Table S5: DNA uncaging conversions quantification at 425 nm evaluated from gels using ImageJ software.

Temp.	Mod.	t1 (min)	Uncaged (%)	t2 (min)	Uncaged (%)	t3 (min)	Uncaged (%)
KpC	**NB**	30	2	120	11	180	28
KpC	**NP**	30	11	120	44	180	49
RsC	**NB**	60	15	180	27	300	42
RsC	**NP**	60	31	180	65	300	65

3.5 PCR

98-mer

The reaction mixture (20 µL) contained KOD XL DNA polymerase (2.5 U/µL, 0.2 µL), dNTPs (5mM, 1.2 µL or modified dCTP 2.4 µL), primers LT20 (10 µM, 2 µL) and LT25TH (10 µM, 2 µL) and 98-mer template FVL-A (1 µM, 0.5 µL), 10x buffer for KOD XL DNA polymerase (2 µL) supplied by the manufacturer and $MnCl_2$ (10 mM, 1 µL). After initial denaturation for 3 min at 94 °C, 30 PCR cycles were run under following conditions: denaturation for 1 min at 94 °C, annealing for 1 min at 51 °C, extension for 2 min at 72 °C. After final extension for 6 min at 72 °C, the reaction was stopped by cooling to 4 °C. PCR products were purified using Agencourt magnetic particles and 6× DNA Loading Dye (Thermo Scientific) was added to samples in ratio 1:5 (loading dye:sample). Samples were analyzed by agarose gel electrophoresis using 2 % agarose gel stained with GelRed™ (Biotium). The gel was run for 40 min at 120 V.

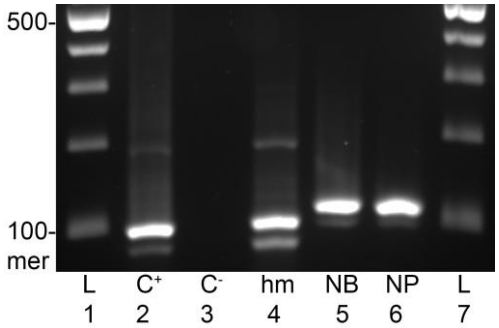


Figure S22: Agarose gel analysis of PCR with FVLA 98-mer template: Lane 1 and 7, L: 100 bp ladder; lane 2, C^+: product of PCR with natural dNTPs; lane 3, C^-: product of PCR in the absence of dCTP; lane 4, hm: product of PCR with modified **dC^{hm}TP** and three natural dNTPs; lane 5, NB: product of PCR with modified **dC^{NB}TP** and three natural dNTPs; lane 6, NP: product of PCR with modified **dC^{NP}TP** and three natural dNTPs;

311-mer

The reaction mixture (20 µL) contained KOD XL DNA polymerase (2.5 U/µL, 1.2 µL), dNTPs (5 mM, 0.6 µL), dCTP (5 mM, 0.6 µL or 1.2 µL for modified dCTP), primers P-FOR-ins-100bp_P2 (20 µM, 3 µL) and P-REV-P/P2 (20 µM, 3 µL), 311-mer template (81 ng/µL, 0.44 µL) and 10x buffer for KOD XL DNA polymerase (2 µL) supplied by the manufacturer. After initial denaturation for 3 min at 94 °C, 40 PCR cycles were run under following conditions: denaturation for 1 min at 94 °C, annealing for 1 min at 68 °C, extension for 1.5 min at 75 °C. After final extension for 5 min at 75 °C, the reaction was stopped by cooling to 4 °C. PCR products were purified using Agencourt magnetic particles and 6× DNA Loading Dye (Thermo Scientific) was added to samples in ratio 1:5 (loading dye:sample). Samples were analyzed by agarose gel electrophoresis using 1.3% agarose gel stained with GelRed™ (Biotium). The gel was run for 70 min at 120 V.

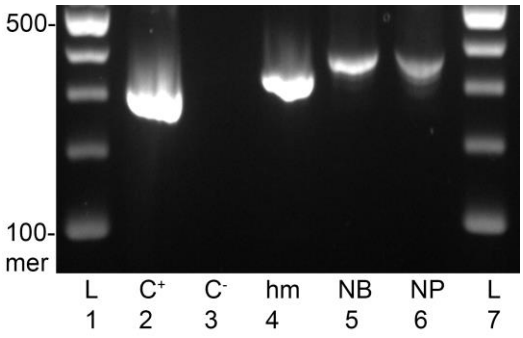
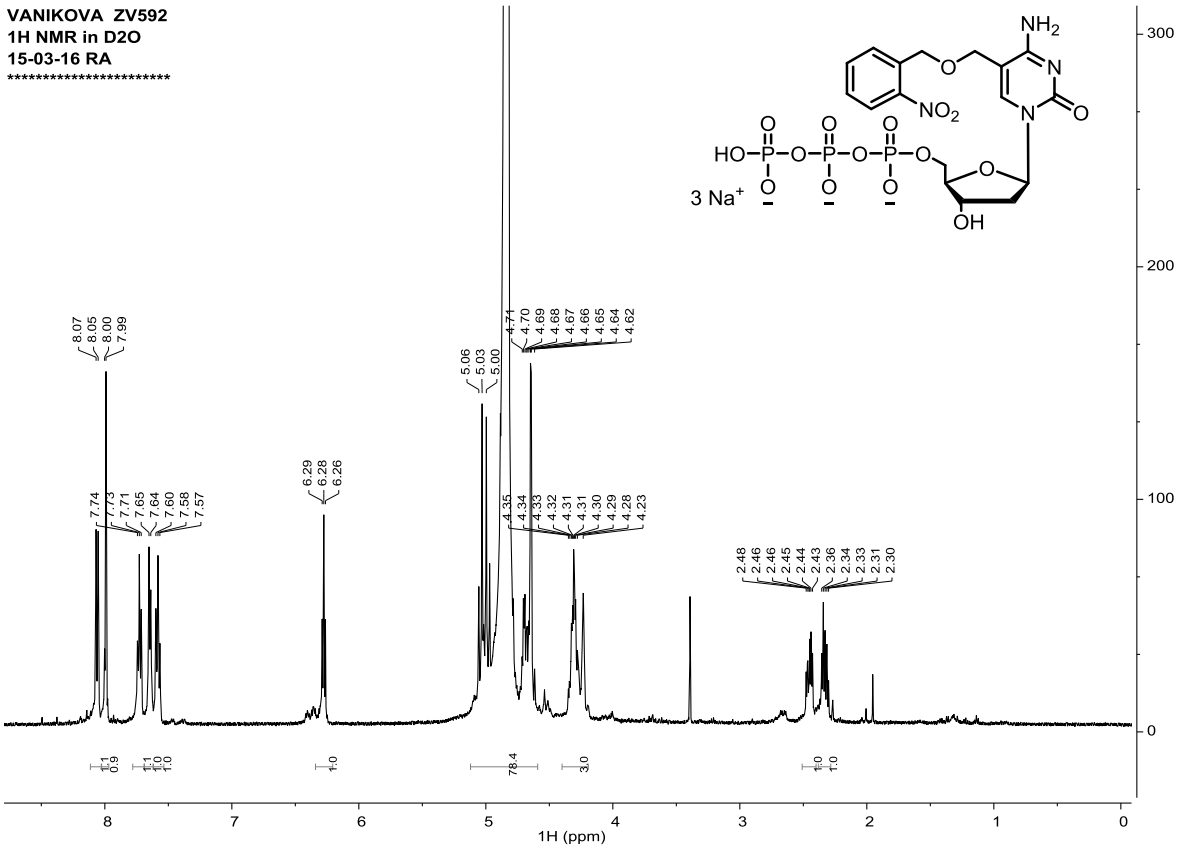
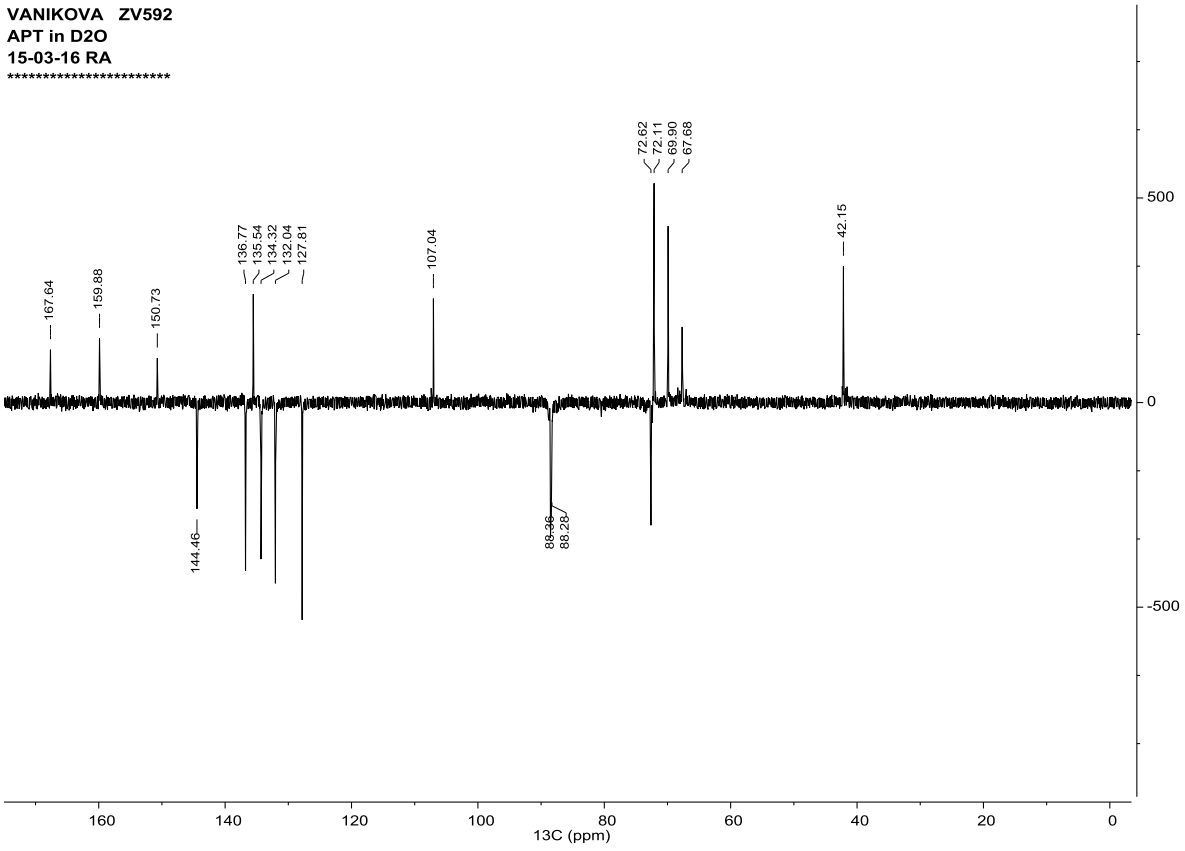
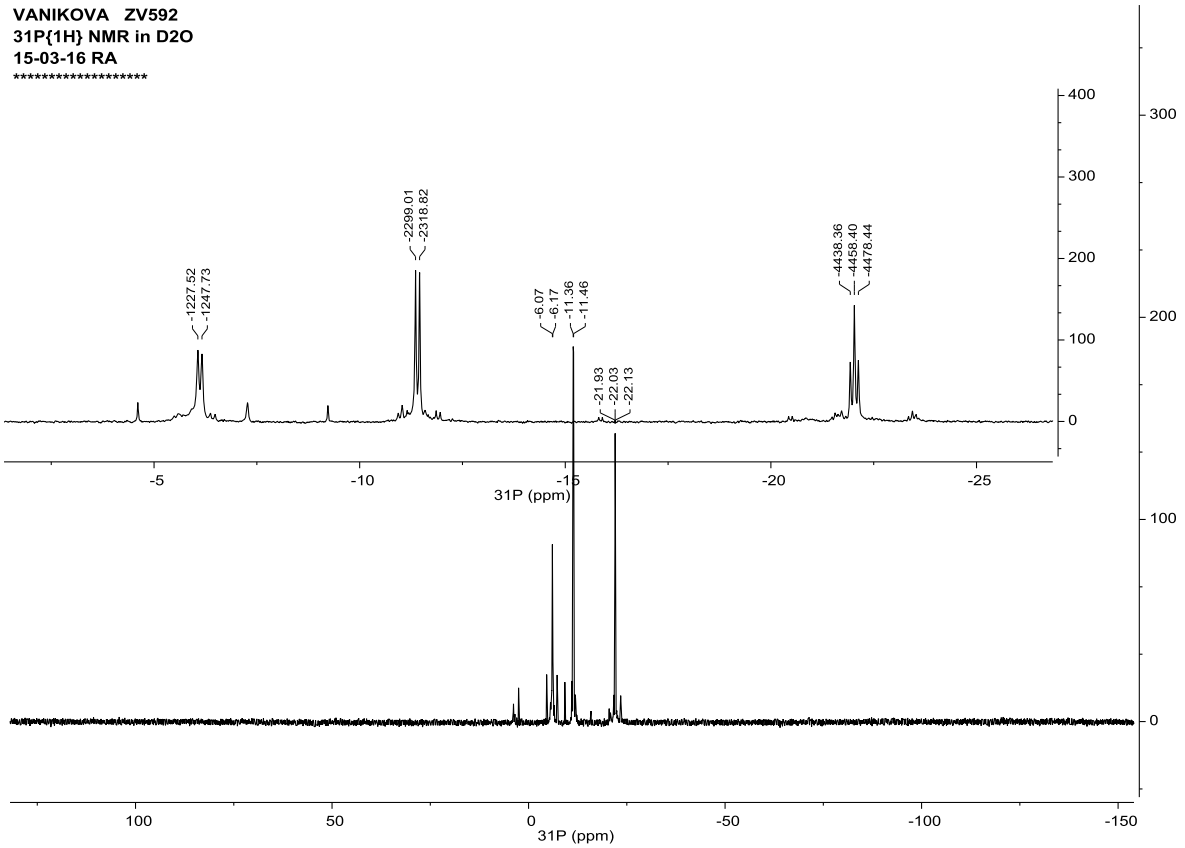


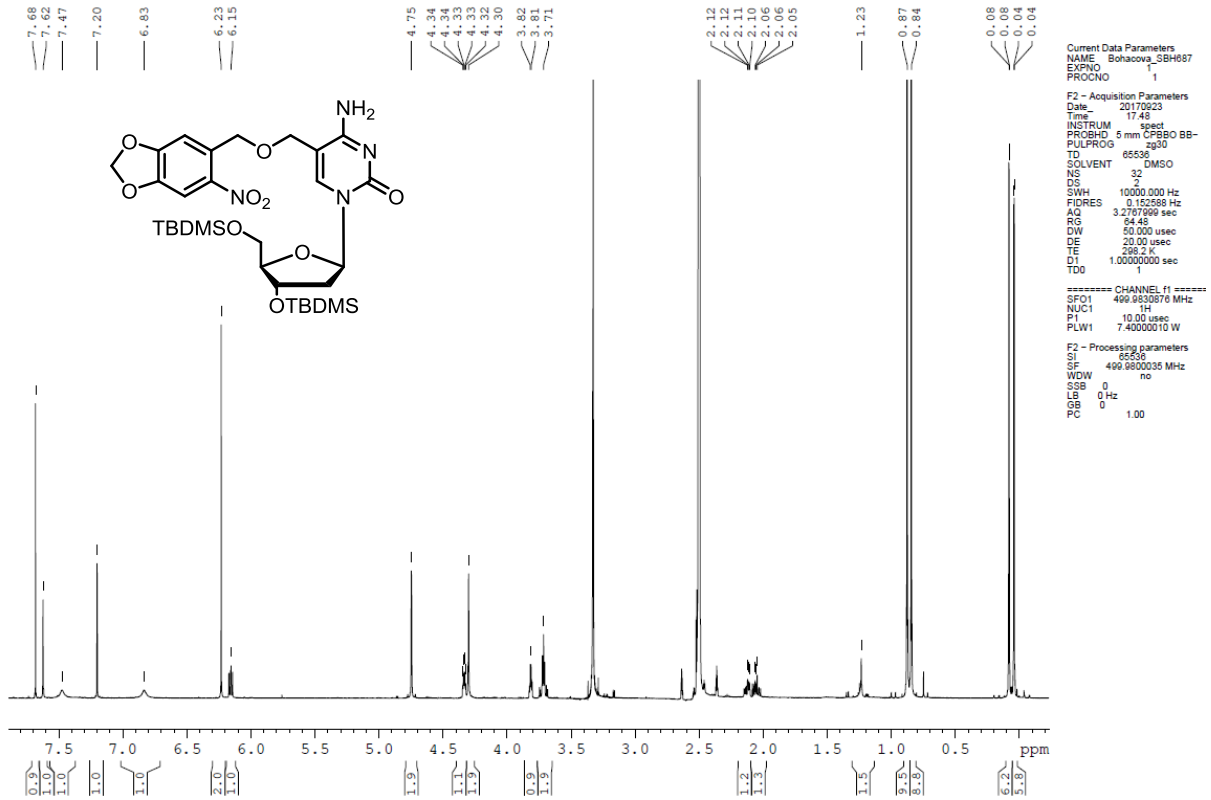
Figure S23: Agarose gel analysis of PCR with 311-mer template: Lane 1 and 7, L: 100 bp ladder; lane 2, C^+: product of PCR with natural dNTPs; lane 3, C^-: product of PCR in the absence of dCTP; lane 4, hm: product of PCR with modified **dC^{hm}TP** and three natural dNTPs; lane 5, NB: product of PCR with modified **dC^{NB}TP** and three natural dNTPs; lane 6, NP: product of PCR with modified **dC^{NP}TP** and three natural dNTPs.

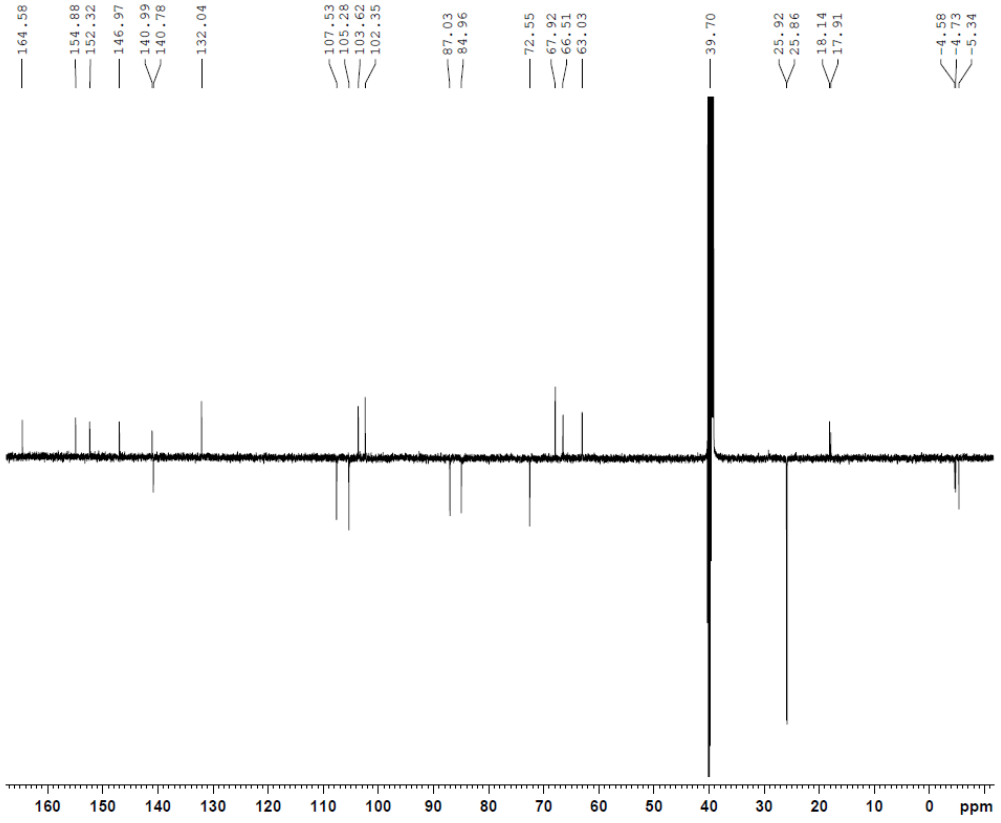
4. Copies of NMR spectra



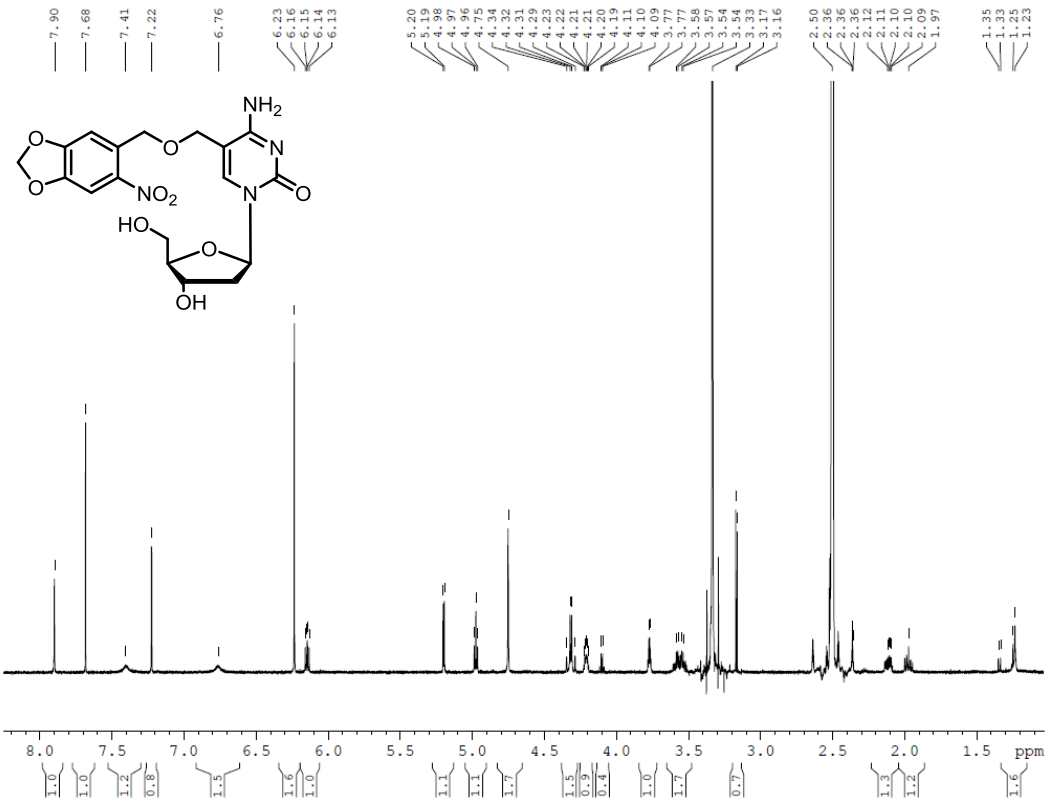



VANIKOVA ZV592
31P{1H} NMR in D2O
15-03-16 RA
****
-1227.52
-1247.73
-2299.01
-2318.82
-6.07
-6.17
-11.36
-11.46
-21.93
-22.03
-22.13
-4438.36
-4458.40
-4478.44
31P (ppm)
31P (ppm)

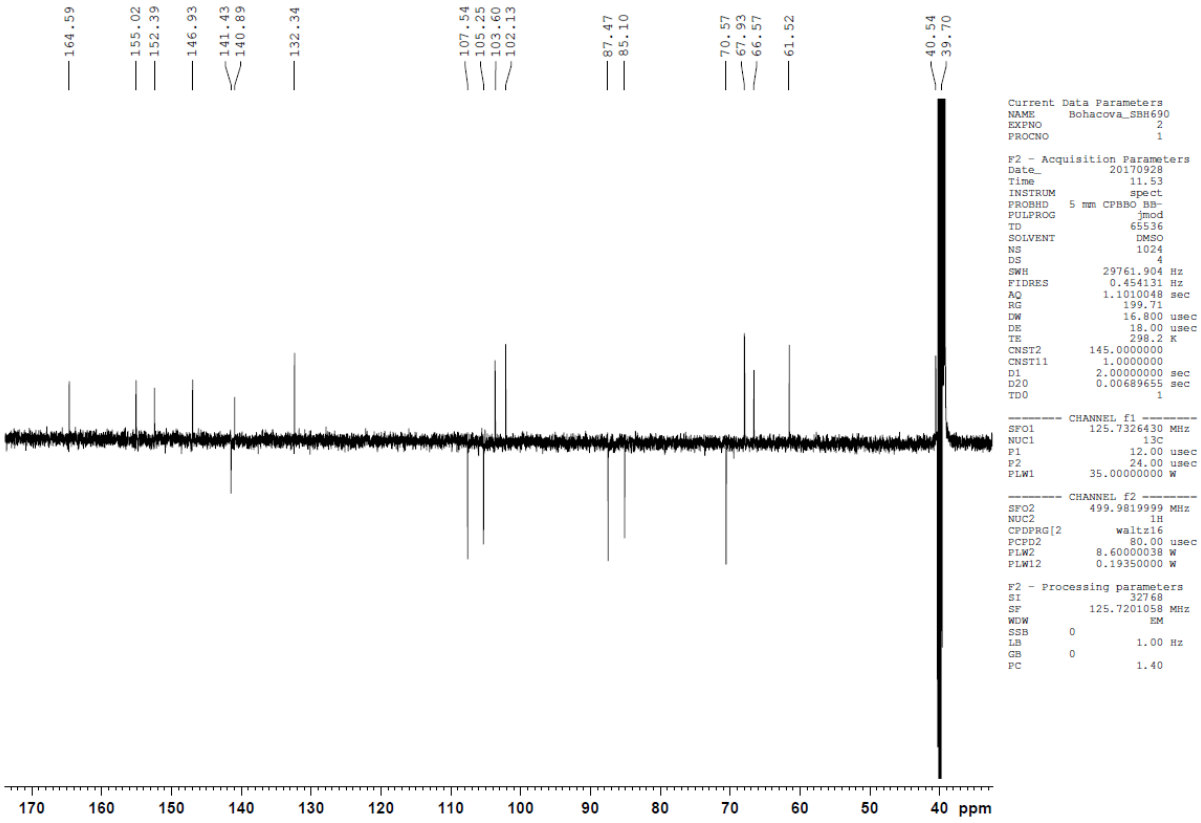

NH2
NO2
TBDMSO
OTBDMS
ppm

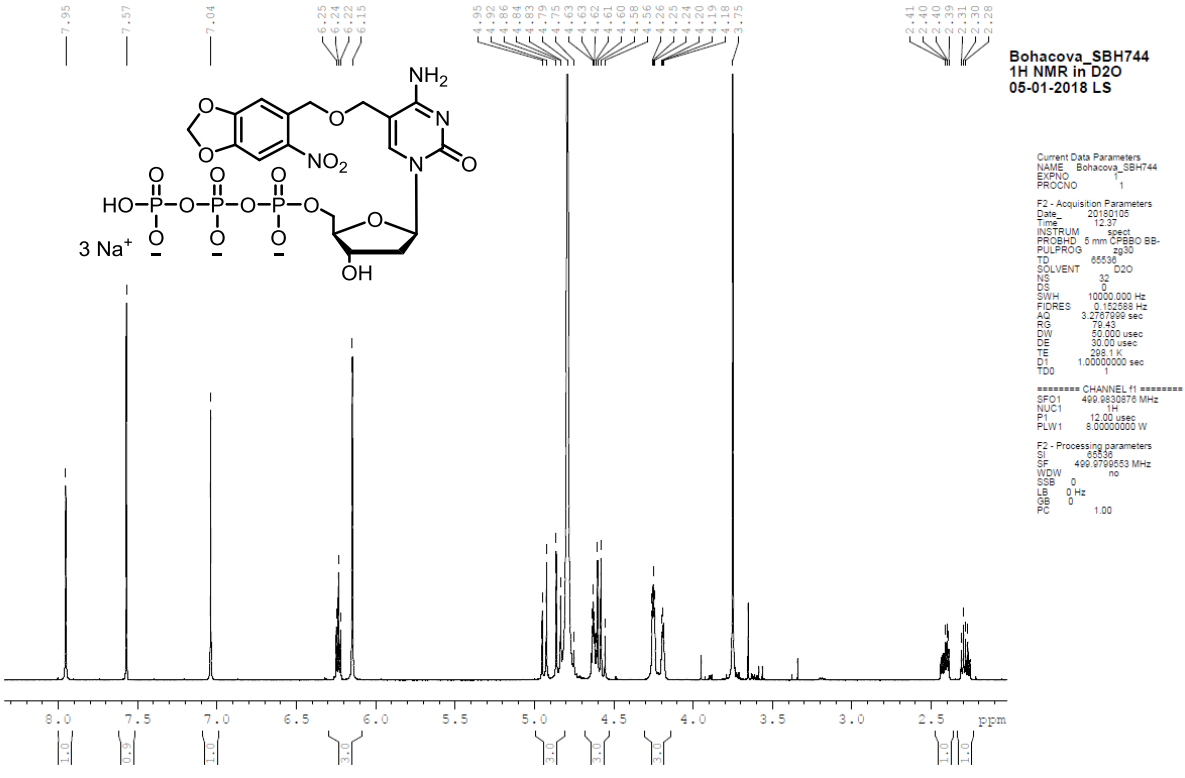


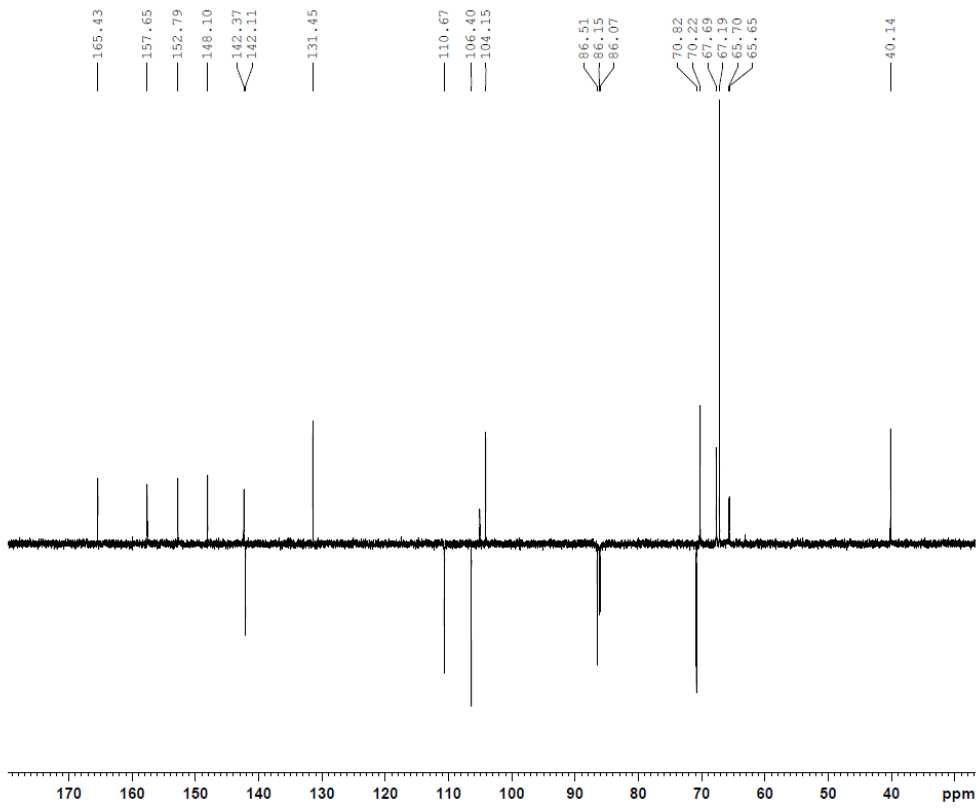

```
Current Data Parameters
NAME      Bohacova_SBH687
EXPNO                   2
PROCNO                  1

F2 - Acquisition Parameters
Date_           20170923
Time              18.42
INSTRUM           spect
PROBHD  5 mm CPBBO BB-
PULPROG           jmod
TD                65536
SOLVENT             DMSO
NS                  1024
DS                     4
SWH           29761.904 Hz
FIDRES         0.454131 Hz
AQ            1.1010048 sec
RG               199.71
DW               16.800 usec
DE               18.00 usec
TE               298.2 K
CNST2         145.0000000
CNST11          1.0000000
D1            2.00000000 sec
D20           0.00689655 sec
TD0                    1

======== CHANNEL f1 ========
SFO1          125.7326430 MHz
NUC1                    13C
P1                   12.00 usec
P2                   24.00 usec
PLW1         35.00000000 W

======== CHANNEL f2 ========
SFO2          499.9819999 MHz
NUC2                    1H
CPDPRG[2           waltz16
PCPD2               80.00 usec
PLW2          8.60000038 W
PLW12         0.19350000 W

F2 - Processing parameters
SI                  32768
SF            125.7201062 MHz
WDW                    EM
SSB       0
LB                1.00 Hz
GB        0
PC                1.40
```




```
Current Data Parameters
NAME    Bohacova_SBH690
EXPNO                 1
PROCNO                1

F2 - Acquisition Parameters
Date_         20170928
Time             10.55
INSTRUM          spect
PROBHD  5 mm CPBBO BB-
PULPROG          zg30
TD              65536
SOLVENT           DMSO
NS                  32
DS                   2
SWH         10000.000 Hz
FIDRES          0.152588 Hz
AQ            3.2767998 sec
RG                31.35
DW               50.000 usec
DE               20.00 usec
TE               298.2 K
D1            1.00000000 sec
TD0                   1

======== CHANNEL f1 ======
SFO1          499.9830876 MHz
NUC1                    1H
P1                   10.00 usec
PLW1          7.40000010 W

F2 - Processing parameters
SI                  65536
SF            499.9800040 MHz
WDW                   no
SSB       0
LB        0 Hz
GB        0
PC                1.00
```

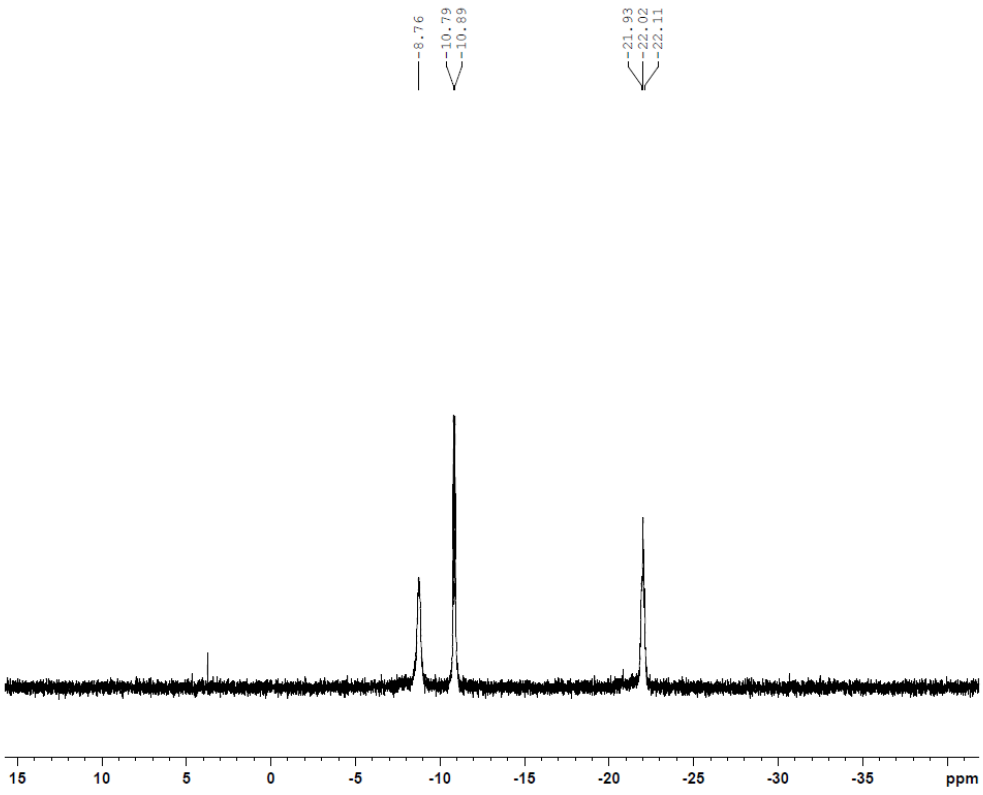

164.59
155.02
152.39
146.93
141.43
140.89
132.34
107.54
105.25
103.60
102.13
87.47
85.10
70.57
67.93
66.57
61.52
40.54
39.70
170 160 150 140 130 120 110 100 90 80 70 60 50 40 ppm
Current Data Parameters
NAME Bohacova_SBH690
EXPNO 2
PROCNO 1
F2 - Acquisition Parameters
Date_ 20170928
Time 11.53
INSTRUM spect
PROBHD 5 mm CPBBO BB-
PULPROG jmod
TD 65536
SOLVENT DMSO
NS 1024
DS 4
SWH 29761.904 Hz
FIDRES 0.454131 Hz
AQ 1.1010048 sec
RG 199.71
DW 16.800 usec
DE 18.00 usec
TE 298.2 K
CNST2 145.0000000
CNST11 1.0000000
D1 2.00000000 sec
D20 0.00689655 sec
TD0 1
======== CHANNEL f1 ========
SFO1 125.7326430 MHz
NUC1 13C
P1 12.00 usec
P2 24.00 usec
PLW1 35.00000000 W
======== CHANNEL f2 ========
SFO2 499.9819999 MHz
NUC2 1H
CPDPRG[2 waltz16
PCPD2 80.00 usec
PLW2 8.60000038 W
PLW12 0.19350000 W
F2 - Processing parameters
SI 32768
SF 125.7201058 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40


7.95
7.57
7.04
6.25
6.24
6.22
6.15
4.95
4.92
4.86
4.84
4.83
4.79
4.75
4.63
4.62
4.61
4.60
4.58
4.56
4.26
4.25
4.24
4.20
4.19
4.18
3.75
2.41
2.40
2.40
2.39
2.31
2.30
2.28
NH2
N
O
O
O
NO2
N
O
HO–P–O–P–O–P–O
O
O
O
O
O
O
3 Na+
O
OH
8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 ppm
1.0
0.9
1.0
3.0
3.0
3.0
3.0
1.0
1.0
Bohacova_SBH744
1H NMR in D2O
05-01-2018 LS
Current Data Parameters
NAME Bohacova_SBH744
EXPNO 1
PROCNO 1
F2 - Acquisition Parameters
Date_ 20180105
Time 12.37
INSTRUM spect
PROBHD 5 mm CPBBO BB-
PULPROG zg30
TD 65536
SOLVENT D2O
NS 32
DS 0
SWH 10000.000 Hz
FIDRES 0.152588 Hz
AQ 3.2767999 sec
RG 79.43
DW 50.000 usec
DE 30.00 usec
TE 298.1 K
D1 1.00000000 sec
TD0 1
======== CHANNEL f1 ========
SFO1 499.9830876 MHz
NUC1 1H
P1 12.00 usec
PLW1 8.00000000 W
F2 - Processing parameters
SI 65536
SF 499.9799653 MHz
WDW no
SSB 0
LB 0 Hz
GB 0
PC 1.00



Bohacova_SBH744
13C NMR in D2O
05-01-2018 LS
****

```
Current Data Parameters
NAME      Bohacova_SBH744
EXPNO                  2
PROCNO                 1

F2 - Acquisition Parameters
Date_           20180105
Time_              13.03
INSTRUM            spect
PROBHD   5 mm CPBBO BB-
PULPROG            zgpg
TD                 65536
SOLVENT              D2O
NS                   540
DS                     4
SWH           29761.904 Hz
FIDRES            0.454131 Hz
AQ             1.1010048 sec
RG                 199.71
DW                16.800 usec
DE                18.00 usec
TE                298.1 K
CNST2         145.0000000
CNST11          1.0000000
D1              2.00000000 sec
D20             0.00689655 sec
TD0                    1

======== CHANNEL f1 ========
SFO1          125.7326430 MHz
NUC1                  13C
P1                 11.00 usec
P2                 22.00 usec
PLW1          35.00000000 W

======== CHANNEL f2 ========
SFO2          499.9819999 MHz
NUC2                   1H
CPDPRG[2           waltz16
PCPD2              80.00 usec
PLW2           8.00000000 W
PLW12          0.18000001 W

F2 - Processing parameters
SI                 32768
SF            125.7199874 MHz
WDW                   EM
SSB       0
LB                 1.00 Hz
GB        0
PC                 1.40
```

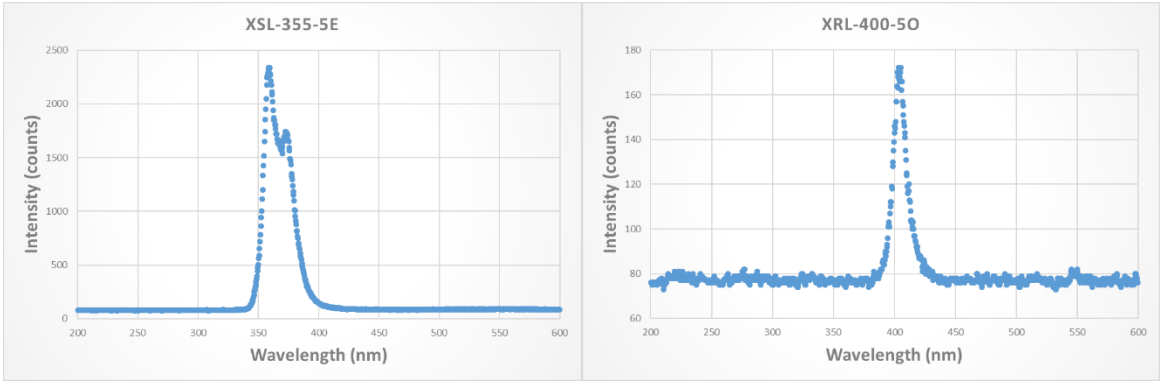


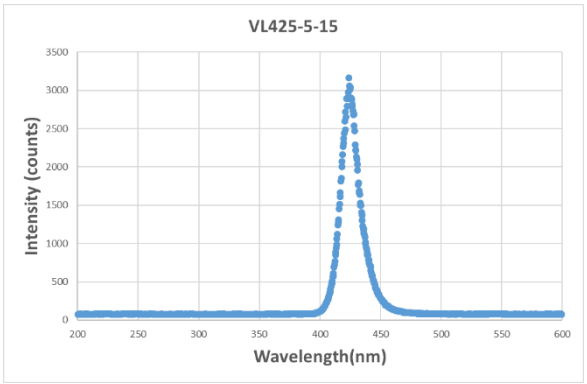
Bohacova_SBH744
31P{1H} in D2O
05-01-2018 LS
****

```
Current Data Parameters
NAME      Bohacova_SBH744
EXPNO                 10
PROCNO                 1

F2 - Acquisition Parameters
Date_           20180105
Time_              13.56
INSTRUM            spect
PROBHD   5 mm CPBBO BB-
PULPROG           zgpg30
TD                 65536
SOLVENT              D2O
NS                    32
DS                     0
SWH           81521.742 Hz
FIDRES            1.243923 Hz
AQ             0.4019541 sec
RG                 199.71
DW                 6.133 usec
DE                20.00 usec
TE                298.1 K
D1              2.00000000 sec
D11             0.03000000 sec
TD0                    1

======== CHANNEL f1 ========
SFO1          202.3864942 MHz
NUC1                  31P
P1                 12.00 usec
PLW1          29.00000000 W

======== CHANNEL f2 ========
SFO2          499.9819999 MHz
NUC2                   1H
CPDPRG[2           waltz16
PCPD2              80.00 usec
PLW2           8.00000000 W
PLW12          0.18000001 W
PLW13          0.09063900 W

F2 - Processing parameters
SI                131072
SF            202.3954671 MHz
WDW                   no
SSB       0
LB        0 Hz
GB        0
PC                 1.40
```

LED emission spectra

Spectra were corrected using fluorescent lamp as a standard.





References

1. V. A. Litosh, W. D. Wu, B. P. Stupi, J. C. Wang, S. E. Morris, M. N. Hersh and M. L. Metzker, *Nucleic Acids Res*, 2011, **39**.
2. B. P. Stupi, H. Li, J. C. Wang, W. D. Wu, S. E. Morris, V. A. Litosh, J. Muniz, M. N. Hersh and M. L. Metzker, *Angew Chem Int Edit*, 2012, **51**, 1724-1727.
3. S. Bohacova, L. Ludvikova, L. Postova Slavetinska, Z. Vanikova, P. Klan, M. Hocek, *Org Biomol Chem* 2018, **16**, 1527-1535.