Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supporting information for

2-Substituted 2'-Deoxyinosine 5'-Triphosphates as Substrates for Polymerase Synthesis of Minor-Groove-Modified DNA and Effects on Restriction Endonuclease Cleavage

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Additional results, schemes, tables and figures

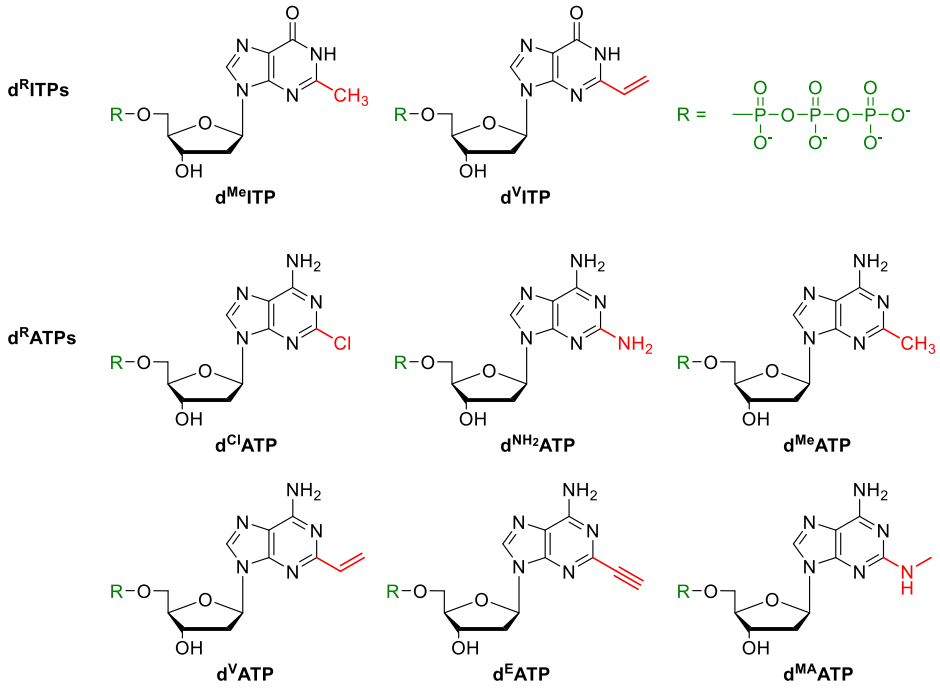


Figure S1. Structures of modified dNTPs used to study the effect of minor-groove modification on cleavage of DNA by restriction endonucleases

Table S1. List of oligonucleotides

Oligonucleotide	Sequence 5'→ 3'	Length
Prim15	CATGGGCGGCATGGG	15-mer
Prim15-FAM [a]	CATGGGCGGCATGGG	15-mer
Prim_KpG-FAM	CATGGGCGGCATGGGG	16-mer
Prim16-FAM	GGAGAAGTGAAAGTGG	16-mer
Temp19 [b]	AAAC *CCCATGCCGCCCATG*	19-mer
Temp19-bio [c]	AAAC *CCCATGCCGCCCATG*	19-mer
Temp31-TINA [d]	CTAGCATGAGCTCAGT *CCCATGCCGCCCATG*	31-mer
Temp31-(bio)TINA [e]	CTAGCATGAGCTCAGT *CCCATGCCGCCCATG*	31-mer
Temp16	C *CCCATGCCGCCCATG*	16-mer
Temp26-bio	GAGGAATTTC *CCACTTTCACTTCTCC*	16-mer
Temp_AfA [f]	AACGACGAC**AGCGCT** *CCCATGCCGCCCATG*	30-mer
Temp_AfG	AAGTAGTAG**AGCGCT** *CCCATGCCGCCCATG*	30-mer
Temp_EcA	AACGACGAC**GAATTC** *CCCATGCCGCCCATG*	30-mer
Temp_EcG	AAGTAGTAG**GAATTC** *CCCATGCCGCCCATG*	30-mer
Temp_KpA	AACGACGAC**GGTACC** *CCCATGCCGCCCATG*	30-mer
Temp_KpG	AAGTAGTAG**GGTAC** ***C****CCCATGCCGCCCATG*	30-mer
Temp_PsA	AACGACGAC**CTGCAG** *CCCATGCCGCCCATG*	30-mer
Temp_PsG	AAGTAGTAG**CTGCAG** *CCCATGCCGCCCATG*	30-mer
Temp_PvA	AACGACGAC**CAGCTG** *CCCATGCCGCCCATG*	30-mer
Temp_PvG	AAGTAGTAG**CAGCTG** *CCCATGCCGCCCATG*	30-mer
Temp_RsA	AACGACGACG**GTAC**G *CCCATGCCGCCCATG*	30-mer
Temp_RsG	AAGTAGTAGT**GTAC**A *CCCATGCCGCCCATG*	30-mer
Temp_SaA	AACGACGAC**GAGCTC** *CCCATGCCGCCCATG*	30-mer
Temp_SaG	AAGTAGTAG**GAGCTC** *CCCATGCCGCCCATG*	30-mer
Temp_ScA	AACGACGAC**AGTACT** *CCCATGCCGCCCATG*	30-mer
Temp_ScG	AAGTAGTAG**AGTACT** *CCCATGCCGCCCATG*	30-mer
ON19_1^{R}H	CATGGGCGGCATGGG^{R}**H**TTT	19-mer
ON31_4^{R}H	CATGGGCGGCATGGGACT^{R}**H**A^{R}**H**CTCAT^{R}**H**CTA^{R}**H**	31-mer
ON26_1^{V}H	GGAGAAGTGAAAGTGG^{V}**H**AAATTCCTC	26-mer

[a] 6-carboxyfluorescein (6-FAM) used for oligonucleotide labeling at 5'-end

[b] primer sequences in template are in italic

[c] template biotinylated at 5'-end

[d] template labeled by *ortho*-TINA at 5'-end

[e] template labeled by *ortho*-TINA at 5'-end and biotinylated at 3'-end

[f] target palindromic sequence for the restriction enzyme are in bold (position of the modification in the product is underlined)

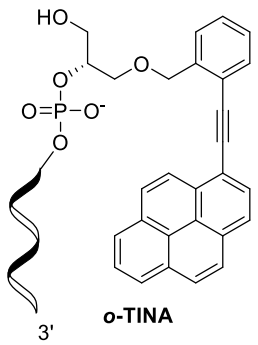


Figure S2. Structure of *ortho*-twisted intercalating nucleic acid (*o*-TINA).

Table S2. MALDI data of modified ONs

ON	*M* (calcd) / Da	*M* (found) $[M+H]^+$ / Da
ON19_1^{Me}H	5914.9	5916.0
ON19_1^{V}H	5926.9	5929.0
ON31_4^{Me}H	9613.3	9614.6
ON31_4^{V}H	9661.3	9662.8
ON19_1^{CM}H	6134.9	6137.6
ON31_4^{CM}H [(a)]	11037.6	11038.3
ON19_1^{Cys}H	6049.0	6050.5
ON31_4^{Cys}H [(b)]	10448.4	10448.7
ON26_1^{V}H	9652.8	9650.6
ON26_1^{pept_1}H	10060.5	10058.4

[(a)] product of reaction of two vinyl groups

[(b)] product of reaction of one vinyl group, found as a $[M+K]^+$

Table S3. Denaturing temperatures of DNA duplexes

DNA	T_m / °C	ΔT_m / °C [(a)]
DNA19_1G	70.9 ± 0.2	–
DNA19_1^{Me}H	67.7 ± 0.1	– 3.2
DNA19_1^{V}H	67.6 ± 0.1	– 3.3
DNA31_4G	78.6 ± 0.1	–
DNA31_4^{Me}H	70.0 ± 0.2	– 2.2
DNA31_4^{V}H	69.9 ± 0.1	– 2.2

[(a)] $\Delta T_m = (T_{m\,mod} - T_{m\,+})/n_{mod}$

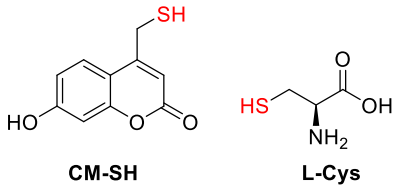


Figure S3. Structures of thiols used for post-synthetic modification of DNA.

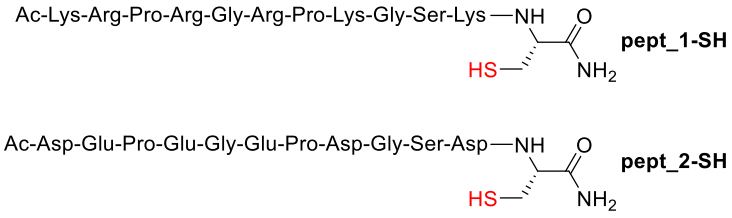


Figure S4. Structures of peptides used for post-synthetic modification of DNA.

Figure S5. PAGE analysis of polymerase synthesis of DNA used for reaction with peptides. Lanes: 1, P: primer; 2, +: 26-mer ON synthesized using naturals dNTPs; 3, G-: negative control experiment, no dGTP used during PEX; 4, ^{V}H: 26-mer ON synthesized using **d^{V}ITP** instead of dGTP.

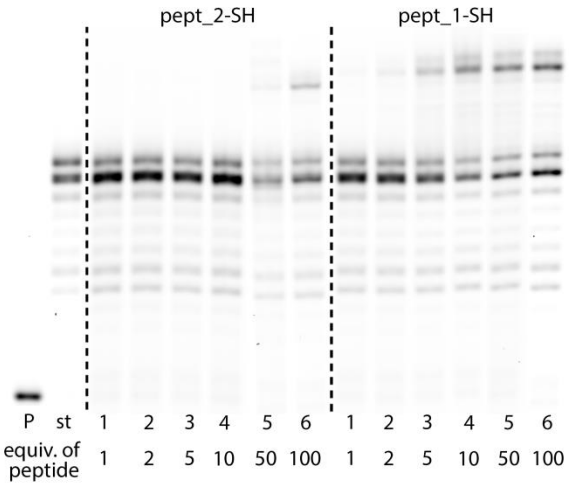


Figure S6. Denaturing PAGE showing results of the optimization of the reaction between peptides containing cysteine and vinyl modified DNA (**DNA26_1^{V}H**). Lanes: P: primer; st: starting **DNA26_1^{V}H**; 1-6: products of reaction of vinyl modified DNA and corresponding peptide with increasing amount of the peptide.

Figure S7. Denaturing PAGE showing results of the optimization of the reaction between peptides containing cysteine and vinyl modified DNA (**DNA26_1^{V}H**). Lanes: P: primer; 1: starting **DNA26_1^{V}H**; 2-3: products of reaction of vinyl modified DNA and corresponding **pept_1-SH** with increased reaction time.

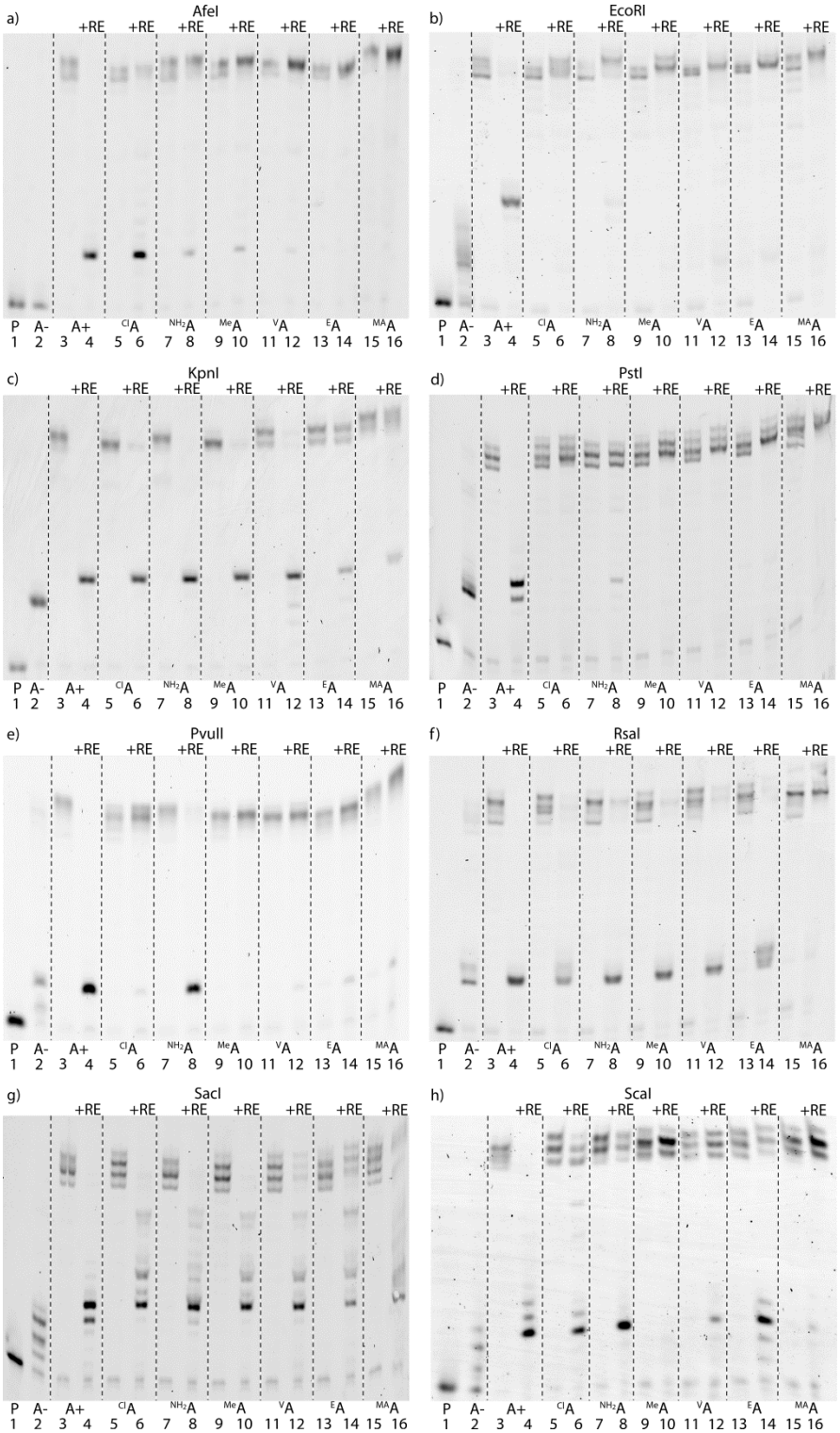


Figure S8. PAGE analysis of DNAs with modified As (**d^{R}ATPs** used in PEX) inside the recognition site of restriction enzymes. Lanes: 1, P: primer; 2, A-: PEX in the presence of dCTP, dGTP and dTTP; 3, 5, 7, 9, 11, 13, 15, A+ or ^{R}A: PEX in the presence of dCTP, dGTP, dTTP and either dATP (A+) or corresponding modified **d^{R}ATP**; 4, 6, 8, 10, 12, 14, 16, +RE: cleavage of DNAs by corresponding restriction enzymes.

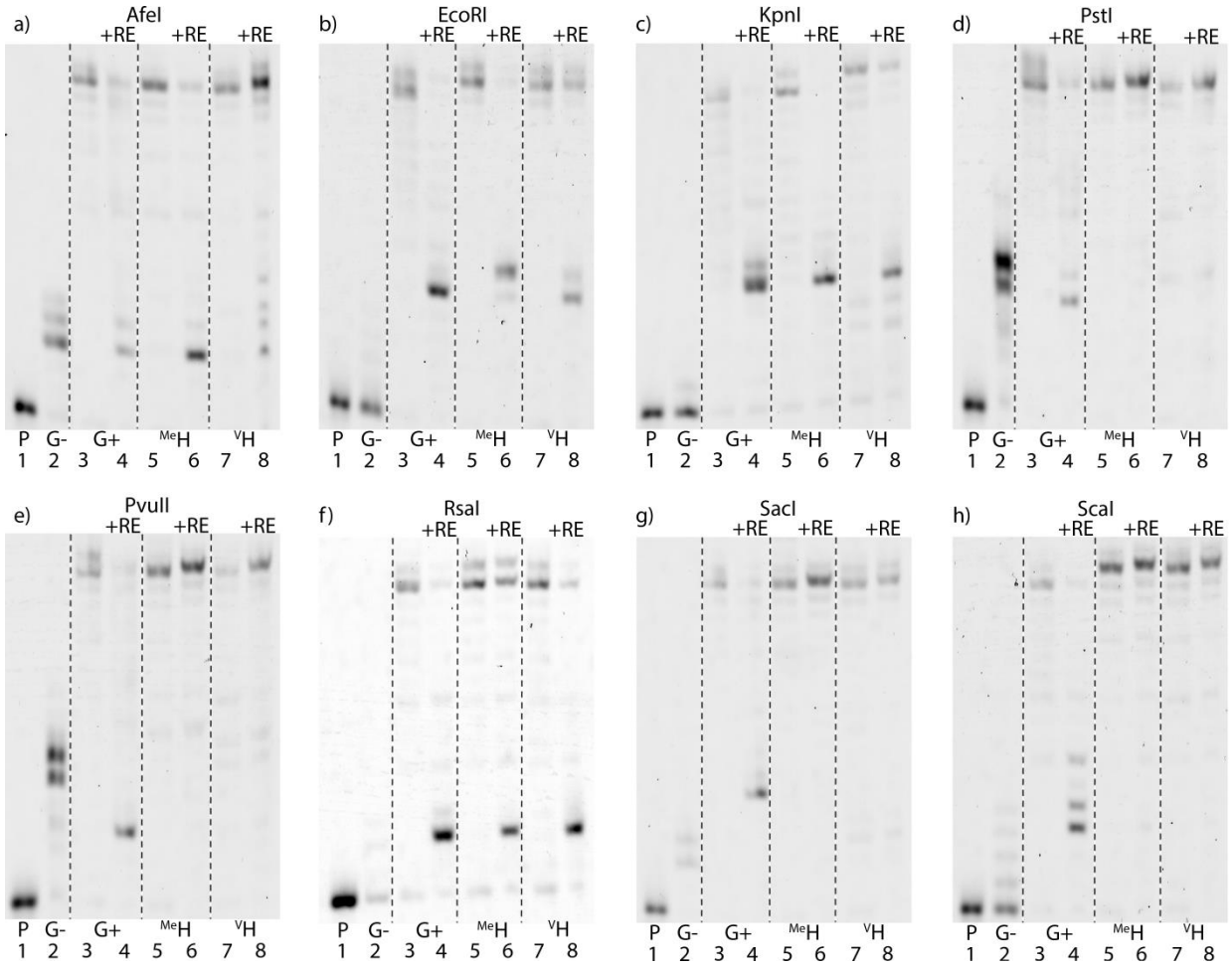


Figure S9. PAGE analysis of DNAs with "modified Gs" (**d^{R}ITPs** used in PEX) inside the recognition site of restriction enzymes. Lanes: 1, P: primer; 2, G-: PEX in the presence of dCTP, dATP and dTTP; 3, 5, 7, G+ or ^{R}H: PEX in the presence of dCTP, dATP, dTTP and either dGTP (G+) or corresponding modified **d^{R}ITP**; 4, 6, 8, +RE: cleavage of DNAs by corresponding restriction enzymes.

Table S4. Summary of the results of cleavage of minor-groove modified DNA by restriction endonucleases. [(a)]

Enzyme	^{Cl}A	^{NH_2}A	^{Me}A	^{V}A	^{E}A	^{MA}A	^{Me}H	^{V}H
AfeI	++	-	-	-	-	-	+++	+
EcoRI	-	-	-	-	-	-	+++	++
KpnI	+++	+++	+++	+++	+	+	+++	+++
PstI	-	-	-	-	-	-	-	-
PvuII	-	+++	-	-	-	-	-	-
RsaI	+++	+++	+++	+++	+++	-	++	+++
SacI	+++	+++	+++	+++	++	++	-	-
ScaI	++	++	-	+	++	-	-	-

[(a)] Approximate yields of cleavage: - = 0-25%; + = 25-50%; ++ = 50-75%; +++ = 75-100%.

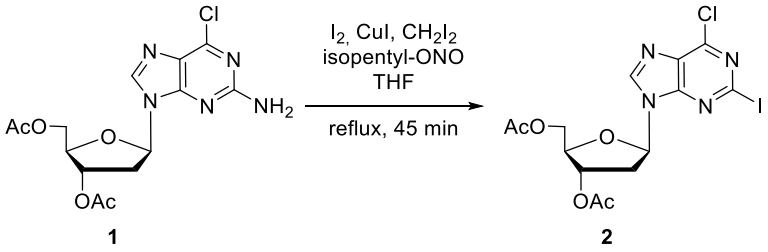
Experimental part

Chemical synthesis

Materials and methods

Reagents and solvents were purchased from Sigma–Aldrich and AlfaAesar. 2'-Deoxyguanosine was purchased from Berry&Associates, 2,6-dichloropurine from Sumika Fine Chemicals. 2-Amino-6-chloro-9-(3',5'-di-*O*-acetyl-2'-deoxy-β-D-ribofuranosyl)purine **1** and 2,6-dichloro-9-[3',5'-di-*O*-(4-methylbenzoyl)-2'-deoxy-β-D-ribofuranosyl]purine **6** were prepared as described previously.[S1,S2] The reactions were monitored by thin-layer chromatography using Merck silica gel 60 F254 plates and visualized by UV (254 nm). Column chromatography was performed using silica gel (40–63 μm). Reversed-phase high-performance flash chromatography (FPLC) purifications were done on an ISCO Combi*Flash* Rf+ apparatus with C-18 columns. Purification of nucleoside triphosphates was performed using HPLC (Waters modular HPLC system) on a column packed with 10 μm C18 reversed phase (Phenomenex, Luna C18 (2) 100 Å) or using a POROS HQ 50 μm packed column. NMR spectra were measured on a Bruker AVANCE 400 (^{1}H at 401.0 MHz, ^{13}C at 100.8 MHz and ^{31}P at 162.0 MHz) and Bruker AVANCE 600 (^{1}H at 600.1 MHz, ^{13}C at 150.9 MHz and ^{31}P at 202.4 MHz) NMR spectrometers in $CDCl_3$, DMSO-d_6 or D_2O solutions at 25 °C. Chemical shifts (in ppm, δ scale) were referenced to the residual solvent signal in ^{1}H spectra (δ($CHCl_3$) = 7.26 ppm, δ((CHD_2)SO(CD_3)) = 2.5 ppm) or to the solvent signal in ^{13}C spectra (δ($CDCl_3$) = 77.0 ppm, δ((CD_3)$_2$SO) = 39.7 ppm). 1,4-Dioxane was used as an internal standard for D_2O solutions (3.75 ppm for ^{1}H and 69.3 ppm for ^{13}C). Coupling constants (*J*) are given in Hz. The complete assignment of ^{1}H and ^{13}C signals was performed by an analysis of the correlated homonuclear H,H-COSY, and heteronuclear H,C-HSQC and H,C-HMBC spectra. Mass spectra were measured with a LCQ classic (Thermo-Finnigan) spectrometer using ESI or a Q-Tof Micro spectrometer (Waters, ESI source, internal calibration with lockspray). High resolution mass spectra were measured on a LTQ Orbitrap XL spectrometer (Thermo Fisher Scientific).

6-Chloro-2-iodo-9-(3',5'-di-*O*-acetyl-2'-deoxy-β-D-ribofuranosyl)purine (2)

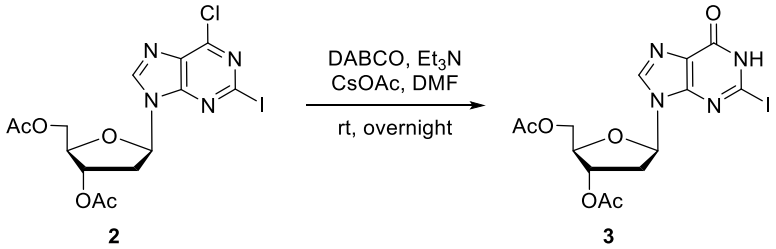


1 **2**

Starting protected nucleoside **1** (3.30 g, 9.0 mmol), I_2 (2.27 g, 9.0 mmol), CuI (1.80 g, 9.4 mmol) and CH_2I_2 (7.4 ml, 92.0 mmol) were suspended in dry THF (90 ml) and isopentyl nitrite (3.7 ml, 27.5 mmol) was added. The resulting mixture was refluxed for 45 min. After cooling it was filtered and most of volatiles were evaporated under reduced pressure. Residue was redissolved in $CHCl_3$ (150 ml) and poured into saturated $Na_2S_2O_3$ solution (100 ml). Mixture was stirred until change of color and then phases were separated. Water phase was extracted with $CHCl_3$ (2 x 100 ml),

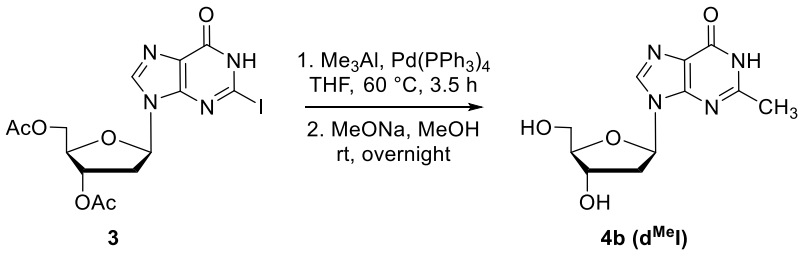
collected organic phases were washed with brine (100 ml), dried over anhydrous Na_2SO_4, filtered and evaporated. The title compound was isolated, after column chromatography (0 → 50% EtOAc in CH_2Cl_2), as a brown solid (2.86 g, 67%).
R_f = 0.32 (50% EtOAc in CH_2Cl_2).
^{1}H NMR (400.1 MHz, $CDCl_3$): 2.09, 2.14 (2 × s, 2 × 3H, C**H**$_3$CO); 2.70 (ddd, 1H, *J* = 14.2, *J* = 6.0, *J* = 2.7, H-2'b); 2.83 (ddd, 1H, *J* = 14.2, *J* = 7.8, *J* = 6.3, H-2'a); 4.36 – 4.39 (m, 3H, H-4', 5′); 5.38 – 5.43 (m, 1H, H-3'); 6.45 (dd, 1H, *J* = 7.8, *J* = 6.0, H-1'); 8.24 (s, 1H, H-8).
^{13}C NMR (100.8 MHz, $CDCl_3$): 20.96, 21.04 (**C**H_3CO); 38.17 (CH_2-2'); 63.65 (CH_2-5'); 74.24 (CH-3'); 83.12 (CH-4'); 85.17 (CH-1'); 116.91 (C-2); 132.29 (C-5); 143.23 (CH-8); 150.89 (C-6); 151.88 (C-4); 170.36, 170.39 (CH_3**C**O).
MS (ESI$^+$): *m/z* (%): 503.0 (100) [(^{35}Cl)M+Na]$^+$, 505.0 (27) [(^{37}Cl)M+Na]$^+$.
HRMS (ESI$^+$): calculated for $C_{14}H_{14}O_5N_4$(^{35}Cl)INa: 502.9590 found: 502.9586.

2-Iodo-3',5'-di-*O*-acetyl-2'-deoxyinosine (3)



To starting nucleoside **2** (2.78 g, 5.8 mmol) and CsOAc (3.33 g, 17.4 mmol) in dry DMF (45 ml), DABCO (0.07 g, 0.6 mmol) and Et_3N (2.4 ml, 17.4 mmol) were added. This mixture was stirred at room temperature (25 °C) overnight, then H_2O (30 ml) was added and stirring continued for another 30 min. Volatiles were evaporated, residue was dissolved in MeOH and coevaporated with silica gel. Purification by column chromatography (0 → 10% MeOH in CH_2Cl_2) afforded the desired product (1.85 g, 69%).
R_f = 0.26 (10% MeOH in CH_2Cl_2).
^{1}H NMR (400.1 MHz, $CDCl_3$): 2.11, 2.15 (2 × s, 2 × 3H, C**H**$_3$CO); 2.65 (ddd, 1H, *J* = 14.2, *J* = 6.1, *J* = 2.6, H-2'b); 2.79 (ddd, 1H, *J* = 14.2, *J* = 7.7, *J* = 6.4, H-2'a); 4.32 – 4.41 (m, 3H, H-4',5'); 5.39 (dt, 1H, *J* = 6.4, *J* = 2.6, H-3'); 6.36 (dd, 1H, *J* = 7.7, *J* = 6.1, H-1'); 7.93 (s, 1H, H-8); 12.06 (bs, 1H, H-1).
^{13}C NMR (100.8 MHz, $CDCl_3$): 20.86, 20.96 (**C**H_3CO); 38.23 (CH_2-2'); 63.64 (CH_2-5'); 74.30 (CH-3'); 82.81 (CH-4'); 84.69 (CH-1'); 104.99 (C-2); 124.77 (C-5); 137.74 (CH-8); 148.33 (C-4); 158.48 (C-6); 170.28, 170.37 (CH_3**C**O).
MS (ESI$^+$): *m/z* (%): 263.1 (64) [nucleobase+H]$^+$, 485.4 (100) [M+Na]$^+$.
HRMS (ESI$^+$): calculated for $C_{14}H_{15}O_6N_4INa$: 484.9929; found: 484.9922.

2-Methyl-2'-deoxyinosine (4b, $d^{Me}I$)



2-Iodo-3',5'-di-*O*-acetyl-2'-deoxyinosine **3** (250 mg, 0.54 mmol) and $Pd(PPh_3)_4$ (63 mg, 0.05 mmol) were dissolved in anhydrous THF (4.2 ml). Me_3Al (1.1 ml, 2.17 mmol, 2 M in toluene) was then added dropwise and this mixture was stirred at 60 °C for 3.5 h. Reaction was carefully stopped by dropwise addition of MeOH (2 ml) and evaporated to dryness. Residue was dissolved in MeOH (5 ml) and MeONa (0.5 ml, 2.19 mmol, 25 wt.% in MeOH) was added. Mixture was stirred at rt (25°C) overnight. Silica gel was added and volatiles were evaporated. Desired product was isolated after RP-FPLC on a C-18 column (0 → 100% MeOH in H_2O) as a white solid (140 mg, 97% over two steps).

R_f = 0.14 (10% MeOH in CH_2Cl_2).

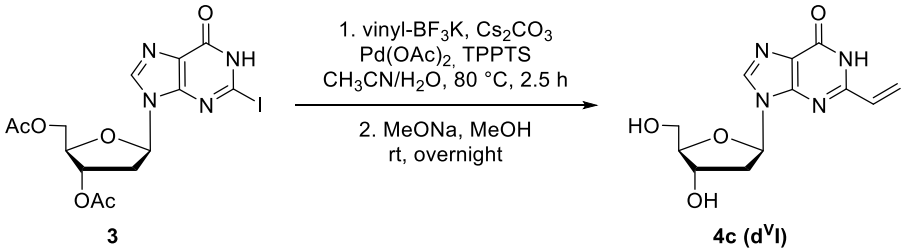
^{1}H NMR (401.0 MHz, DMSO-d_6): 2.17 (ddd, 1H, *J* = 13.0, *J* = 5.9, *J* = 2.2, H-2'b); 2.26 (s, 3H, CH_3); 2.69 (ddd, 1H, *J* = 13.0, *J* = 8.5, *J* = 5.4, H-2'a); 3.52 (dd, 1H, *J* = 12.1, *J* = 3.3, H-5'a); 3.62 (dd, 1H, *J* = 12.1, *J* = 3.3, H-5'b); 3.90 (td, 1H, *J* = 3.3, *J* = 2.2, H-4'); 4.39 (td, 1H, *J* = 5.4, *J* = 2.2, H-3'); 6.23 (dd, 1H, *J* = 8.5, *J* = 5.9, H-1'); 7.97 (s, 1H, H-8).

^{13}C NMR (100.8 MHz, DMSO-d_6): 23.33 (CH_3); 39.62 (CH_2-2'); 62.30 (CH_2-5'); 71.40 (CH-3'); 84.66 (CH-1'); 88.35 (CH-4'); 122.78 (C-5); 136.88 (CH-8); 148.70 (C-4); 158.64 (C-2); 162.93 (C-6).

MS (ESI$^+$): *m/z* (%): 289.1 (100) $[M+Na]^+$, 555.3 (54) $[2M+Na]^+$.

HRMS (ESI$^+$): calculated for $C_{11}H_{14}O_4N_4Na$: 289.0907; found: 289.0905.

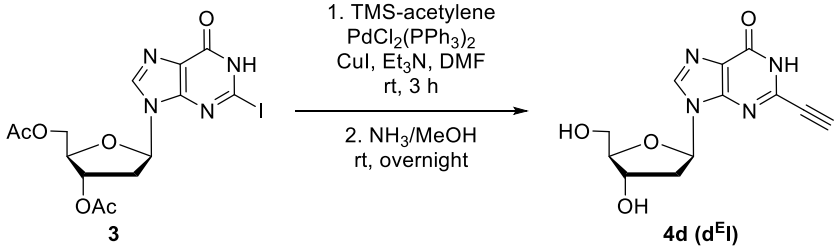
2-Vinyl-2'-deoxyinosine (4c, $d^{V}I$)



A flask containing 2-Iodo-3',5'-di-*O*-acetyl-2'-deoxyinosine **3** (180 mg, 0.39 mmol), potassium vinyltrifluoroborate (78 mg, 0.58 mmol), Cs_2CO_3 (380 mg, 1.17 mmol), $Pd(OAc)_2$ (4 mg, 0.05 mmol) and TPPTS [3,3′,3″-Phosphanetriyltris(benzenesulfonic acid) trisodium salt, 28 mg, 0.125 mmol] was put under argon and a mixture of CH_3CN/H_2O (2/1, 5 ml) was added. Mixture was stirred at 80°C for 2.5 h. After cooling, volatiles were evaporated and residue was dissolved in MeOH (5 ml) and MeONa (0.3 ml, 1.31 mmol, 25 wt.% in MeOH) was added. Resulting solution was stirred at rt (25°C) overnight. Silica gel was added and after evaporation the product was

purified by RP-FPLC on a C-18 column (0 → 100% MeOH in H_2O). Product was isolated as a white solid (80 mg, 74% over two steps).
R_f = 0.12 (10% MeOH in CH_2Cl_2).
^{1}H NMR (401.0 MHz, DMSO-d_6): 2.11 (ddd, 1H, *J* = 12.9, *J* = 5.8, *J* = 1.7, H-2'b); 2.78 (ddd, 1H, *J* = 12.9, *J* = 9.0, *J* = 5.3, H-2'a); 3.48 – 3.57 (bm, 1H, H-5'a); 3.64 (dd, 1H, *J* = 12.0, *J* = 2.9, H-5'b); 3.90 (td, 1H, *J* = 3.0, *J* = 1.4, H-4'); 4.40 (d, 1H, *J* = 5.2, H-3'); 5.24 (bs, 1H, OH-5'); 5.30 (dd, 1H, *J* = 10.4, *J* = 2.7, CH=C**H$_a$**H$_b$); 6.19 (dd, 1H, *J* = 17.3, *J* = 2.7, CH=CH$_a$**H$_b$**); 6.23 (dd, 1H, *J* = 9.0, *J* = 5.8, H-1'); 6.33 (bs, 1H, OH-3'); 6.44 (dd, 1H, *J* = 17.3, *J* = 10.4, C**H**=CH$_2$); 7.83 (s, 1H, H-8).
^{13}C NMR (100.8 MHz, DMSO-d_6): 39.42 (CH$_2$-2'); 62.62 (CH$_2$-5'); 71.77 (CH-3'); 85.05 (CH-1'); 88.30 (CH-4'); 118.70 (CH=**C**H$_2$); 124.50 (C-5); 136.49 (CH-8); 138.07 (**C**H=CH$_2$); 149.04 (C-4); 155.84 (C-6); 158.13 (C-2).
MS (ESI$^+$): *m/z* (%): 301.0 (100) [M+Na]$^+$.
HRMS (ESI$^+$): calculated for $C_{12}H_{14}O_4N_4Na$: 301.0907; found: 301.0902.

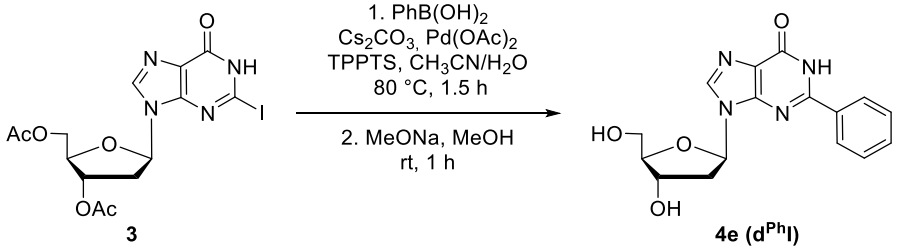
2-Ethynyl-2'-deoxyinosine (4d, d^{E}I)



2-Iodo-3',5'-di-*O*-acetyl-2'-deoxyinosine **3** (183 mg, 0.40 mmol), $PdCl_2(PPh_3)_2$ (42 mg, 0.06 mmol) and CuI (8 mg, 0.04 mmol) were placed in a flask and dry DMF (5.0 ml) was added. The flask was purge-and-refilled with argon six times, trimethylsilylacetylene (0.45 ml, 3.17 mmol) and Et_3N (0.55 ml, 4.0 mmol) were added and the mixture was stirred at rt (25 °C) for 3 h. Volatiles were evaporated, residue was dissolved in MeOH and silica gel was added. After evaporation of MeOH, the 2-trimethylsilylethynyl-3',5'-di-*O*-acetyl-2'-deoxyinosine was partially purified by column chromatography (0 → 10% MeOH in CH_2Cl_2). The crude product was dissolved in MeOH saturated (at 0 °C) with ammonia (5.0 ml) and this mixture was stirred at rt (25 °C) overnight. Silica gel was added and, after evaporation of all volatiles, the desired product was purified by RP-FPLC on a C-18 column (0 → 100% MeOH in H_2O). The title compound was isolated as a white solid (80 mg, 73% over two steps). The compound slowly decomposes on the bench, therefore long term storage at -20 °C is necessary.
R_f = 0.11 (10% MeOH in CH_2Cl_2).
^{1}H NMR (600.1 MHz, DMSO-d_6): 2.13 (ddd, 1H, *J* = 12.9, *J* = 5.9, *J* = 2.2, H-2'b); 2.67 (ddd, 1H, *J* = 12.9, *J* = 8.5, *J* = 5.5, H-2'a); 3.50 (ddd, 1H, *J* = 11.9, *J* = 8.0, *J* = 3.6, H-5'b); 3.56 – 3.63 (m, 1H, H-5'a); 3.59 (s, 1H, HC≡C-); 3.86 (td, 1H, *J* = 3.6, *J* = 2.2, H-4'); 4.35 – 4.38 (m, 1H, H-3'); 5.25 (bd, 1H, *J* = 3.0, OH-5'); 5.66 (dd, 1H, *J* = 7.4, *J* = 4.1, OH-3'); 6.19 (dd, 1H, *J* = 8.5, *J* = 5.9, H-1'); 7.90 (s, 1H, H-8).

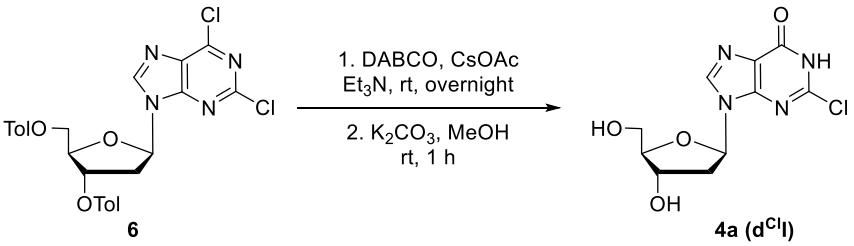
^{13}C NMR (150.9 MHz, DMSO-d_6): 39.63 (CH_2-2'); 62.48 (CH_2-5'); 71.53 (CH-3'); 72.33 (H**C**≡C-); 84.41 (CH-1'); 85.03 (-**C**≡CH); 88.22 (CH-4'); 125.63 (C-5); 136.54 (CH-8); 145.98 (C-2); 148.81 (C-4); 166.36 (C-6).
MS (ESI$^+$): *m/z* (%): 299.1 (100) $[M+Na]^+$, 575.2 (33) $[2M+Na]^+$.
HRMS (ESI$^+$): calculated for $C_{12}H_{12}O_4N_4Na$: 299.0751; found: 299.0750.

2-Phenyl-2'-deoxyinosine (4e, d^{Ph}I)



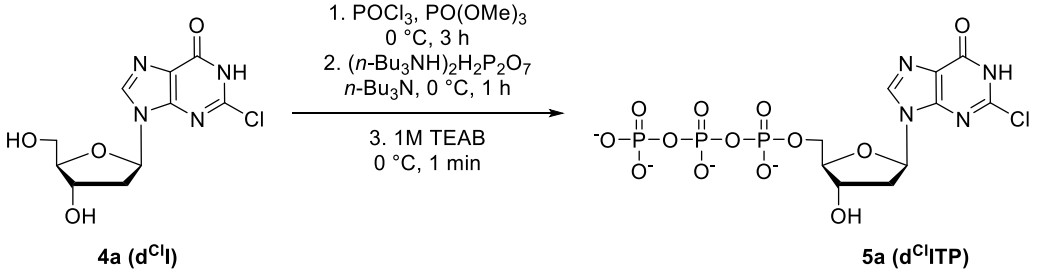
To a flask containing 2-Iodo-3',5'-di-*O*-acetyl-2'-deoxyinosine **3** (200 mg, 0.43 mmol), $PhB(OH)_2$ (79 mg, 0.65 mmol), Cs_2CO_3 (423 mg, 1.30 mmol), $Pd(OAc)_2$ (5 mg, 0.02 mmol) and TPPTS [3,3′,3″-Phosphanetriyltris(benzenesulfonic acid) trisodium salt, 31 mg, 0.05 mmol] a mixture of CH_3CN/H_2O (2/1, 5.4 ml) was added under argon. This mixture was heated at 80 °C for 1.5 h. After cooling, volatiles were evaporated and residue was dissolved in MeOH (5 ml) and MeONa (0.4 ml, 1.75 mmol, 25 wt.% in MeOH) was added. This mixture was stirred at rt (25 °C) for 1 h. Silica gel was added, volatiles were evaporated and the title compound was obtained, after purification by RP-FPLC on a C-18 column (0 → 100 % MeOH in H_2O), as a white solid (130 mg, 92% over two steps). NMR data were in good agreement with those published previously.[S3]
R_f = 0.14 (10% MeOH in CH_2Cl_2).
^{1}H NMR (401.0 MHz, DMSO-d_6): 2.33 (ddd, 1H, *J* = 13.2, *J* = 6.3, *J* = 3.3, H-2'b); 2.71 (ddd, 1H, *J* = 13.2, *J* = 7.9, *J* = 5.9, H-2'a); 3.53 (dd, 1H, *J* = 11.7, *J* = 4.7, H-5'a); 3.60 (dd, 1H, *J* = 11.7, *J* = 4.7, H-5'b); 3.88 (td, 1H, *J* = 4.7, *J* = 2.8, H-4'); 4.43 (td, 1H, *J* = 5.9, *J* = 3.3, H-3'); 4.94 (bs, 1H, OH-5'); 5.39 (bs, 1H, OH-3'); 6.40 (dd, 1H, *J* = 7.9, *J* = 6.3, H-1'); 7.50-7.60 (m, 3H, H-*m*,*p*-Ph); 8.12 (dd, 2H, *J* = 8.1, *J* = 1.7, H-*o*-Ph); 8.32 (s, 1H, H-8); 12.53 (bs, 1H, H-1).
^{13}C NMR (100.8 MHz, DMSO-d_6): 39.59 (CH_2-2'); 61.67 (CH_2-5'); 70.76 (CH-3'); 83.41 (CH-1'); 87.92 (CH-4'); 122.99 (C-5); 127.85 (CH-*o*-Ph); 128.64 (CH-*m*-Ph); 131.25 (CH-*p*-Ph); 132.48 (C-*i*-Ph); 139.06 (CH-8); 148.36 (C-4); 153.55 (C-2); 157.72 (C-6).
MS (ESI$^+$): *m/z* (%): 351.1 (100) $[M+Na]^+$.
HRMS (ESI$^+$): calculated for $C_{16}H_{16}O_4N_4Na$: 351.1064; found: 351.1064.

2-Chloro-2'-deoxyinosine (4a, d^{Cl}I)



Starting protected nucleoside **6** (200 mg, 0.40 mmol), DABCO (4 mg, 0.04 mmol) and CsOAc (213 mg, 1.10 mmol) were suspended in dry DMF (3.7 ml). Et_3N (0.16 ml, 1.10 mmol) was added this mixture was stirred at rt (25 °C) overnight. H_2O (2 ml) was added and stirring continued for another 30 min, after which $CHCl_3$ (25 ml) and H_2O (20 ml) were added. Phases were separated, water phase was extracted with $CHCl_3$ (2 x 25 ml). Collected organic phases were dried over anhydrous Na_2SO_4, filtered and evaporated. Residue was dissolved in MeOH (3.7 ml) and K_2CO_3 (128 mg, 0.90 mmol) was added. This mixture was stirred at rt (25 °C) for 1 h, silica gel was added and volatiles were evaporated. Purification by RP-FPLC on a C-18 column (0 → 100% MeOH in H_2O) afforded the desired product as a white solid (76 mg, 72% over two steps).
R_f = 0.12 (10% MeOH in CH_2Cl_2).
^{1}H NMR (401.0 MHz, DMSO-d_6): 2.15 (ddd, 1H, *J* = 13.0, *J* = 6.0, *J* = 2.5, H-2'b); 2.62 (ddd, 1H, *J* = 13.0, *J* = 8.4, *J* = 5.6, H-2'a); 3.49 (dd, 1H, *J* = 11.9, *J* = 4.0, H-5'a); 3.59 (dd, 1H, *J* = 11.9, *J* = 4.0, H-5'b); 3.84 (td, 1H, *J* = 4.0, *J* = 2.1, H-4'); 4.35 (td, 1H, *J* = 5.6, *J* = 2.1, H-3'); 5.29 (bs, 2H, OH-3', OH-5'); 6.15 (dd, 1H, *J* = 8.4, *J* = 6.0, H-1'); 7.85 (s, 1H, H-8).
^{13}C NMR (100.8 MHz, DMSO-d_6): 39.38 (CH_2-2'); 62.15 (CH_2-5'); 71.18 (CH-3'); 83.82 (CH-1'); 87.89 (CH-4'); 123.94 (C-5); 135.70 (CH-8); 149.76 (C-4); 153.74 (C-2); 166.17 (C-6).
MS (ESI$^+$): *m/z* (%): 309.1 (100) [(^{35}Cl)M+Na]$^+$, 311.1 (31) [(^{37}Cl)M+Na]$^+$, 325.1 (32) [(^{35}Cl)M+K]$^+$, 327.1 (10) [(^{37}Cl)M+K]$^+$.
HRMS (ESI$^+$): calculated for $C_{10}H_{11}O_4N_4$(^{35}Cl)Na: 309.0361 found: 309.0362.

2-Chloro-2'-deoxyinosine-5'-*O*-triphosphate (5a, d^{Cl}ITP)



Starting 2-chloro-2'-deoxyinosine **4a** (**d^{Cl}I**, 21 mg, 0.07 mmol) was dried in vacuo overnight. Then it was dissolved in dry $PO(OMe)_3$ (0.84 ml) and cooled down to 0 °C. Freshly distilled $POCl_3$ (8 µl, 0.09 mmol) was added dropwise and this mixture was stirred at 0 °C until no more starting material could be observed by TLC (ca. 3 hours). Then an ice-cold solution of (n-$Bu_3NH)_2H_2P_2O_7$ (197 mg, 0.36 mmol) and n-Bu_3N (75 µl, 0.29 mmol) in dry DMF (0.54 ml) was added dropwise.

The mixture was stirred for another 1 hour at 0 ºC and then stopped by the addition of cold 2 M TEAB (0.54 ml). The mixture was concentrated on a rotavap; the residue was co-evaporated with distilled water three times. The crude product was dissolved in water (ca 3 ml); the aqueous solution was purified by semi-preparative HPLC using a linear gradient of methanol (5→100%) in 0.1 M TEAB buffer. The appropriate fractions were combined and evaporated on a rotavap. The viscous oil was coevaporated with distilled water three times. The product was converted to sodium salt on an ion-exchange column (Dowex 50WX8 in Na^+ cycle) and freeze-dried. The title compound was obtained as a white solid (8 mg, 21%).

^{1}H NMR (401.0 MHz, D_2O, ref(dioxane) = 3.75 ppm): 2.55 (ddd, 1H, *J* = 14.0, *J* = 6.3, *J* = 3.8, H-2'b); 2.77 (ddd, 1H, *J* = 14.0, *J* = 7.3, *J* = 6.2, H-2'a); 4.15 (ddd, 1H, *J* = 11.5, *J* = 5.3, *J* = 3.8, H-5'b); 4.23 (ddd, 1H, *J* = 11.5, *J* = 6.3, *J* = 3.8, H-5'a); 4.28 (qd, 1H, *J* = 3.8, *J* = 2.4, H-4'); 4.80 (dt, 1H, *J* = 6.2, *J* = 3.8, *J* = 3.7, H-3'); 6.38 (dd, 1H, *J* = 7.3, 6.3, H-1'); 8.28 (s, 1H, H-8).

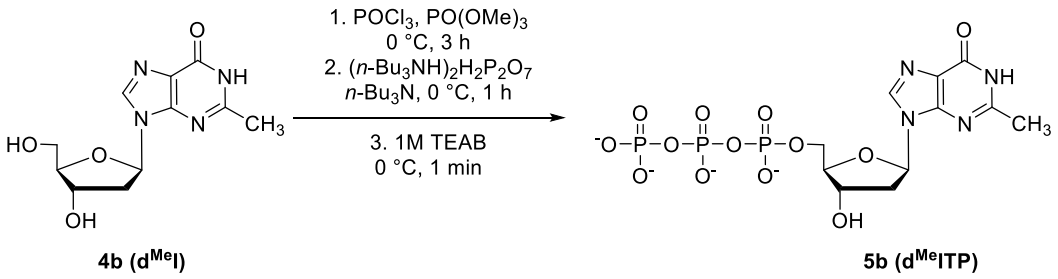
^{13}C NMR (100.8 MHz, D_2O, ref(dioxane) = 69.3 ppm): 41.75 (CH_2-2'); 67.99 (d, *J* = 5.5, CH_2-5'); 73.53 (CH-3'); 86.04 (CH-1'); 88.41 (d, *J* = 8.8, CH-4'); 124.96 (C-5); 140.80 (CH-8); 153.18 (C-4); 156.75 (C-2); 170.16 (C-6).

^{31}P{^{1}H} NMR (162.3 MHz, D_2O): -18.86 (t, *J* = 19.2, P$_\beta$); -8.21 (d, *J* = 19.2, P$_\alpha$); -3.51 (d, *J* = 19.2, P$_\gamma$).

MS (ESI$^-$): *m/z* (%): 365.0 (100) [(^{35}Cl)M–$H_3P_2O_6$]$^-$, 367.0 (24) [(^{37}Cl)M–$H_3P_2O_6$]$^-$, 445.0 (38) [(^{35}Cl)M–H_2PO_3–H]$^-$, 467.0 (33) [(^{35}Cl)M–H–H_2PO_3+Na]$^-$, 524.9 (67) [(^{35}Cl)M–H]$^-$, 526.9 (14) [(^{37}Cl)M–H]$^-$.

HRMS (ESI$^-$): calculated for $C_{10}H_{13}O_{13}N_4(^{35}Cl)P_3$: 524.9386; found: 524.9383; calculated for $C_{10}H_{12}O_{13}N_4(^{35}Cl)P_3Na$: 546.9205; found: 546.9202.

2-Methyl-2'-deoxyinosine-5'-*O*-triphosphate (5b, d^{Me}ITP)



2-Methyl-2'-deoxyinosine-5'-*O*-triphosphate **5b** (**d^{Me}ITP**) was prepared from nucleoside **4b** (**d^{Me}I**, 25 mg, 0.10 mmol) following the procedure described for the preparation of **5a** (**d^{Cl}ITP**). The desired triphosphate was isolated as a white lyophilizate (27 mg, 56%).

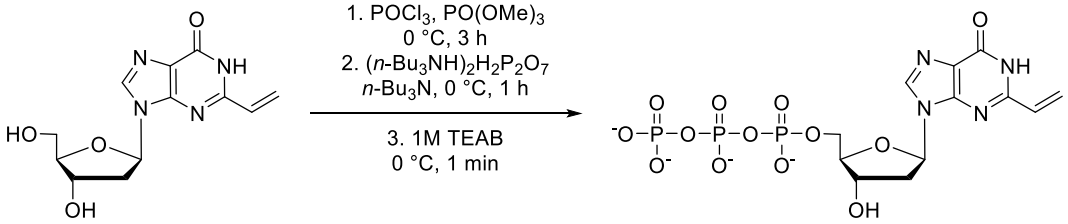
^{1}H NMR (401.0 MHz, D_2O, ref(dioxane) = 3.75 ppm): 2.53 (s, 3H, CH_3); 2.60 (ddd, 1H, *J* = 14.0, *J* = 6.5, *J* = 4.3, H-2'b); 2.78 (ddd, 1H, *J* = 14.0, *J* = 7.1, *J* = 5.7, H-2'a); 4.14 – 4.24 (m, 2H, H-5'a, H-5'b); 4.31 (qd, 1H, *J* = 3.4, *J* = 1.2, H-4'); 4.76 – 4.80 (m, 1H, H-3'); 6.45 (dd, 1H, *J* = 7.1, *J* = 6.5, H-1'); 8.36 (s, 1H, H-8).

^{13}C NMR (100.8 MHz, D_2O, ref(dioxane) = 69.3 ppm): 23.33 (CH_3); 41.79 (CH_2-2'); 67.89 (d, *J* = 5.4, CH_2-5'); 73.34 (CH-3'); 86.42 (CH-1'); 88.58 (d, *J* = 8.2, CH-4'); 124.16 (C-5); 142.05 (CH-8); 151.83 (C-4); 159.95 (C-2); 162.09 (C-6).

^{31}P{^{1}H} NMR (162.3 MHz, D_2O): -17.78 (t, *J* = 18.5, P$_\beta$); -7.85 (d, *J* = 18.2, P$_\alpha$); -2.29 (d, *J* = 19.0, P$_\gamma$).

MS (ESI$^-$): *m/z* (%): 365.0 (75) [M–$H_3P_2O_6$]$^-$, 425.0 (100) [M–H_2PO_3]$^-$, 447.0 (53) [M–H–H_2PO_3+Na]$^-$, 469.0 (31) [M–2H–H_2PO_3+2Na]$^-$, 549.0 (28) [M–3H+2Na]$^-$.
HRMS (ESI$^-$): calculated for $C_{11}H_{16}O_{13}N_4P_3$: 504.9932; found: 504.9931; calculated for $C_{11}H_{15}O_{13}N_4P_3Na$: 526.9752; found: 526.9750.

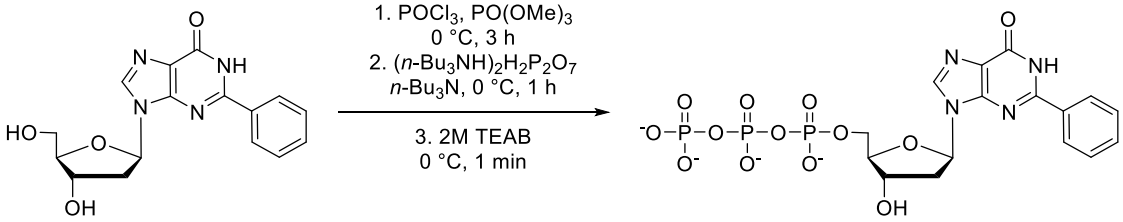
2-Vinyl-2'-deoxyinosine-5'-*O*-triphosphate (5c, d^{V}ITP)



4c (d^{V}I) **5c (d^{V}ITP)**

2-Vinyl-2'-deoxyinosine-5'-*O*-triphosphate **5c** (**d^{V}ITP**) was prepared from nucleoside **4c** (**d^{V}I**, 20 mg, 0.07 mmol) following the procedure described for the preparation of **5a** (**d^{Cl}ITP**). The desired triphosphate was isolated as a white lyophilizate (11.4 mg, 31%).
^{1}H NMR (401.0 MHz, D_2O, ref(dioxane) = 3.75 ppm): 2.57 (ddd, 1H, *J* = 13.9, *J* = 6.3, *J* = 3.9, H-2'b); 2.83 (ddd, 1H, *J* = 13.9, *J* = 7.6, *J* = 5.9, H-2'a); 4.14 – 4.26 (m, 2H, H-5'a, H-5'b); 4.28 (qd, 1H, *J* = *J* = 4.0, *J* = 1.7, H-4'); 4.76 – 4.79 (m, 1H, H-3'); 5.68 (dd, 1H, *J* = 10.7, *J* = 1.3, CH_a**H$_b$**=CH); 6.33 (dd, 1H, *J* = 17.4, *J* = 1.3, C**H$_a$**H$_b$=CH); 6.51 (dd, 1H, *J* = 7.6, 6.3, H-1'); 6.68 (dd, 1H, *J* = 17.4, *J* = 10.7, CH_2=C**H**); 8.32 (s, 1H, H-8).
^{13}C NMR (100.8 MHz, D_2O, ref(dioxane) = 69.3 ppm): 41.83 (CH_2-2'); 68.10 (d, *J* = 5.9, CH_2-5'); 73.63 (CH-3'); 85.91 (CH-1'); 88.27 (d, *J* = 8.3, CH-4'); 124.87 (C-5); 125.54 (**C**H_2=CH); 137.70 (**C**H=CH_2); 141.22 (CH-8); 153.00 (C-4); 162.53 (C-2); 167.88 (C-6).
^{31}P{^{1}H} NMR (162.0 MHz, D_2O): -20.29 (t, *J* = 20.6, P_β); -8.97 (d, *J* = 20.0, P_α); -4.06 (d, *J* = 21.0, P_γ).
MS (ESI$^-$): *m/z* (%): 357.1 (73) [M–$H_3P_2O_6$]$^-$, 437.0 (100) [M–H_2PO_3]$^-$, 517.0 (57) [M–H]$^-$, 539.0 (39) [M–2H+Na]$^-$.
HRMS (ESI$^-$): calculated for $C_{12}H_{16}O_{13}N_4P_3$: 516.9932; found: 516.9929; calculated for $C_{12}H_{15}O_{13}N_4P_3Na$: 538.9752; found: 538.9749.

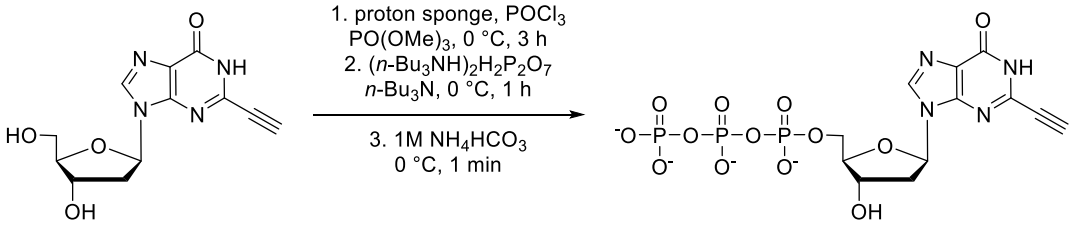
2-Phenyl-2'-deoxyinosine-5'-*O*-triphosphate (5e, d^{Ph}ITP)



4e (d^{Ph}I) **5e (d^{Ph}ITP)**

2-Phenyl-2'-deoxyinosine-5'-*O*-triphosphate **5e** (**d^{Ph}ITP**) was prepared from nucleoside **4e** (**d^{Ph}I**, 24 mg, 0.07 mmol) following the procedure described for the preparation of **5a** (**d^{Cl}ITP**). The desired triphosphate was isolated as a white lyophilizate (16 mg, 39%).
^{1}H NMR (401.0 MHz, D_2O, ref(dioxane) = 3.75 ppm): 2.60 (ddd, 1H, *J* = 13.9, *J* = 6.4, *J* = 3.9, H-2'b); 2.84 (ddd, 1H, *J* = 13.9, *J* = 7.6, *J* = 7.0, H-2'a); 4.17 – 4.29 (m, 2H, H-5'a, H-5'b); 4.31 (qd, 1H, *J* = 3.7, *J* = 1.5, H-4'); 4.83 (dt, 1H, *J* = 7.0, *J* = 3.8, H-3'); 6.60 (dd, 1H, *J* = 7.6, *J* = 6.4, H-1'); 7.50 – 7.53 (m, 3H, H-*m*,*p*-Ph); 8.06 – 8.09 (m, 2H, H-*o*-Ph); 8.34 (s, 1H, H-8).
^{13}C NMR (100.8 MHz, D_2O, ref(dioxane) = 69.3 ppm): 41.84 (CH_2-2'); 68.14 (d, *J* = 5.6, CH_2-5'); 73.65 (CH-3'); 85.92 (CH-1'); 88.25 (d, *J* = 8.6, CH-4'); 124.54 (C-5); 130.90 (CH-*o*-Ph); 131.22 (CH-*m*-Ph); 132.69 (CH-*p*-Ph); 140.65 (CH-*i*-Ph); 141.15 (CH-8); 153.52 (C-4); 164.24 (C-6); 170.27 (C-2).
^{31}P{^{1}H} NMR (162.3 MHz, D_2O): -18.23 (t, *J* = 18.9, P$_\beta$); -8.03 (d, *J* = 18.9, P$_\alpha$); -2.77 (d, P$_\gamma$).
MS (ESI$^-$): *m/z* (%): 407.1 (57) [M–$H_3P_2O_6$]$^-$, 487.0 (100) [M–H_2PO_3]$^-$, 567.0 (50) [M–H]$^-$, 589.0 (30) [M–2H+Na]$^-$.
HRMS (ESI$^-$): calculated for $C_{16}H_{18}O_{13}N_4P_3$: 567.0089; found: 567.0087; calculated for $C_{16}H_{17}O_{13}N_4P_3Na$: 588.9908; found: 588.9905.

2-Ethynyl-2'-deoxyinosine-5'-*O*-triphosphate (5d, d^{E}ITP)



4d (d^{E}I) **5d (d^{E}ITP)**

2-Ethynyl-2'-deoxyinosine **4d** (**d^{E}I**, 19 mg, 0.07 mmol) and proton sponge (17 mg, 0.08 mmol) were dried in vacuo overnight. Dry $PO(OMe)_3$ (0.8 ml) was added and resulting mixture was cooled down to 0 °C. Freshly distilled $POCl_3$ (8 µl, 0.09 mmol) was added dropwise and this mixture was stirred at 0 °C until no more starting material could be observed by TLC (ca 3 hours). Then an ice-cold solution of (*n*-$Bu_3NH)_2H_2P_2O_7$ (185 mg, 0.34 mmol) and *n*-Bu_3N (70 µl, 0.27 mmol) in dry DMF (0.5 ml) was added dropwise. This mixture was stirred for another 1 hour at 0 ºC and then stopped by the addition of cold 1 M NH_4HCO_3 (1 ml). The mixture was concentrated on a rotavap; the residue was co-evaporated with distilled water three times. The crude product was dissolved in water (ca 3 ml); the aqueous solution was purified by semi-preparative HPLC using a linear gradient of methanol (5 → 100%) in 0.1 M NH_4HCO_3 buffer and further purified by ion-exchange HPLC on POROS HQ with linear gradient of 0.4 M NH_4HCO_3 (0 → 100%) in H_2O. The appropriate fractions were combined and evaporated on a rotavap. The viscous oil was coevaporated with distilled water three times. The product was converted to sodium salt on an ion-exchange column (Dowex 50WX8 in Na^+ cycle) and freeze-dried. The title compound was obtained as a white lyophilizate (6 mg, 17%).
^{1}H NMR (600.1 MHz, DMSO-d_6): 2.60 (ddd, 1H, *J* = 13.6, *J* = 6.0, *J* = 3.7, H-2'b); 2.78 (ddd, 1H, *J* = 13.6, *J* = 7.1, *J* = 5.7, H-2'a); 4.15 – 4.24 (m, 2H, H-5'); 3.56 – 3.63 (m, 1H, H-5'a); 4.27 –

4.31 (m, 1H, H-4'); 4.74 – 4.78 (m, 1H, H-3'); 6.46 (dd, 1H, *J* = 7.1, *J* = 6.0, H-1'); 8.49 (bs, 1H, H-8).
^{13}C NMR (150.9 MHz, DMSO-d_6): 42.02 (CH_2-2'); 68.23 (d, *J* = 4.4, CH_2-5'); 73.70 (CH-3'); 77.71 (H**C**≡C-); 83.70 (-**C**≡CH); 86.40 (CH-1'); 88.26 (d, *J* = 8.4, CH-4'). Multiple aromatic signals missing in the spectra, most probably due to tautomerism.
^{31}P{^{1}H} NMR (202.4 MHz, D_2O): -19.09 (bs, P_β); -10.34 (s, P_α); -5.45 (bs, P_γ).
MS (ESI$^-$): *m/z* (%): 355.0 (100) $[M–H_3P_2O_6]^-$, 457.0 (33) $[M–H–H_2PO_3+Na]^-$.
HRMS (ESI$^-$): calculated for $C_{12}H_{14}O_{13}N_4P_3$: 514.9776; found: 514.9776; calculated for $C_{12}H_{13}O_{13}N_4P_3Na$: 536.9595; found: 536.9600.

Enzymatic synthesis and characterization of modified DNA

Materials and methods

Oligonucleotides were purchased from Generi Biotech (Czech Republic) or Eurofins Genomics (Germany). *KOD XL* DNA polymerase and corresponding reaction buffer were obtained from Merck Millipore. *Therminator* DNA polymerase and all of the restriction enzymes, corresponding reaction buffers, as well as natural nucleoside triphosphates (dATP, dCTP, dGTP, dTTP), were purchased from New England Biolabs. Streptavidin magnetic beads were obtained from Roche. All solutions for biochemical reactions were prepared using Milli-Q water. The gels were visualized by a fluorescence scanner (Typhoon FLA 9500, GE Healthcare). Concentration of DNA solutions was calculated using A_{260} values measured on a Nanodrop and values obtained with OligoCalc.[S4] Mass spectra of oligonucleotides were measured by MALDI-TOF, on UltrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Germany), with 1 kHz smartbeam II laser. QIAquick nucleotide removal kit was purchased from QIAGEN.

Analytical primer extension

Incorporation of one modified $d^{R}ITP$

Reaction mixture (20 μl) was prepared by mixing primer Prim15-FAM (0.150 μM), template Temp19 (0.225 μM), *Therminator* DNA polymerase (0.1 U), natural dTTP (1.5 μM), either natural dGTP or modified $d^{R}ITP$ (8 μM for $d^{Cl}ITP$ and $d^{Me}ITP$ or 20 μM for $d^{V}ITP$, $d^{E}ITP$ and $d^{Ph}ITP$) and reaction buffer (10×, 2 μl). The reaction was incubated at 60 °C for 60 min (30 min in case of the reaction with natural dGTP). The reaction was stopped by addition of PAGE stop solution (20 μl; 80% [v/v] formamide, 20 mM EDTA, 0.025% [w/v] bromophenol blue, 0.025% [w/v] xylene cyanol) and heated at 95 °C for 5 min. Aliquots (3.5 μl) were subjected to separation by denaturing polyacrylamide gel electrophoresis (12.5%) containing 1 × TBE buffer (pH 8.0) and 7 M urea at 42 mA for 1 hour. The gel was visualized by a fluorescent scanner.

Incorporation of four modified $d^{R}ITP$

Reaction mixture (20 μl) was prepared by mixing primer Prim15-FAM (0.150 μM), template Temp31-TINA (0.225 μM), *Therminator* DNA polymerase (0.2 U), natural dNTPs (dTTP, dATP, dCTP, 20 μM), either natural dGTP or modified $d^{R}ITP$ (100 μM) and reaction buffer (10×, 2 μl). The reaction was incubated at 60 °C for 60 min. The reaction was stopped by addition of PAGE stop solution (20 μl; 80% [v/v] formamide, 20 mM EDTA, 0.025% [w/v] bromophenol blue, 0.025% [w/v] xylene cyanol) and heated at 95 °C for 5 min. Aliquots (3.5 μl) were subjected to separation by denaturing polyacrylamide gel electrophoresis (12.5%) containing 1 × TBE buffer (pH 8.0) and 7 M urea at 42 mA for 1 hour. The gel was visualized by a fluorescent scanner.

Kinetic study of incorporation of $d^{Me}ITP$ and $d^{V}ITP$

Multiple PEX reaction mixtures (10 μl, 6 samples for each nucleotide used in the reactions, 18 in total) containing *Therminator* DNA polymerase (0.2 U), primer Prim15-FAM (0.150 μM),

template Temp16 (0.225 μM), natural dGTP or modified dITPs (200 μM) and reaction buffer (10×, 1 μl) were incubated at 60 °C for specific time intervals (0.5, 1, 2, 5, 10 and 30 minutes) and then the reaction was stopped by the addition of PAGE stop solution (10 μl) and immediate heating to 95 °C for 5 minutes. Aliquots (3.5 μl) were subjected to vertical electrophoresis in 12.5% denaturing polyacrylamide gel containing 1 × TBE buffer (pH 8.0) and 7 M urea at 42 mA for 1 hour. The gel was visualized by a fluorescent scanner.

Incorporation of one $d^{V}ITP$ into the 26-mer DNA used for reaction between peptide and DNA

Reaction mixture (20 μl) was prepared by mixing primer Prim16-FAM (0.150 μM), template Temp26 (0.225 μM), *Therminator* DNA polymerase (0.05 U), natural dNTPs (dATP, dCTP, dTTP, 10 μM), either natural dGTP or modified $d^{V}ITP$ (20 μM) and reaction buffer (10×, 2 μl). The reaction was incubated at 60 °C for 60 min (30 min in case of the reaction with natural dGTP). The reaction was stopped by addition of PAGE stop solution (20 μl; 80% [v/v] formamide, 20 mM EDTA, 0.025% [w/v] bromophenol blue, 0.025% [w/v] xylene cyanol) and heated at 95 °C for 5 min. Aliquots (3.5 μl) were subjected to separation by denaturing polyacrylamide gel electrophoresis (12.5%) containing 1 × TBE buffer (pH 8.0) and 7 M urea at 42 mA for 1 hour. The gel was visualized by a fluorescent scanner.

Preparation of modified oligonucleotides by PEX on a semi-preparative scale followed by magnetoseparation

General remarks

Streptavidine magnetic particles (Roche, 50 μl) were washed with binding buffer (3 × 200 μl, 10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.5). Finished PEX reactions were stopped by cooling to 4 °C. Then the PEX solution (20 μl) and binding buffer (50 μl) were added to the magnetic beads. The mixture was incubated for 30 min at 15 °C and 1100 rpm. The magnetic beads were collected on a magnet (DynaMagTM-2, Invitrogen) and washed with washing buffer (3 × 200 μl, 10 mM Tris, 1 mM EDTA, 500 mM NaCl, pH 7.5) and water (5 × 200 μl). Then water (50 μl) was added and the sample was denatured for 2 min at 55 °C and 900 rpm. The beads were collected on a magnet and the solution was transferred into a clean vial and analyzed by MALDI-TOF MS (for copies of mass spectra see **Figures S10-S13, S18**).

Reaction with $d^{Me}ITP$ and template Temp19-bio

Reaction mixture (20 μl) contained *Therminator* DNA polymerase (0.6 U), primer Prim15 (8 μM), template Temp19-bio (10 μM), modified $d^{Me}ITP$ (40 μM), natural dTTP (72 μM) and Thermopol reaction buffer (10×, 2 μl). The reaction mixture was incubated for 30 min at 60 °C in a thermal cycler.

Reaction with $d^{V}ITP$ and template Temp19-bio

Reaction mixture (20 μl) contained *Therminator* DNA polymerase (0.6 U), primer Prim15 (8 μM), template Temp19-bio (10 μM), modified $d^{V}ITP$ (100 μM), natural dTTP (150 μM) and Thermopol

reaction buffer (10×, 2 μl). The reaction mixture was incubated for 90 min at 60 °C in a thermal cycler.

Reaction with d^{Me}ITP and template Temp31-(bio)TINA

Reaction mixture (20 μl) contained *Therminator* DNA polymerase (0.6 U), primer Prim 15 (8 μM), template Temp31-(bio)TINA (10 μM), modified d^{Me}ITP (200 μM), natural dNTPs (dATP, dCTP, dTTP, 200 μM) and Thermopol reaction buffer (10×, 2 μl). The reaction mixture was incubated for 30 min at 60 °C in a thermal cycler.

Reaction with d^{V}ITP and template Temp31-(bio)TINA

Reaction mixture (20 μl) contained *Therminator* DNA polymerase (1 U), primer Prim15 (8 μM), template Temp31-(bio)TINA (10 μM), modified d^{V}ITP (1000 μM), natural dNTPs (dATP, dCTP, dTTP, 600 μM) and Thermopol reaction buffer (10×, 2 μl). The reaction mixture was incubated for 120 min at 60 °C in a thermal cycler.

Reaction with d^{V}ITP and template Temp26-bio

Reaction mixture (20 μl) contained *Therminator* DNA polymerase (0.75 U), primer Prim16-FAM (8 μM), template Temp26-bio (10 μM), modified d^{V}ITP (300 μM), natural dNTPs (dATP, dCTP, dTTP, 200 μM) and Thermopol reaction buffer (10×, 2 μl). The reaction mixture was incubated for 60 min at 60 °C in a thermal cycler.

Semi-preparative preparation of modified DNA

PEX reactions were performed as described above for preparation of modified DNA followed by magnetoseparation, with the exception of using non-biotinylated templates (for sequences see Table S1). Reactions were stopped by cooling to 4 °C. Prepared modified dsDNA was purified with a QIAquick nucleotide removal kit (QIAGEN) following the provided procedure. Products were eluted with 50 μl of H_2O and concentration was determined using NanoDrop and values obtained from OligoCalc.[S4]

Natural DNA for control experiments was prepared by preparing a reaction mixture (20 μl) containing *Therminator* DNA polymerase (0.3 U), primer Prim-15 (8 μM), template (either Temp19 or Temp31-TINA, 10 μM), natural dNTPs (120 μM) and Thermopol reaction buffer (10×, 2 μl). Reaction mixtures were incubated at 60 °C for 40 min. Prepared dsDNA was purified as described above.

Primer extension experiments for studies of cleavage by restriction endonucleases

Reaction mixtures (20 μl) were prepared by mixing primer (0.150 μM), template (0.225 μM), *Therminator* or *KOD XL* DNA polymerases, natural or modified dNTPs and corresponding reaction buffer (10×, 2 μl). The reactions were incubated at 60 °C for 30-100 min. The reaction

mixtures were then divided into two portions. The first portion (11.5 µl) was used directly for PAGE analysis and the second portion (8.5 µl) was used for DNA cleavage studies.

Incorporation of modified d^{R}ATPs within the recognition sequence of AfeI restriction endonuclease
PEX was performed using *KOD XL* DNA polymerase (0.05 U), natural dNTPs (dGTP, dCTP, dTTP, 10 µM), natural dATP or modified d^{R}ATPs (40 µM), primer Prim15-FAM and template Temp_AfA. Reaction mixtures were incubated at 60 °C for 60 min. Restriction endonuclease AfeI (5 U) and NEB 4 reaction buffer (10×, 1 µl) were added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

Incorporation of modified d^{R}ITPs within the recognition sequence of AfeI restriction endonuclease
PEX was performed using *Therminator* DNA polymerase (0.16 U), natural dNTPs (dATP, dCTP, dTTP, 16 µM), natural dGTP or modified d^{R}ITPs (40 µM), primer Prim15-FAM and template Temp_AfG. Reaction mixtures were incubated at 60 °C for 100 min. Restriction endonuclease AfeI (5 U) and NEB 4 reaction buffer (10×, 1 µl) were added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

Incorporation of modified d^{R}ATPs within the recognition sequence of EcoRI restriction endonuclease
PEX was performed using *Therminator* DNA polymerase (0.1 U), natural dNTPs (dGTP, dCTP, dTTP, 140 µM), natural dATP or modified d^{R}ATPs (200 µM), primer Prim15-FAM and template Temp_EcA. Reaction mixtures were incubated at 60 °C for 30 min. Restriction endonuclease EcoRI-HF (10 U) and CutSmart reaction buffer (10×, 1 µl) were added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

Incorporation of modified d^{R}ITPs within the recognition sequence of EcoRI restriction endonuclease
PEX was performed using *Therminator* DNA polymerase (0.04 U), natural dNTPs (dATP, dCTP, dTTP, 10 µM), natural dGTP or modified d^{R}ITPs (40 µM), primer Prim15-FAM and template Temp_EcG. Reaction mixtures were incubated at 60 °C for 100 min. Restriction endonuclease EcoRI-HF (10 U) and CutSmart reaction buffer (10×, 1 µl) were added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

Incorporation of modified d^{R}ATPs within the recognition sequence of KpnI restriction endonuclease
PEX was performed using *KOD XL* DNA polymerase (0.05 U), natural dNTPs (dGTP, dCTP, dTTP, 10 µM), natural dATP or modified d^{R}ATPs (40 µM), primer Prim15-FAM and template Temp_KpA. Reaction mixtures were incubated at 60 °C for 60 min. Restriction endonuclease KpnI (5 U) and NEB 1.1 reaction buffer (10×, 1 µl) were added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

Incorporation of modified d^{R}ITPs within the recognition sequence of KpnI restriction endonuclease
PEX was performed using *Therminator* DNA polymerase (0.04 U), natural dNTPs (dATP, dCTP, dTTP, 10 µM), natural dGTP or modified d^{R}ITPs (40 µM), primer Prim_KpG-FAM and template Temp_KpG. Reaction mixtures were incubated at 60 °C for 100 min. Restriction endonuclease

KpnI (5 U) and NEB 1.1 reaction buffer (10×, 1 μl) were added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

Incorporation of modified d^RATPs within the recognition sequence of PstI restriction endonuclease
PEX was performed using *KOD XL* DNA polymerase (0.025 U), natural dNTPs (dGTP, dCTP, dTTP, 20 μM), natural dATP or modified d^RATPs (20 μM), primer Prim15-FAM and template Temp_PsA. Reaction mixtures were incubated at 60 °C for 30 min. Restriction endonuclease PstI-HF (10 U) and CutSmart reaction buffer (10×, 1 μl) were added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

Incorporation of modified d^RITPs within the recognition sequence of PstI restriction endonuclease
PEX was performed using *Therminator* DNA polymerase (0.14 U), natural dNTPs (dATP, dCTP, dTTP, 16 μM), natural dGTP or modified d^RITPs (40 μM), primer Prim15-FAM and template Temp_PsG. Reaction mixtures were incubated at 60 °C for 100 min. Restriction endonuclease PstI-HF (10 U) and CutSmart reaction buffer (10×, 1 μl) were added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

Incorporation of modified d^RATPs within the recognition sequence of PvuII restriction endonuclease
PEX was performed using *KOD XL* DNA polymerase (0.063 U), natural dNTPs (dGTP, dCTP, dTTP, 100 μM), natural dATP or modified d^RATPs (100 μM), primer Prim15-FAM and template Temp_PvA. Reaction mixtures were incubated at 60 °C for 30 min. Restriction endonuclease PvuII (5 U) and NEB 3.1 reaction buffer (10×, 1 μl) were added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

Incorporation of modified d^RITPs within the recognition sequence of PvuII restriction endonuclease
PEX was performed using *Therminator* DNA polymerase (0.16 U), natural dNTPs (dATP, dCTP, dTTP, 16 μM), natural dGTP or modified d^RITPs (40 μM), primer Prim15-FAM and template Temp_PvG. Reaction mixtures were incubated at 60 °C for 100 min. Restriction endonuclease PvuII (5 U) and NEB 3.1 reaction buffer (10×, 1 μl) were added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

Incorporation of modified d^RATPs within the recognition sequence of RsaI restriction endonuclease
PEX was performed using *KOD XL* DNA polymerase (0.025 U), natural dNTPs (dGTP, dCTP, dTTP, 100 μM), natural dATP or modified d^RATPs (200 μM), primer Prim15-FAM and template Temp_RsA. Reaction mixtures were incubated at 60 °C for 30 min. Restriction endonuclease RsaI (5 U) and NEB 4 reaction buffer (10×, 1 μl) were added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

Incorporation of modified d^RITPs within the recognition sequence of RsaI restriction endonuclease
PEX was performed using *Therminator* DNA polymerase (0.04 U), natural dNTPs (dATP, dCTP, dTTP, 10 μM), natural dGTP or modified d^RITPs (40 μM), primer Prim15-FAM and template

Temp_RsG. Reaction mixtures were incubated at 60 °C for 90 min. Restriction endonuclease RsaI (5 U) and NEB 4 reaction buffer (10×, 1 μl) were added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

Incorporation of modified d^RATPs within the recognition sequence of SacI restriction endonuclease PEX was performed using *KOD XL* DNA polymerase (0.025 U), natural dNTPs (dGTP, dCTP, dTTP, 20 μM), natural dATP or modified d^RATPs (20 μM), primer Prim15-FAM and template Temp_SaA. Reaction mixtures were incubated at 60 °C for 30 min. Restriction endonuclease SacI-HF (10 U) and CutSmart reaction buffer (10×, 1 μl) were added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

Incorporation of modified d^RITPs within the recognition sequence of SacI restriction endonuclease PEX was performed using *Therminator* DNA polymerase (0.16 U), natural dNTPs (dATP, dCTP, dTTP, 16 μM), natural dGTP or modified d^RITPs (40 μM), primer Prim15-FAM and template Temp_SaG. Reaction mixtures were incubated at 60 °C for 100 min. Restriction endonuclease SacI-HF (10 U) and CutSmart reaction buffer (10×, 1 μl) were added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

Incorporation of modified d^RATPs within the recognition sequence of ScaI restriction endonuclease PEX was performed using *KOD XL* DNA polymerase (0.075 U), natural dNTPs (dGTP, dCTP, dTTP, 20 μM), natural dATP or modified d^RATPs (20 μM), primer Prim15-FAM and template Temp_ScA. Reaction mixtures were incubated at 60 °C for 40 min. Restriction endonuclease ScaI-HF (10 U) and CutSmart reaction buffer (10×, 1 μl) were added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

Incorporation of modified d^RITPs within the recognition sequence of ScaI restriction endonuclease PEX was performed using *Therminator* DNA polymerase (0.04 U), natural dNTPs (dATP, dCTP, dTTP, 10 μM), natural dGTP or modified d^RITPs (40 μM), primer Prim15-FAM and template Temp_ScG. Reaction mixtures were incubated at 60 °C for 100 min. Restriction endonuclease ScaI-HF (10 U) and CutSmart reaction buffer (10×, 1 μl) were added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

Before loading of the PAGE gel, PAGE stop solution (either 11.5 or 8.5 μl, depending on the original portion of the PEX reaction) was added and samples were denatured by heating at 95 °C for 5 min. Aliquots (3.5 μl) were analyzed by denaturing PAGE (12.5%). The gels were visualized by a fluorescent scanner. Conversion of the cleavage was determined using ImageJ software by comparison of the ratios of the intensity of the cleavage product and sum of intensities of the cleavage product and the rest of the products.[S5]

Post-synthetic modification of DNA

Materials and methods

The oligonucleotides for these experiments were prepared by PEX on a large scale and purified with a QIAquick nucleotide removal kit (QIAGEN). Fluorescent thiol (**CM-SH**) was prepared as described previously.[S6] L-cysteine was obtained from Sigma-Aldrich. Dodecapeptides were purchased at IOCB Prague. Samples were concentrated on a CentriVap Vacuum Concentrator system (Labconco). Fluorescence spectra were measured on a Fluoromax 4 spectrofluorimeter (HORIBA Scientific). Conversion of the reactions was determined using ImageJ software.

Fluorescent labelling of vinyl modified DNAs

Reaction mixture was prepared by dissolving vinyl modified DNA (**DNA19_1^{V}H** or **DNA31_4^{V}H**) (0.5 nmol) in H_2O (12.5 μl). To this, thiol [**CM-SH**, (500 mM in 0.5 M TEAA buffer, pH = 8.0), 12.5 μl] was added and the reaction mixture was kept at 37 °C and 500 rpm for 3 days.
Steady-state fluorescence measurements of the products of thiol-ene reaction were performed with DNA after thiol-ene reaction and purification with QIAquick nucleotide removal kit (QIAGEN). DNA concentration was determined on a Nanodrop and recalculated using values from OligoCalc.[S4] Emission spectra were recorded using a 0.5 μM solution of DNA in phosphate buffer (20 mM, pH = 7.0, 1 M NaCl) using 100 μl quartz cuvette. Solution was equilibrated for 2 min in a thermal holder (25 °C) before the fluorescence spectrum was recorded. Excitation wavelength was 390 nm and the range of the emission spectra was 410-650 nm. Control experiments were performed using natural non-modified DNA following the same procedure (thiol-ene reaction, purification and emission spectra measurement).
To obtain single-stranded DNA for MALDI analysis, the thiol-ene reaction was performed as described above using DNA after PEX with biotinylated template. Reaction mixture was first purified using QIAquick nucleotide removal kit (QIAGEN) and subsequently ssDNAs were generated by magnetoseparation. Thus a solution of modified DNA obtained after elution from the spin column (50 μl of miliQ H_2O) was diluted by 50 μl of binding buffer (10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.5). Streptavidine magnetic particles (Roche, 100 μl) were washed with binding buffer (3 × 200 μl). The diluted modified DNA sample (100 μl) was then added to the magnetic particles. Mixture was incubated at 15 °C and 1100 rpm for 30 min. The magnetic beads were collected on a magnet (DynaMagTM-2, Invitrogen) and washed with washing buffer (3 × 200 μl, 10 mM Tris, 1 mM EDTA, 500 mM NaCl, pH 7.5) and water (5 × 200 μl). Then water (50 μl) was added and the sample was denatured for 2 min at 55 °C and 900 rpm. The beads were collected on a magnet and the solution was transferred into a clean vial and analyzed by MALDI-TOF MS (for copies of mass spectra see **Figure S14-S15**).
To analyze the product of thiol-ene reaction using PAGE, the reaction was performed as described above using DNA after PEX with Prim15-FAM and Temp19. The product of thiol-ene reaction was purified with QIAquick nucleotide removal kit (QIAGEN) two times. Concentration of DNA was determined on a Nanodrop and recalculated using values from OligoCalc. An aliquot was taken and diluted with miliQ H_2O to a total volume of 20 μl of approximately 500 nM concentration. PAGE stop solution (20 μl) was added and sample was denaturated at 95 °C for 5 min. Aliquot (3.5 μl) was subjected to vertical electrophoresis in 20% denaturing polyacrylamide gel containing

1×TBE buffer (pH 8.0) and 7 M urea at 42 mA for 120 min. The gel was visualized by a fluorescent scanner.

Reaction of vinyl modified DNAs and L-cysteine

Reaction mixture was prepared by dissolving vinyl modified DNA (**DNA19_1^{V}H** or **DNA31_4^{V}H**) (0.5 nmol) in H_2O (12.5 µl). To this, L-cysteine (500 mM in 0.5 M TEAA buffer, pH = 8.0, 12.5 µl) was added and the reaction mixture was kept at 50 °C and 500 rpm overnight.
To obtain single-stranded DNA for MALDI analysis, reaction mixture was first purified using QIAquick nucleotide removal kit (QIAGEN) and subsequently ssDNAs were generated by magnetoseparation as described above (for copies of mass spectra see **Figure S16-S17**).
To analyze the product of thiol-ene reaction using PAGE, the reaction was performed as described above using DNA after PEX with Prim15-FAM and Temp19. The PAGE analysis was performed as described above.

Reaction of vinyl modified DNA and dodecapeptides

DNA for these reactions was prepared using primer Prim16-FAM and template Temp26-bio. Reaction mixture was prepared as follows: in a PCR eppendorf, 2 µl of vinyl modified DNA solution (**DNA26_1^{V}H-FAM**, 26 µM in TEAA buffer, pH = 8.0) was mixed with the corresponding amount of peptide (1-100 eq., solutions in TEAA buffer). The final concentration was of DNA was adjusted to 5 µM with TEAA buffer. Reactions were kept at 20 °C for 1 h and then the temperature was increased over the course of 2 h to 37 °C. Reactions were kept at this temperature for 1 day (in one case reaction was run for 2 days). Stop solution (3 µl) and reaction mixtures (3 µl) were then combined and this mixture was denatured at 95 °C for 12 min. Residues were subjected to vertical electrophoresis in 12.5 % denaturing polyacrylamide gel containing 1×TBE buffer (pH 8.0) and 7 M urea at 42 mA for 80 min. The gel was visualized by a fluorescent scanner.
To obtain DNA suitable for MALDI analysis, reaction was performed on a bigger scale using 30 µl of the aforementioned DNA solution and 10 eq. of peptide_pos. After completion of the reaction, ssDNA was obtained by magnetoseparation as described above using 100 µl of magnetic beads (for copy of mass spectra see **Figure S19**).

Thermal denaturation studies

The oligonucleotides for these experiments were prepared by PEX on a semi-preparative scale. Concentration of DNA was determined on a Nanodrop and recalculated using values from OligoCalc. Samples were concentrated and then redissolved in phosphate buffer (20 mM, pH = 7.0; 1 M NaCl) to have A_{260} above 0.1. Thermal denaturation studies were performed on a Cary 100 UV-Vis spectrometer equipped with temperature controller (Agilent Technologies) monitoring the absorbance values at 260 nm between 35–95 °C. Data were collected from 6 independent heating-cooling cycles. Annealing temperatures (T_m / °C) were obtained by plotting temperature versus absorbance and applying a sigmodial curve fit.

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Copies of MALDI spectra

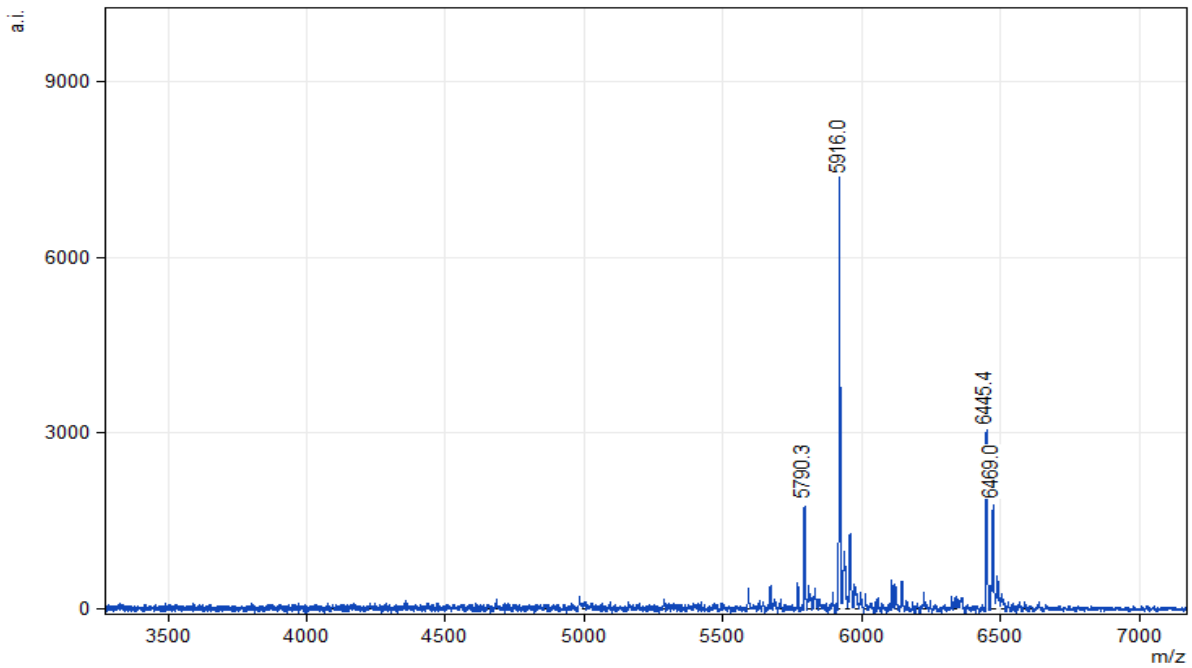


Figure S10: MALDI-TOF MS spectrum of **ON19_1^{Me}H** after PEX with Temp19-bio and **d^{Me}ITP**, calculated for $[M+H]^+$: 5914.9 Da; found: 5916.0.

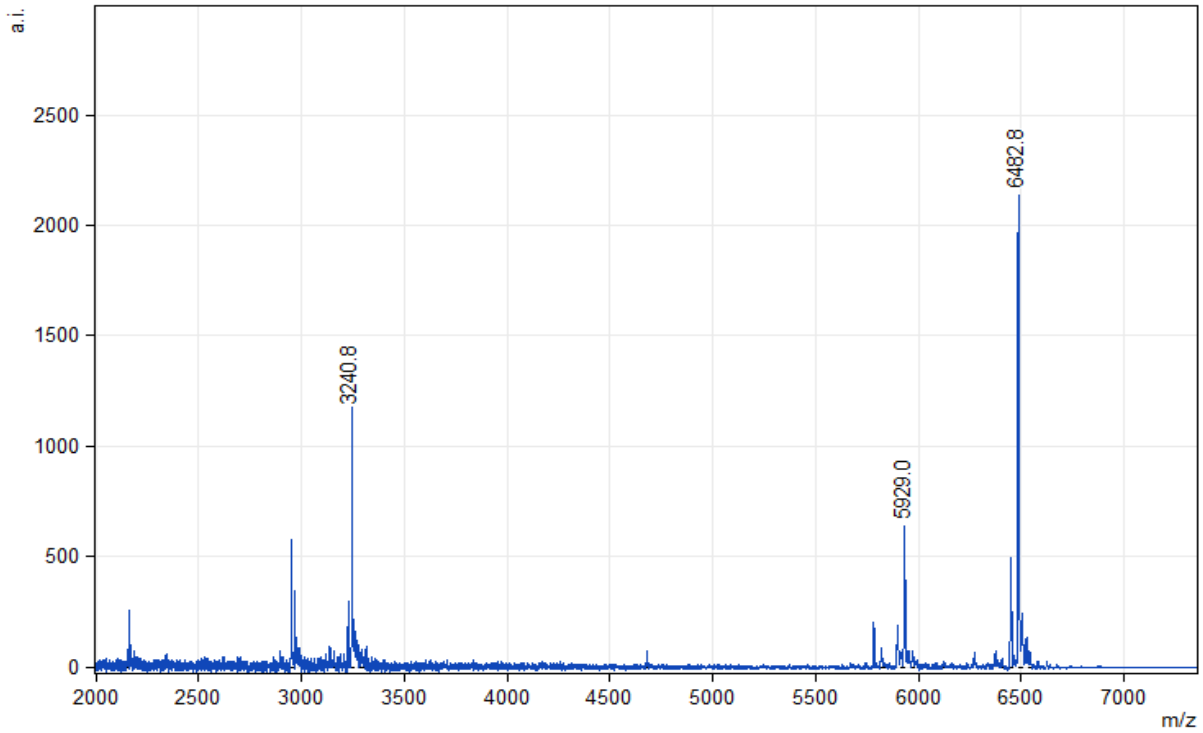


Figure S11: MALDI-TOF MS spectrum of **ON19_1^{V}H** after PEX with Temp19-bio and **d^{V}ITP**, calculated for $[M+H]^+$: 5926.9 Da; found: 5929.0.

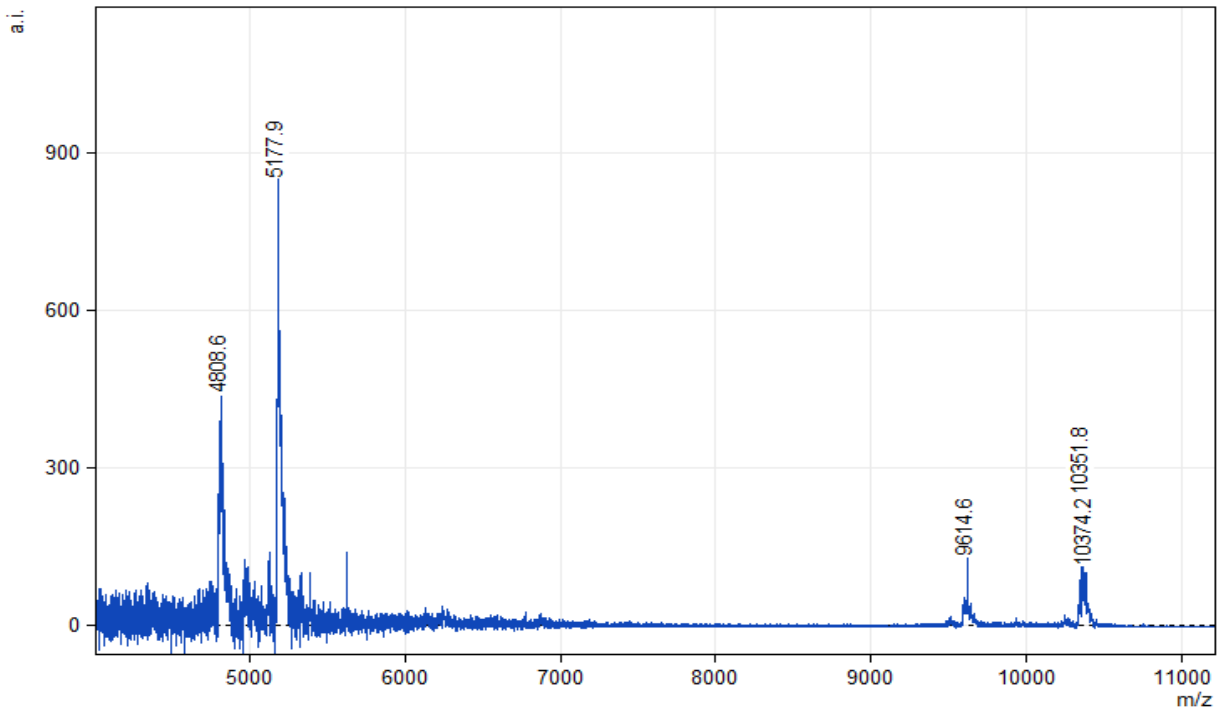


Figure S12: MALDI-TOF MS spectrum of **ON31_4^{Me}H** after PEX with Temp31-(bio)TINA and **d^{Me}ITP**, calculated for $[M+H]^+$: 9613.3 Da; found: 9614.6.

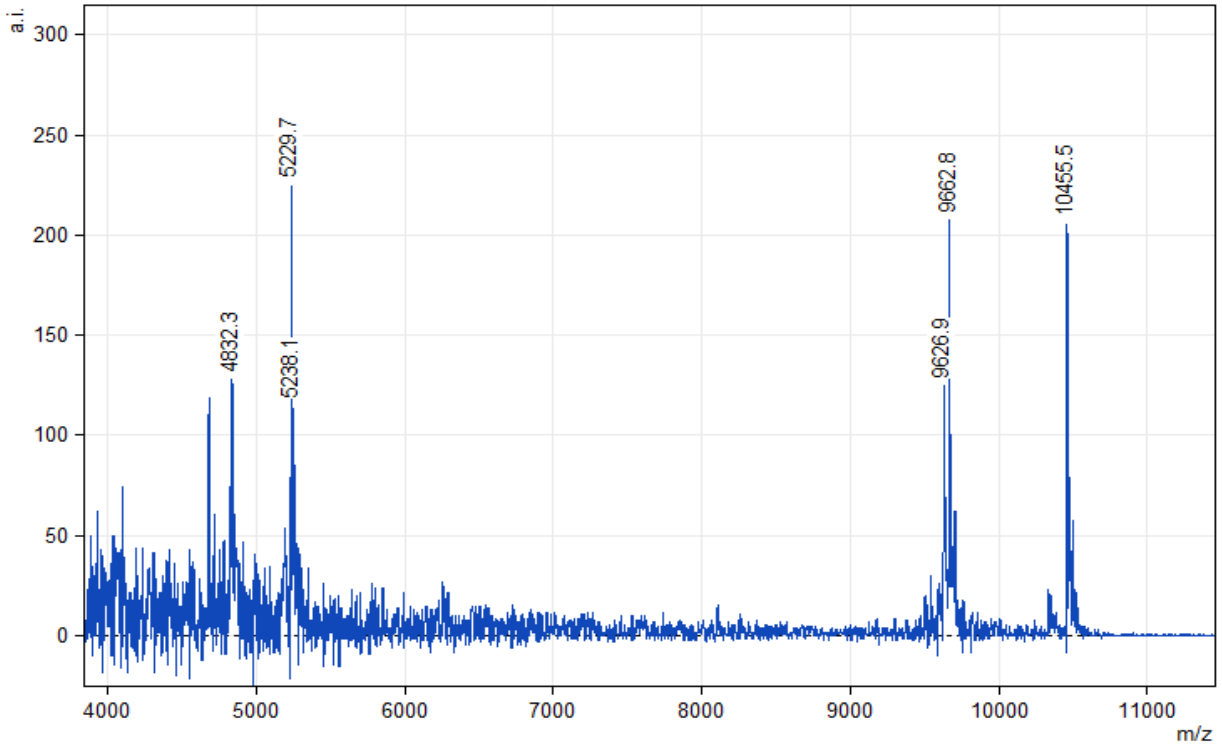


Figure S13: MALDI-TOF MS spectrum of **ON31_4^{V}H** after PEX with Temp31-(bio)TINA and **d^{V}ITP**, calculated for $[M+H]^+$: 9661.3 Da; found: 9662.8.

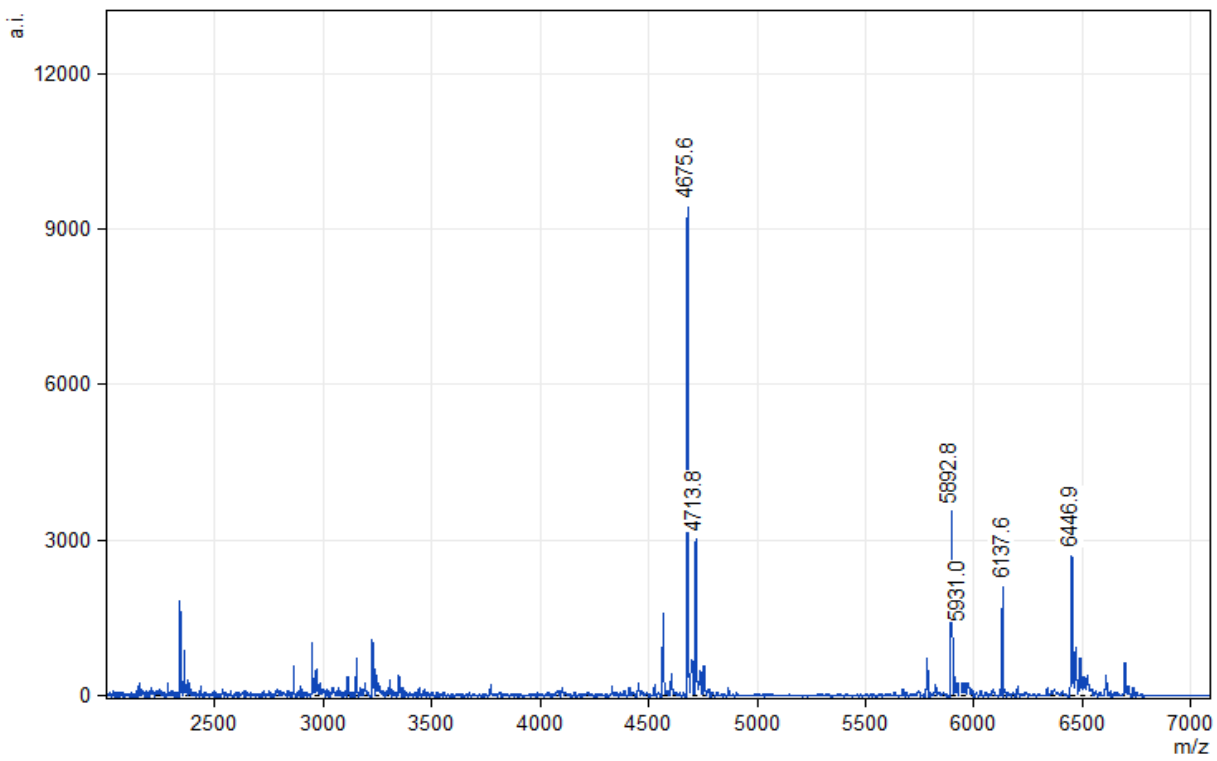


Figure S14: MALDI-TOF MS spectrum of **ON19_1^{CM}H** after thiol-ene reaction of **DNA19_1^{V}H** and **CM-SH**, calculated for $[M+H]^+$: 6134.9 Da; found: 6137.6. Peak at 5931.0 Da represents the starting vinyl modified DNA, 4675.6 Da is the signal of unreacted primer.

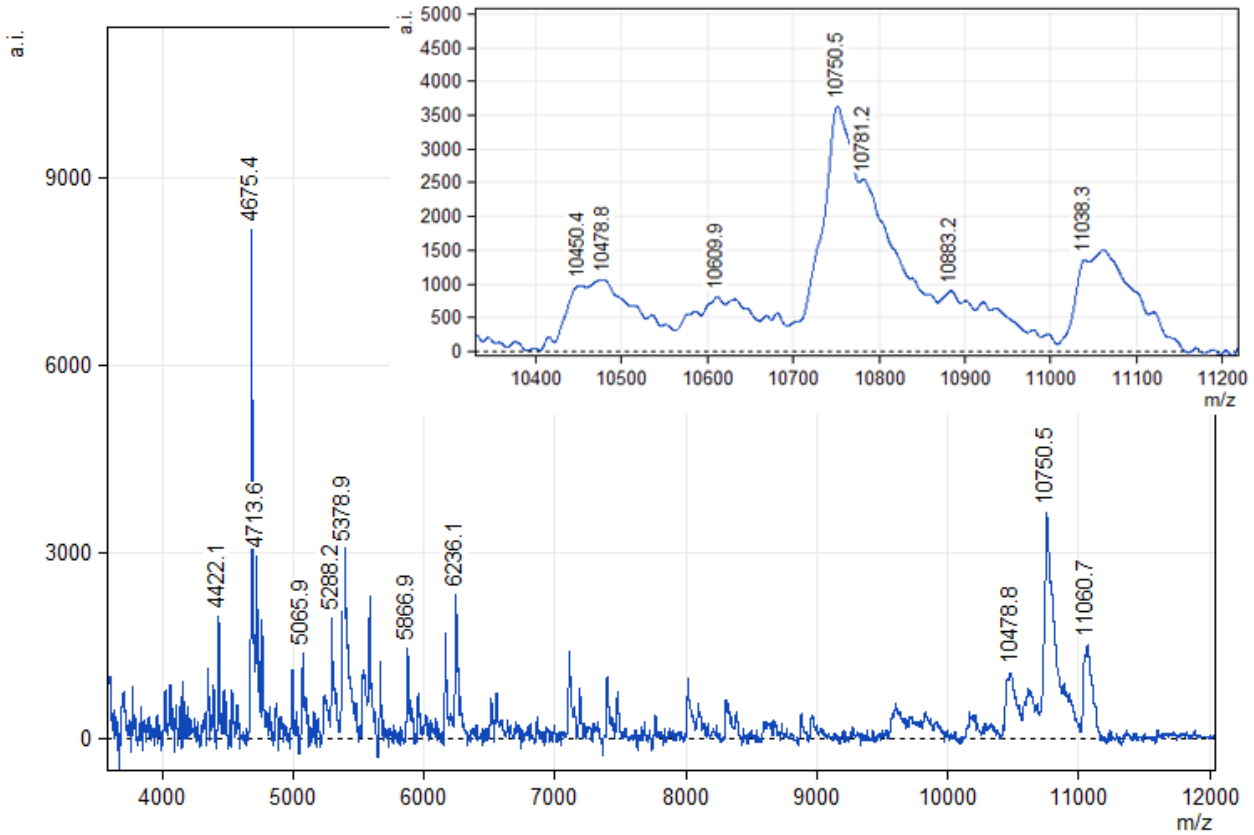


Figure S15: MALDI-TOF MS spectrum of **ON31_4^{CM}H** after thiol-ene reaction of **DNA31_4^{V}H** and **CM-SH**. Starting vinyl modified DNA was extended by one or two or three additional As at the 3' end due to non-templated extension. Products of thiol-ene reaction: $[M+2As+2CM+K]^+$: 10756.4, found 10750.5 – product of reaction of two vinyl groups, $[M+3As+2CM+Na]^+$: 11037.6, found 11038.3, etc.

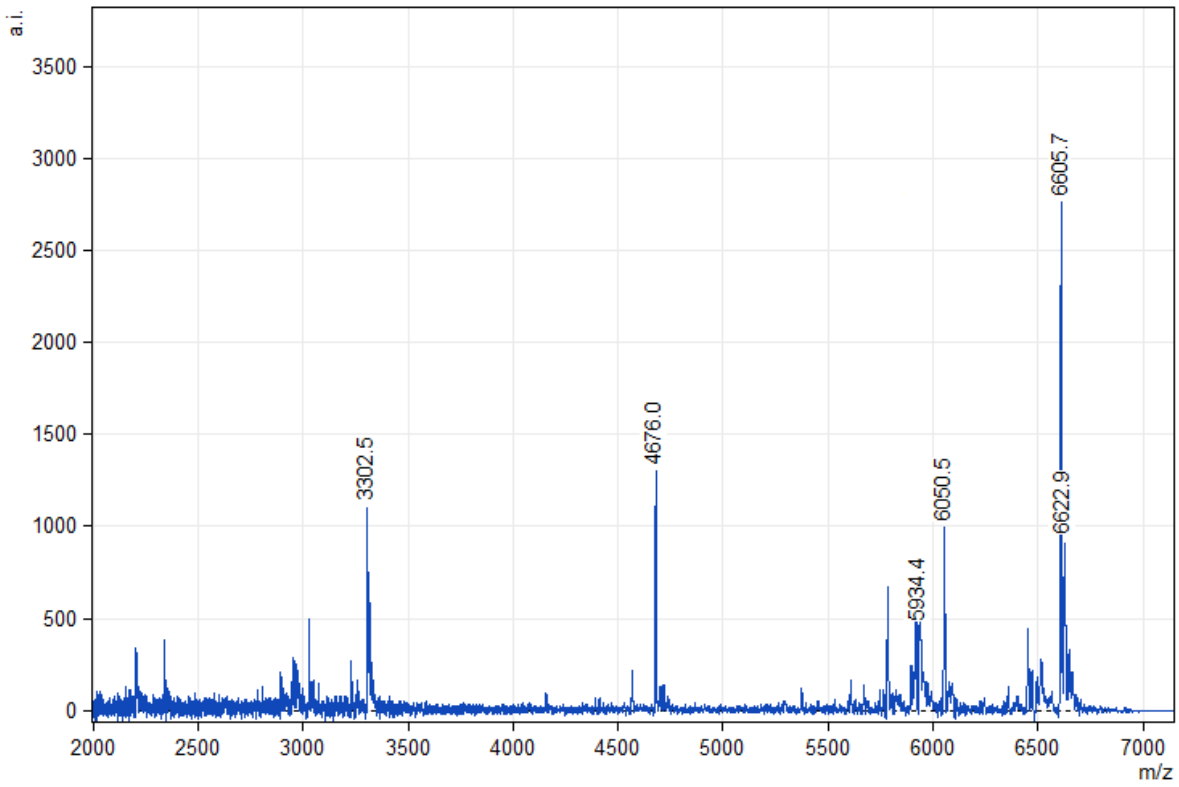


Figure S16: MALDI-TOF MS spectrum of **ON19_1^{Cys}H** after thiol-ene reaction of **DNA19_1^{V}H** and **L-cysteine**, calculated for $[M+H]^+$: 6049.0 Da; found: 6050.5. Peak at 5934.4 Da represents the starting vinyl modified DNA.

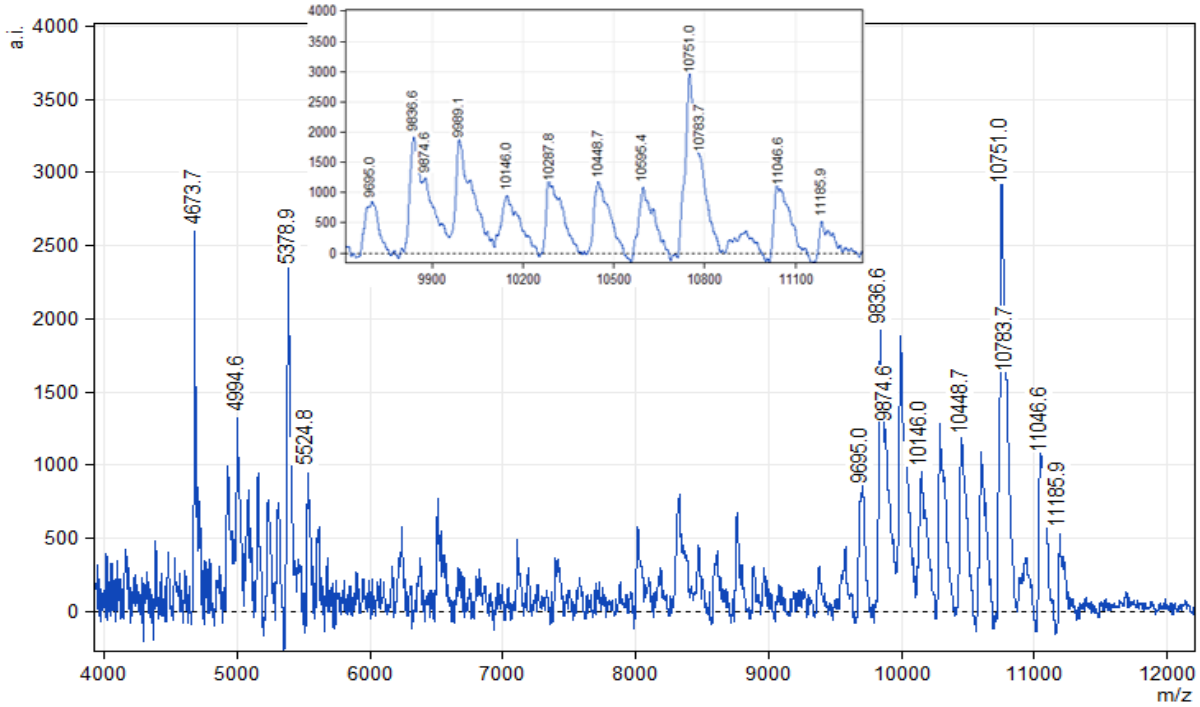


Figure S17: MALDI-TOF MS spectrum of **ON31_4^{Cys}H** after thiol-ene reaction of **DNA31_4^{V}H** and **L-cysteine**. Starting vinyl modified DNA was extended by one, two or three additional As at the 3' end due to non-templated extension (calculated for $[M+2As+H]^+$: 10287.4 Da; found: 10287.8; $[M+3As+H]^+$ 10600.6; found 10595.4). Products of thiol-ene reaction: $[M+2As+cys+K]^+$: 10448.4, found 10448.7 – product of reaction of one vinyl group; $[M+3As+cys+Na]^+$: 10746.4, found 10751.0 – product of reaction of one vinyl group, etc.

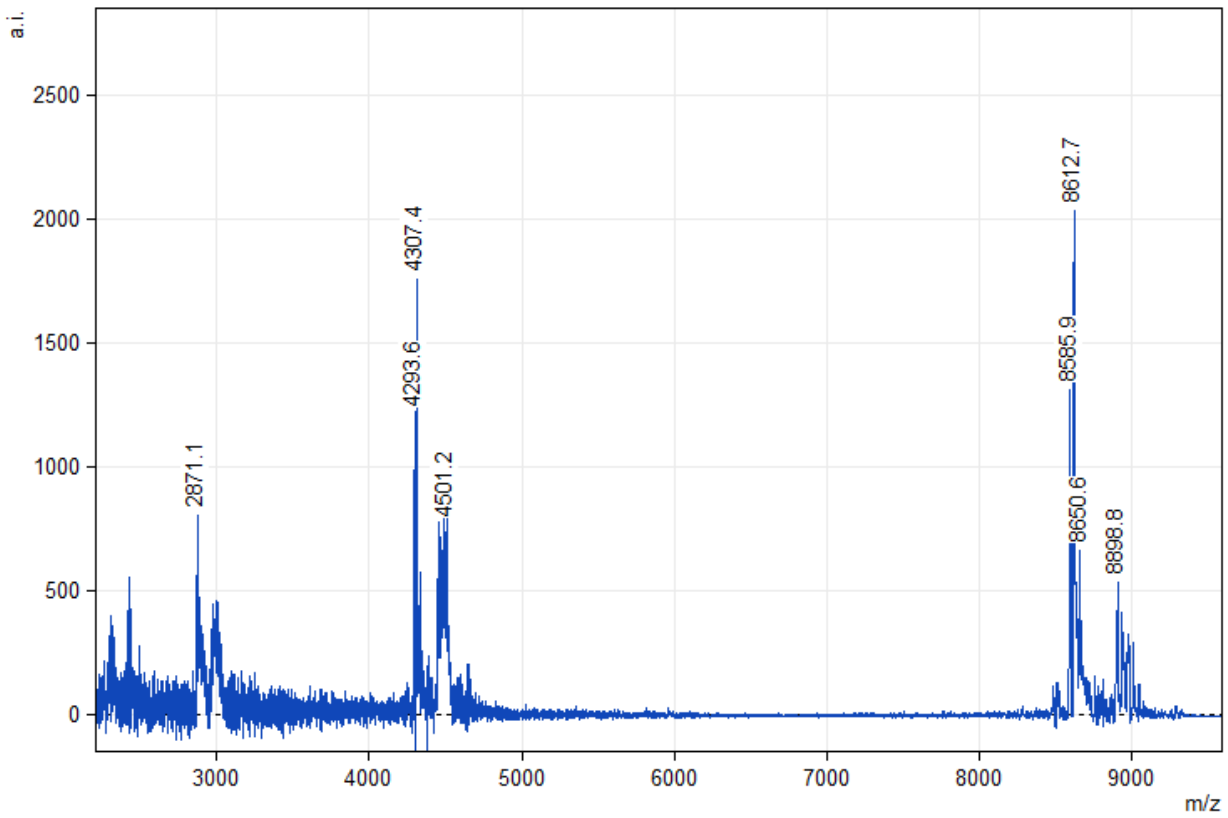


Figure S18: MALDI-TOF MS spectrum of **ON26_1^{V}H** after PEX with Temp26-bio, Prim16-FAM and **d^{V}ITP**, calculated for $[M+H]^+$: 8652.5 Da; found: 8650.6.

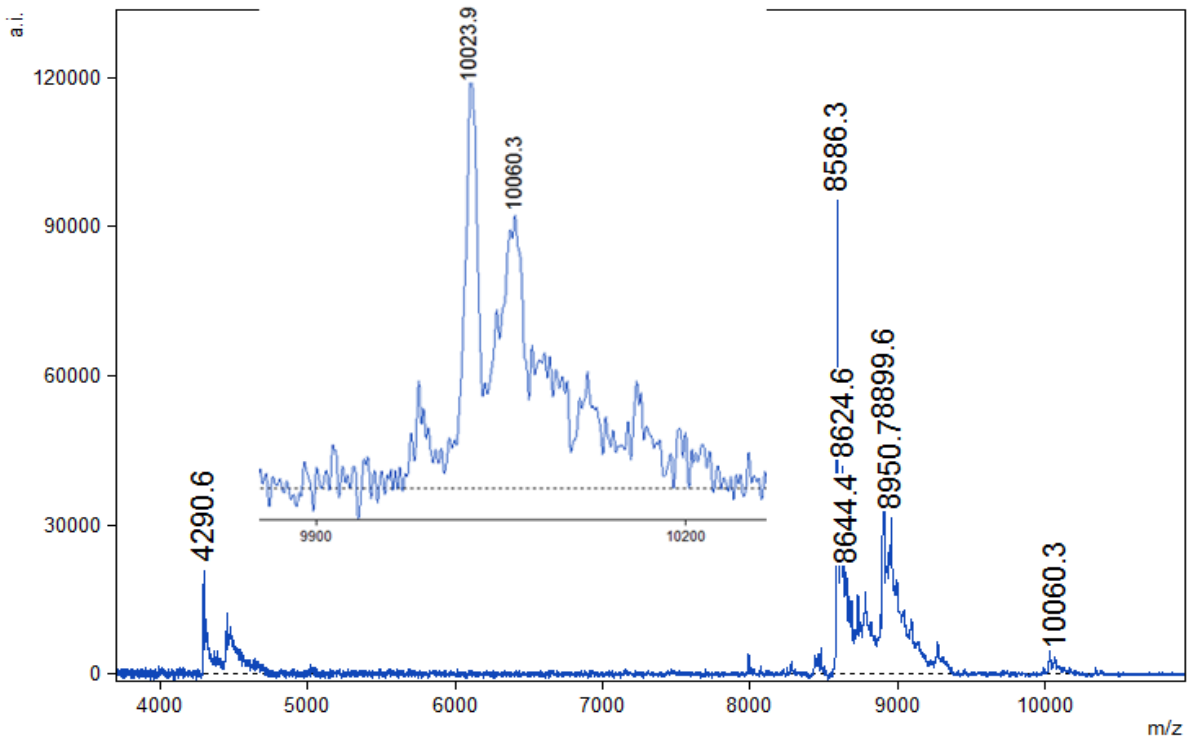
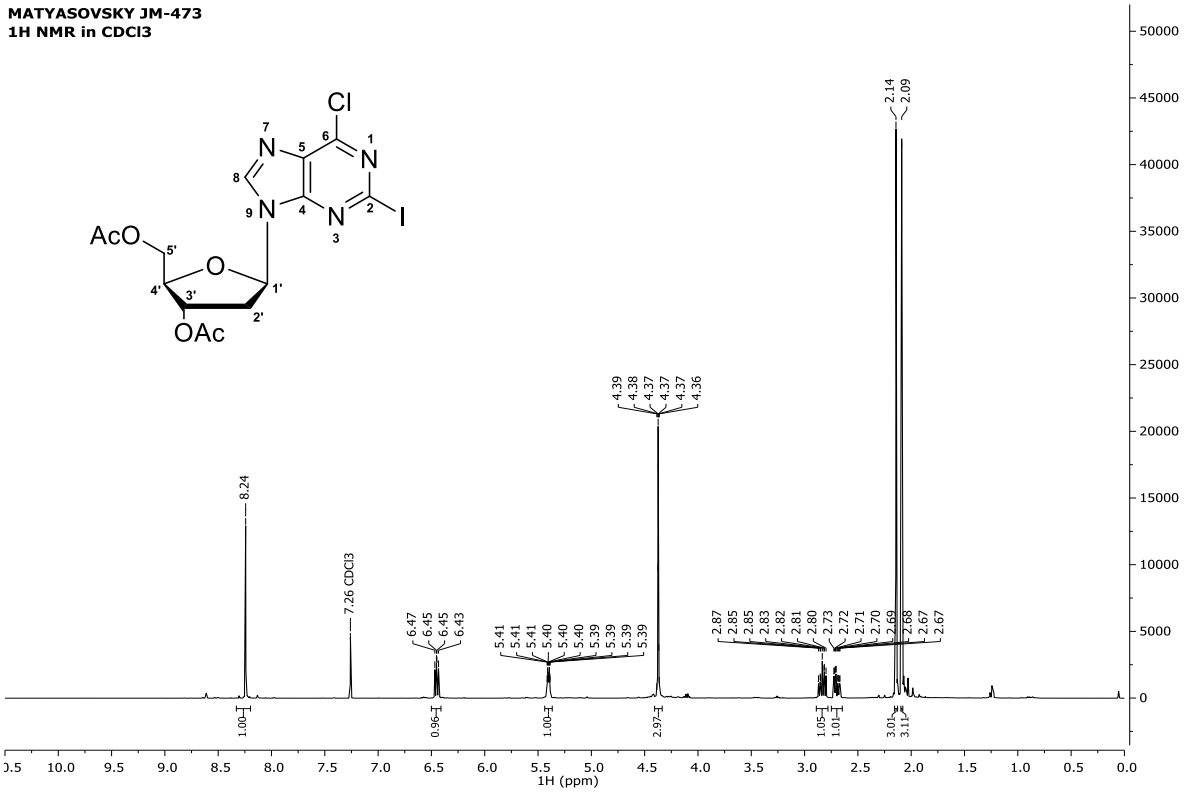
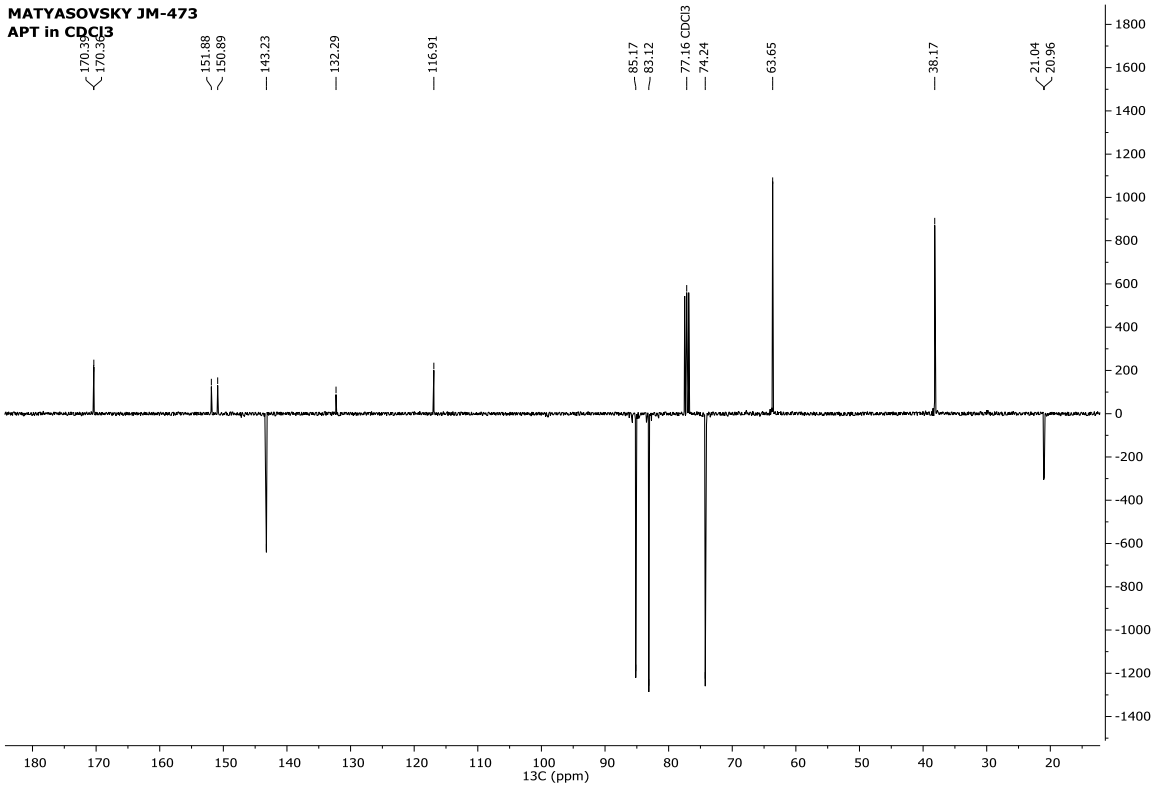
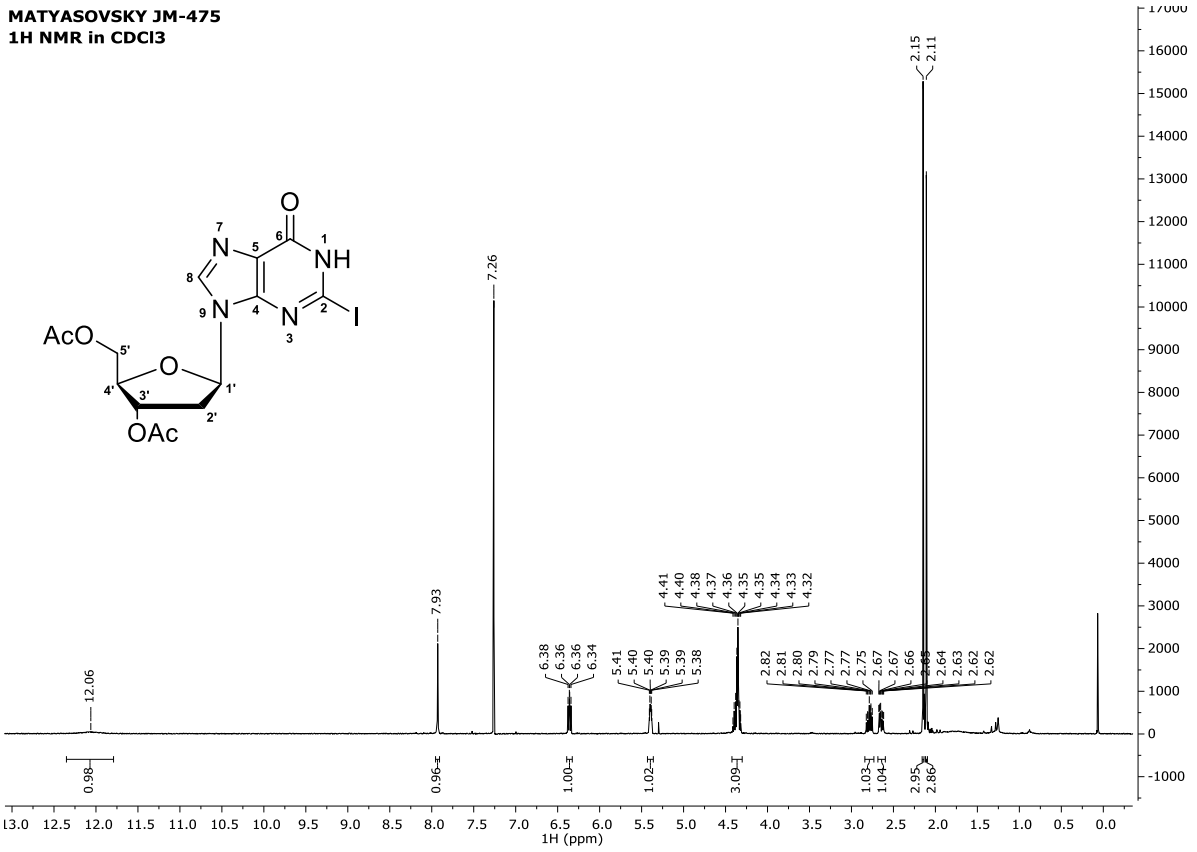


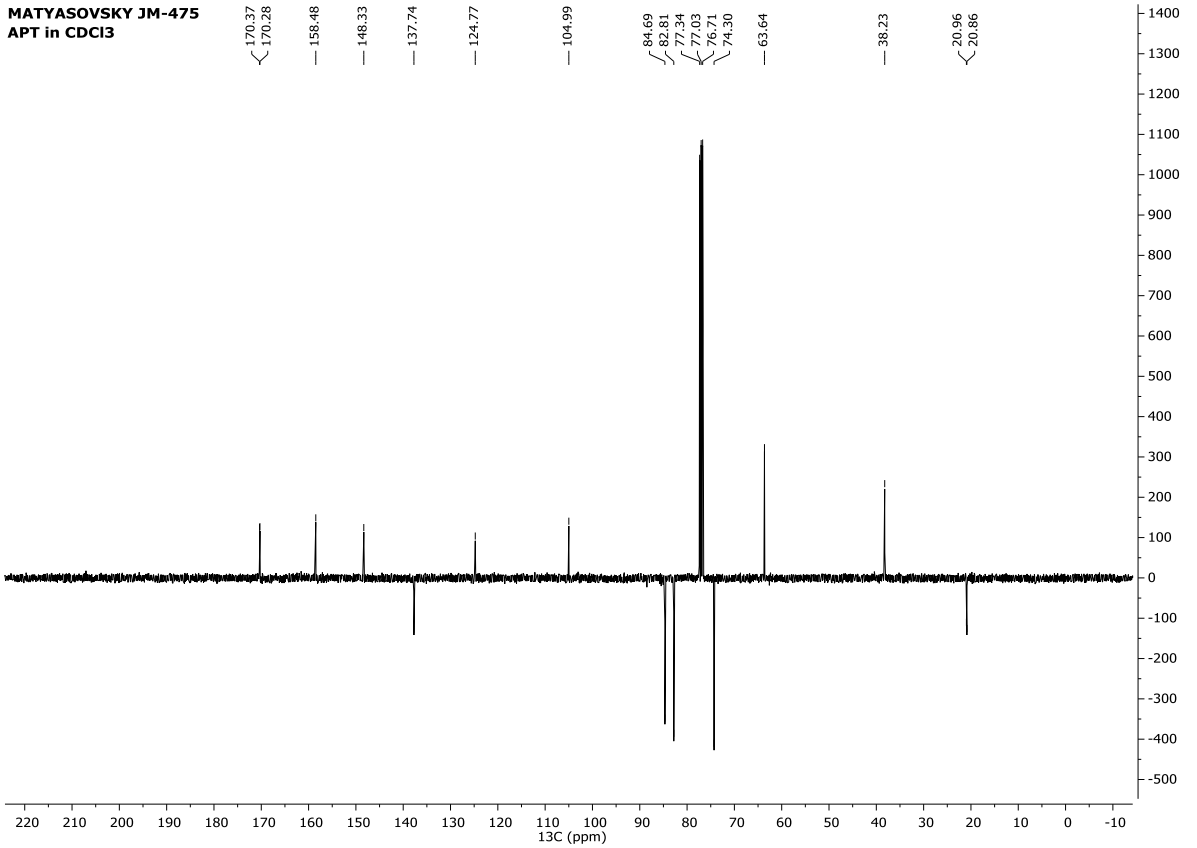
Figure S19: MALDI-TOF MS spectrum of **ON26_1^{pept_pos}H**, acquired by reaction of **DNA26_1^{V}H** with **peptide_pos**, calculated for $[M+H]^+$: 10060.5 Da; found: 10060.3, $[M-Ac+H]^+$ = 10018.5, found 10023.9.

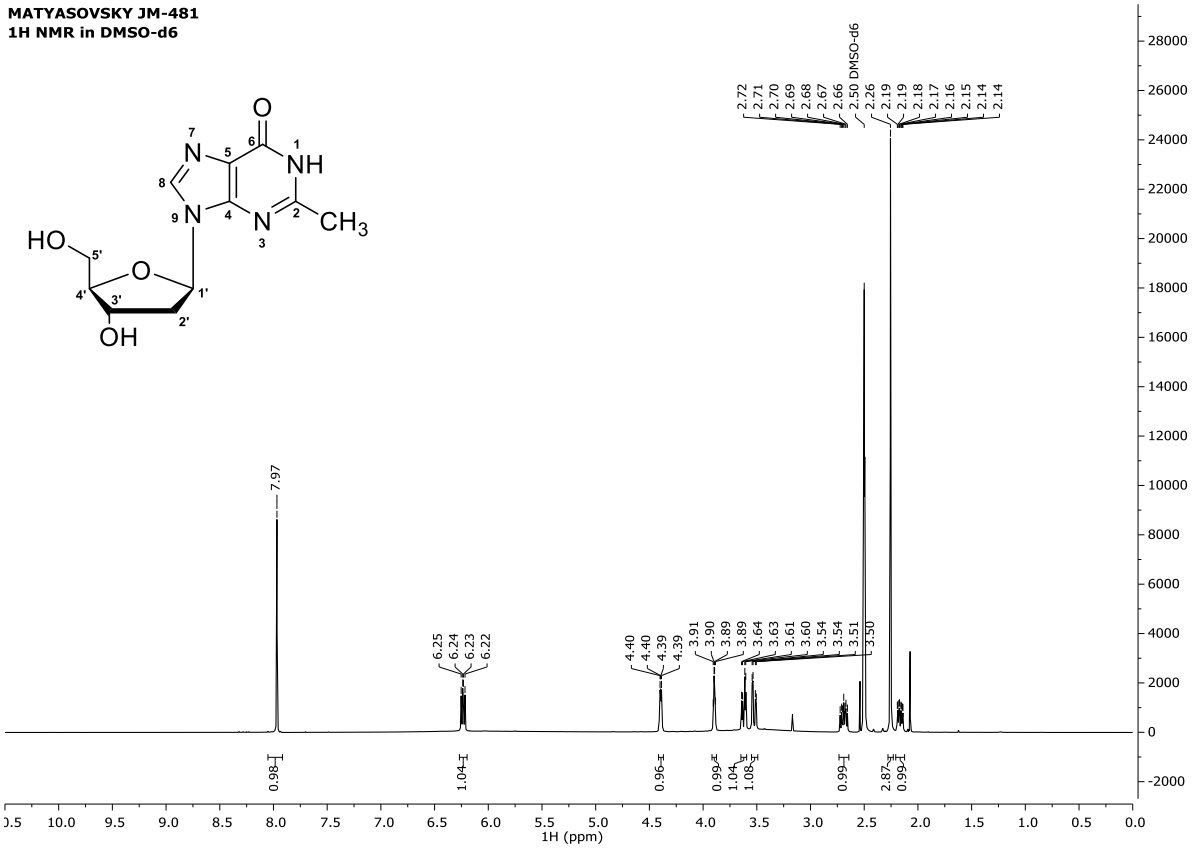
Copies of NMR spectra

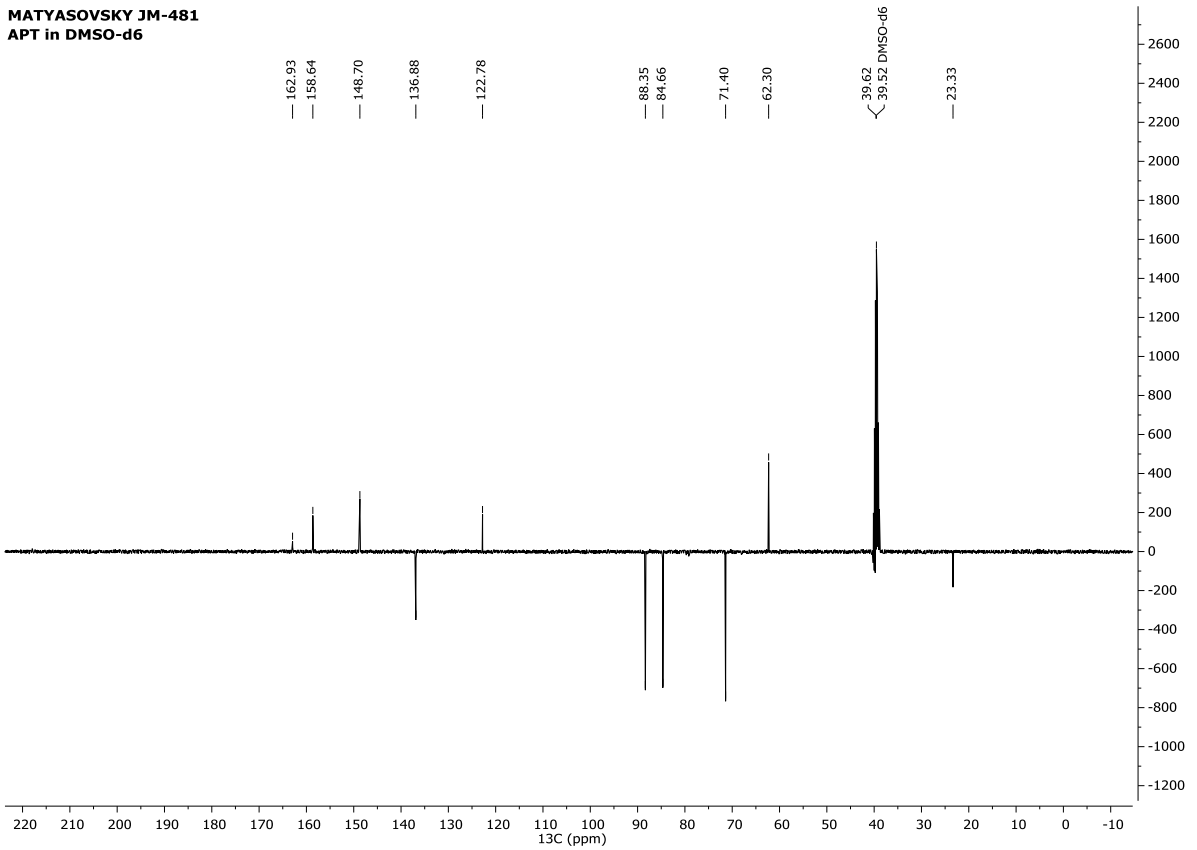


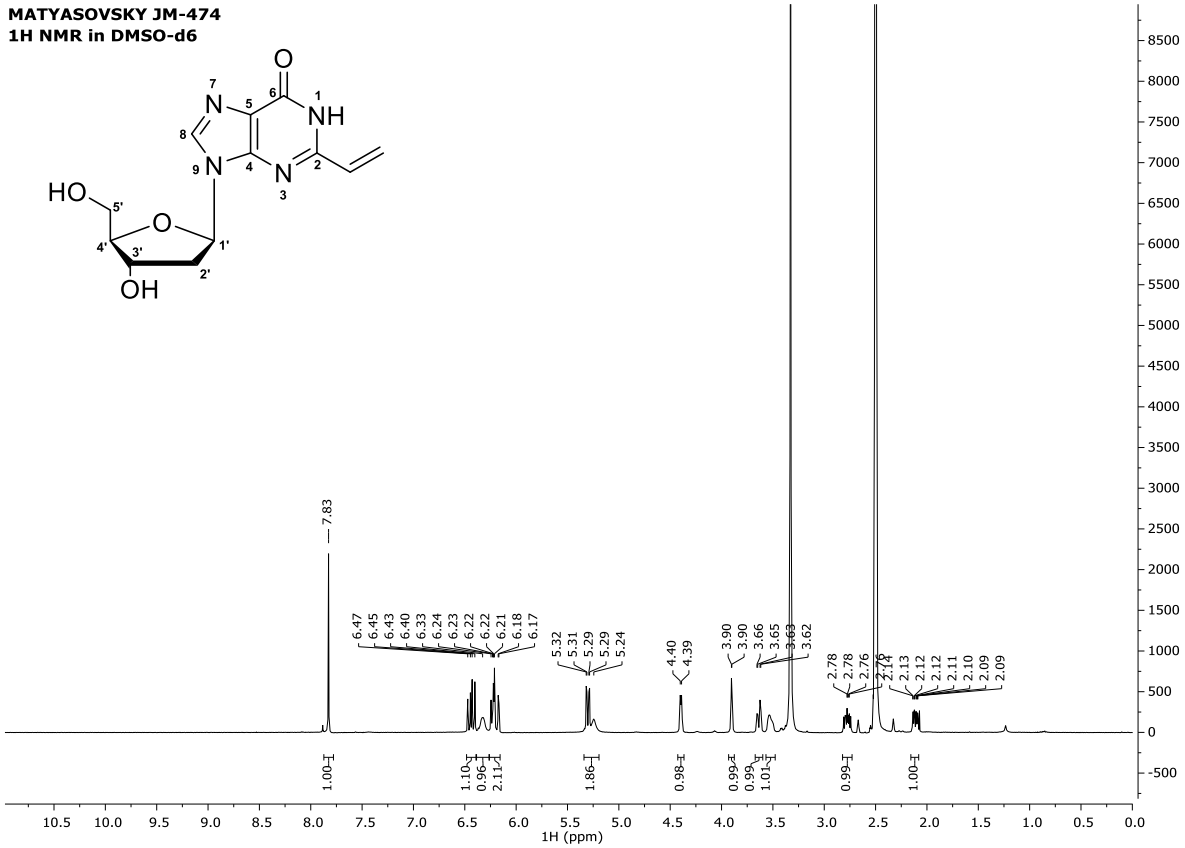


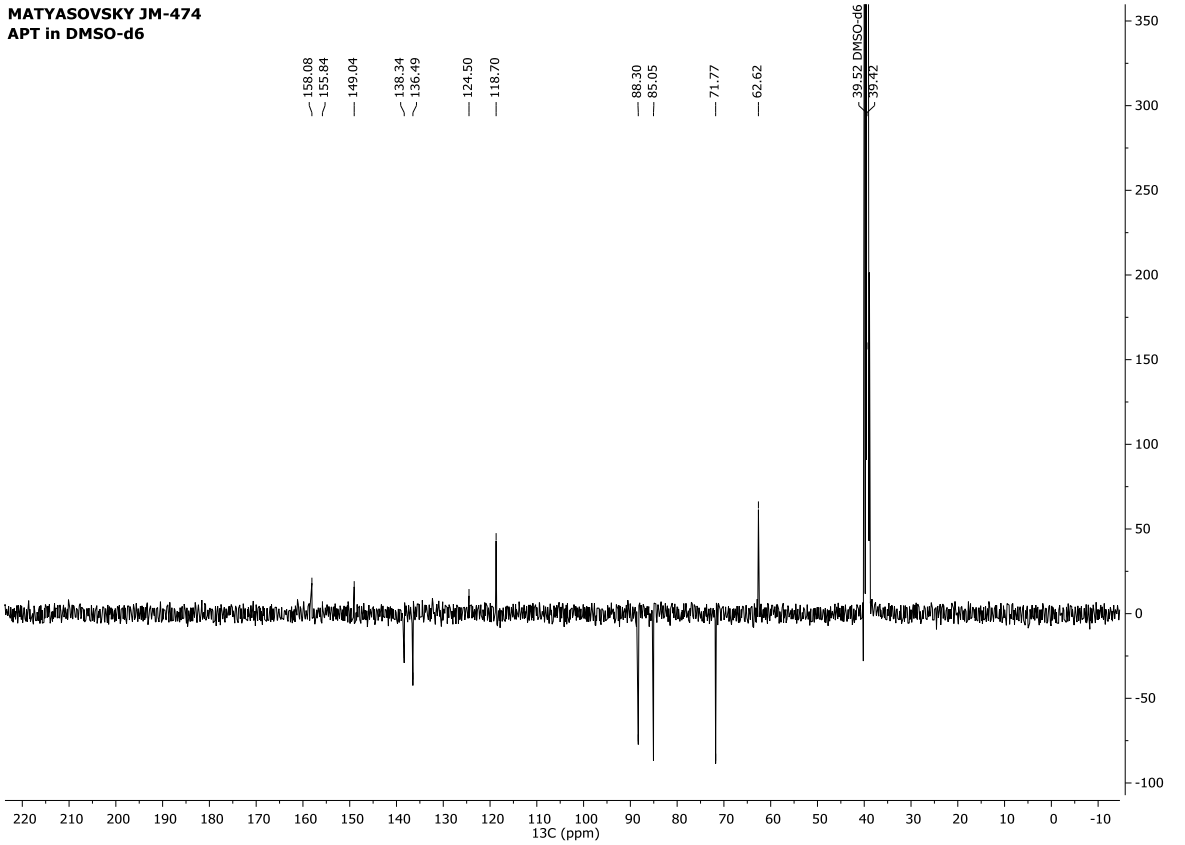

MATYASOVSKY JM-475
1H NMR in CDCl3
AcO
OAc
O
NH
N
I
12.06
7.93
7.26
6.38
6.36
6.36
6.34
5.41
5.40
5.40
5.39
5.39
5.38
4.41
4.40
4.38
4.37
4.36
4.35
4.35
4.34
4.33
4.32
2.82
2.81
2.80
2.79
2.77
2.77
2.75
2.67
2.67
2.66
2.64
2.63
2.62
2.62
2.15
2.11
0.98
0.96
1.00
1.02
3.09
1.03
1.04
2.95
2.86
1H (ppm)

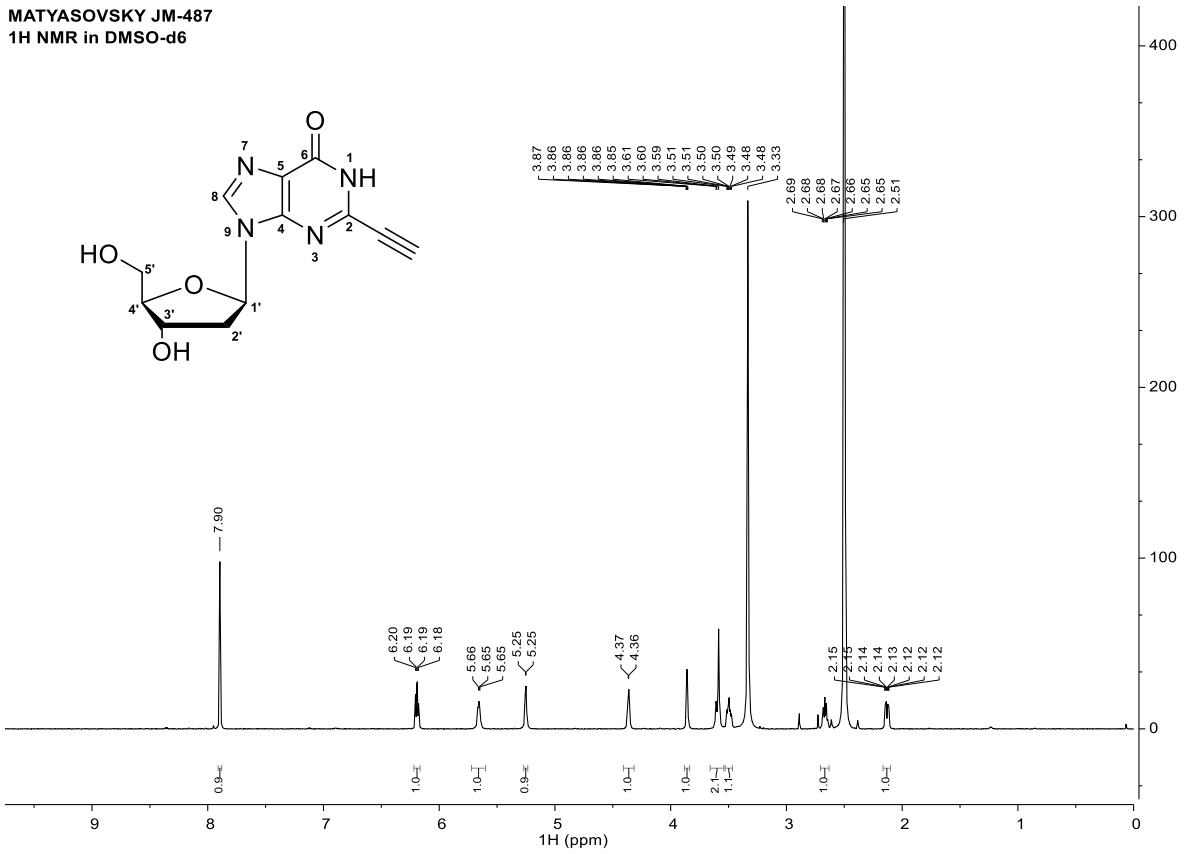

MATYASOVSKY JM-475
APT in CDCl3
170.37
170.28
158.48
148.33
137.74
124.77
104.99
84.69
82.81
77.34
77.03
76.71
74.30
63.64
38.23
20.96
20.86
13C (ppm)

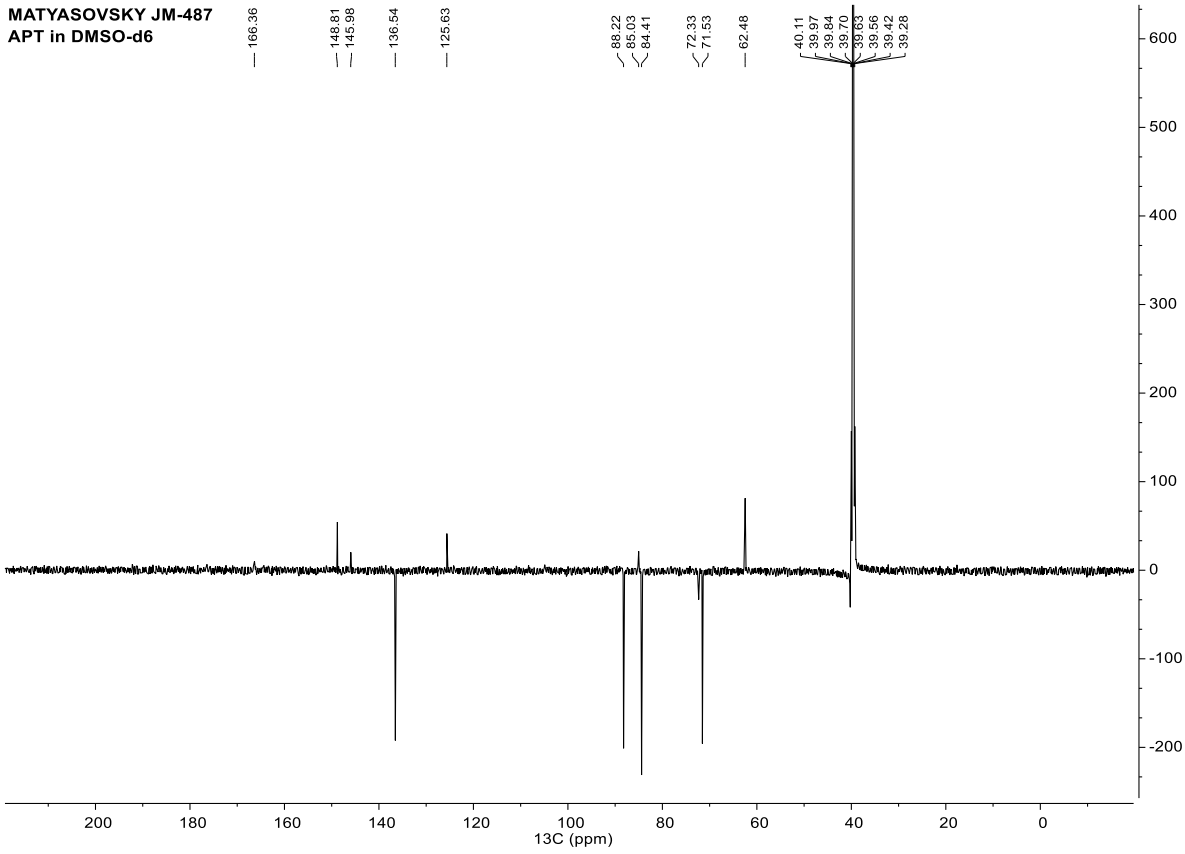

MATYASOVSKY JM-481
1H NMR in DMSO-d6
HO
O
NH
N
CH3
OH
2.50 DMSO-d6
7.97
6.25
6.24
6.23
6.22
4.40
4.40
4.39
4.39
3.91
3.90
3.89
3.89
3.64
3.63
3.61
3.60
3.54
3.54
3.51
3.50
2.72
2.71
2.70
2.69
2.68
2.67
2.66
2.26
2.19
2.19
2.18
2.17
2.16
2.15
2.14
2.14
0.98
1.04
0.96
0.99
1.04
1.08
0.99
2.87
0.99
1H (ppm)

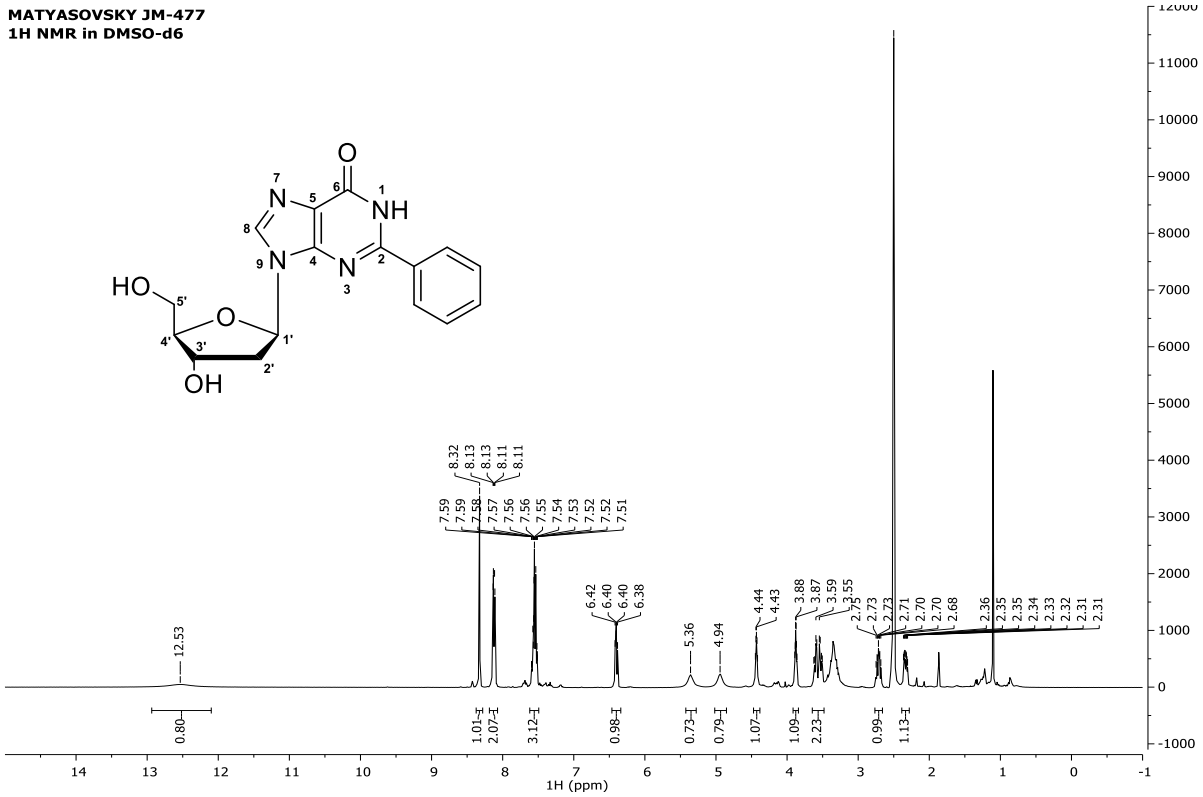

MATYASOVSKY JM-481
APT in DMSO-d6
162.93
158.64
148.70
136.88
122.78
88.35
84.66
71.40
62.30
39.62
39.52 DMSO-d6
23.33
13C (ppm)

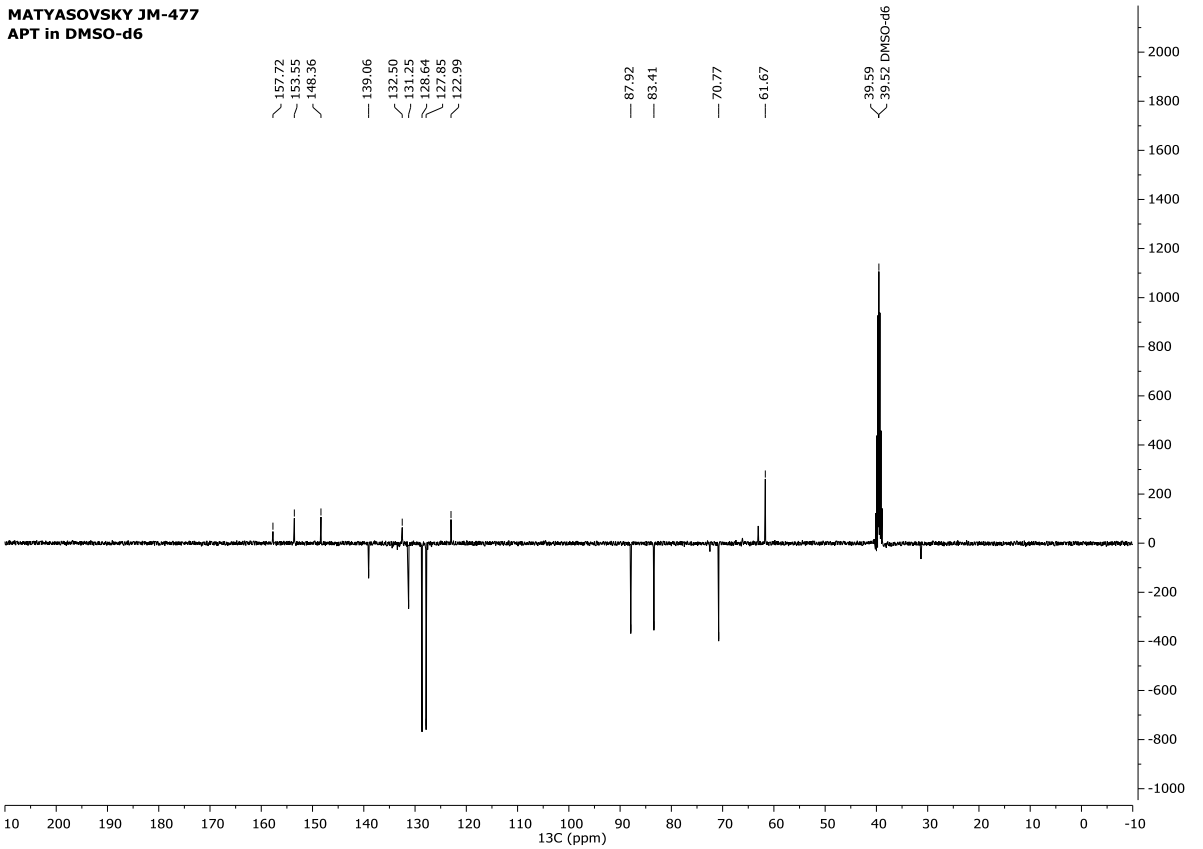

MATYASOVSKY JM-474
1H NMR in DMSO-d6
7.83
6.47
6.45
6.43
6.40
6.33
6.24
6.23
6.22
6.22
6.21
6.18
6.17
5.32
5.31
5.29
5.29
5.24
4.40
4.39
3.90
3.90
3.66
3.65
3.63
3.62
2.78
2.78
2.76
2.76
2.14
2.13
2.12
2.12
2.11
2.10
2.09
2.09
1.00
1.10
0.96
2.11
1.86
0.98
0.99
0.99
1.01
0.99
1.00
1H (ppm)

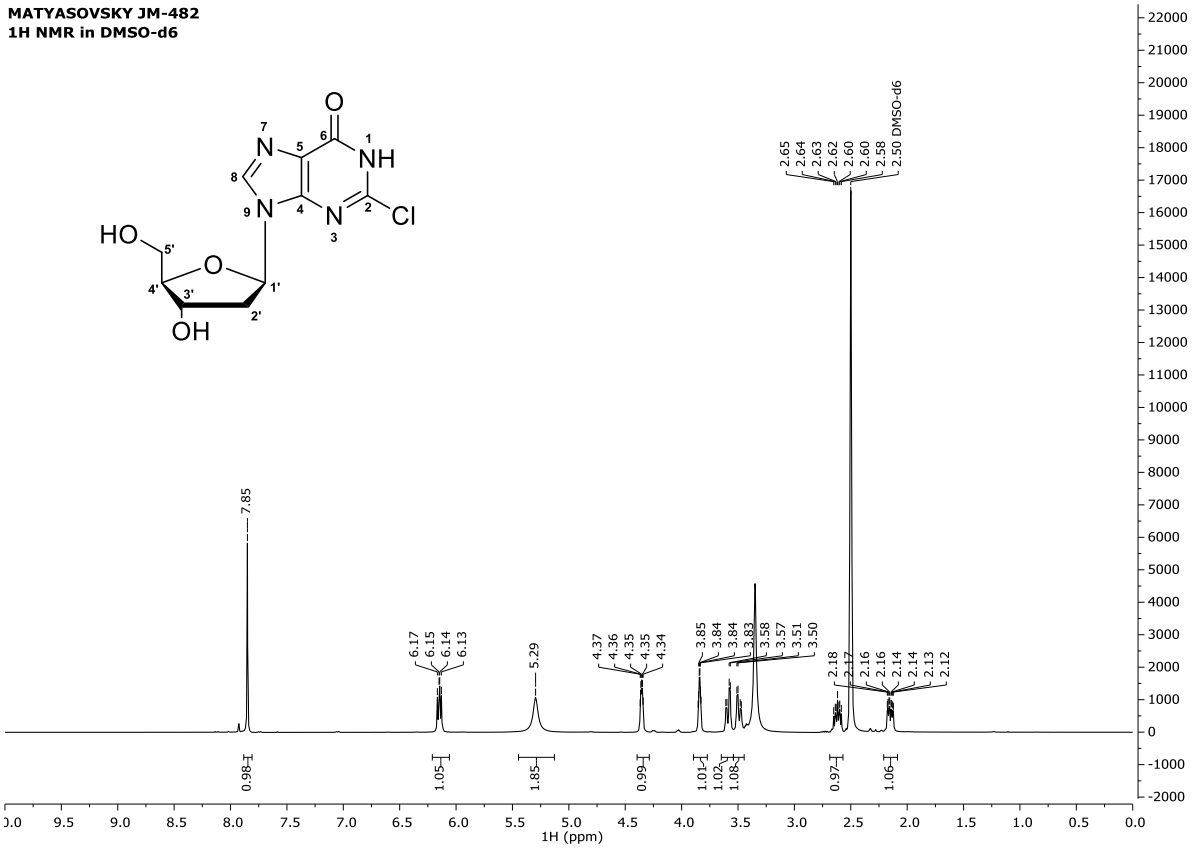

MATYASOVSKY JM-474
APT in DMSO-d6
158.08
155.84
149.04
138.34
136.49
124.50
118.70
88.30
85.05
71.77
62.62
39.52 DMSO-d6
39.42
13C (ppm)

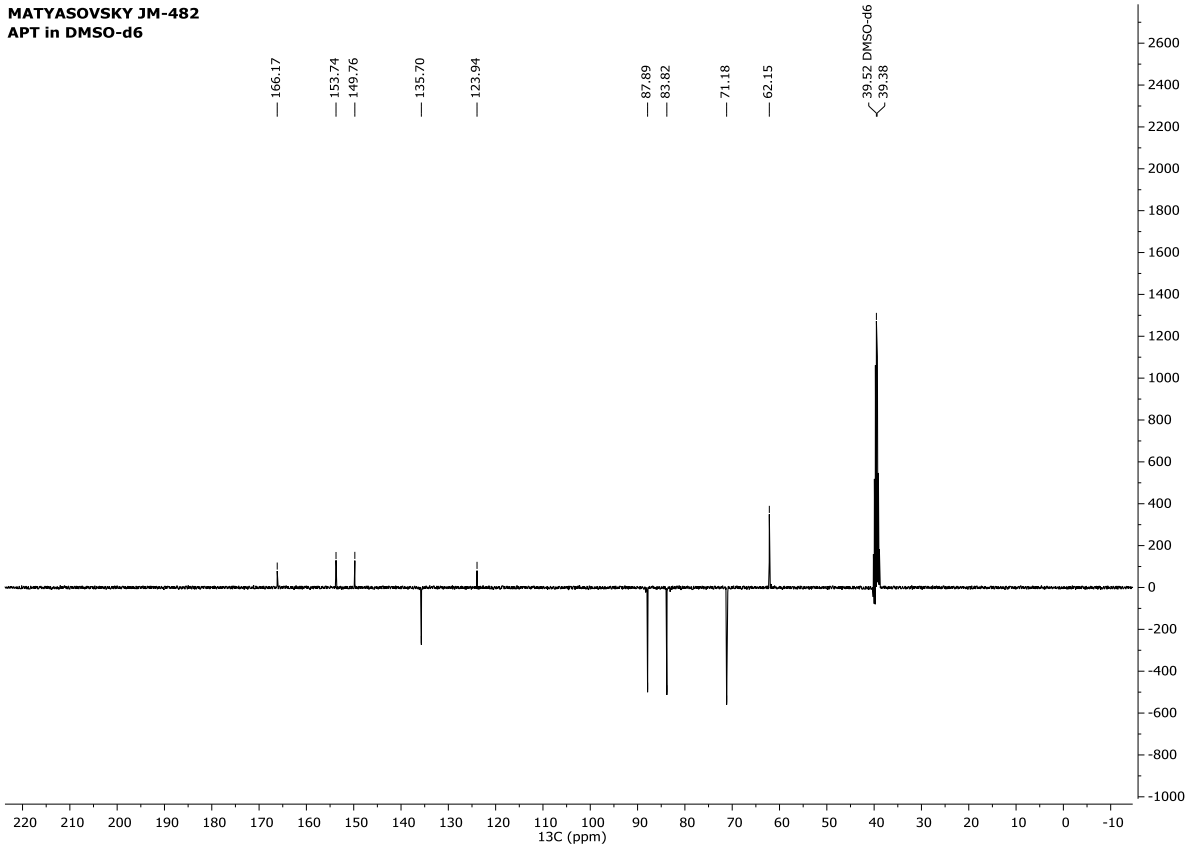

MATYASOVSKY JM-487
1H NMR in DMSO-d6
1H (ppm)

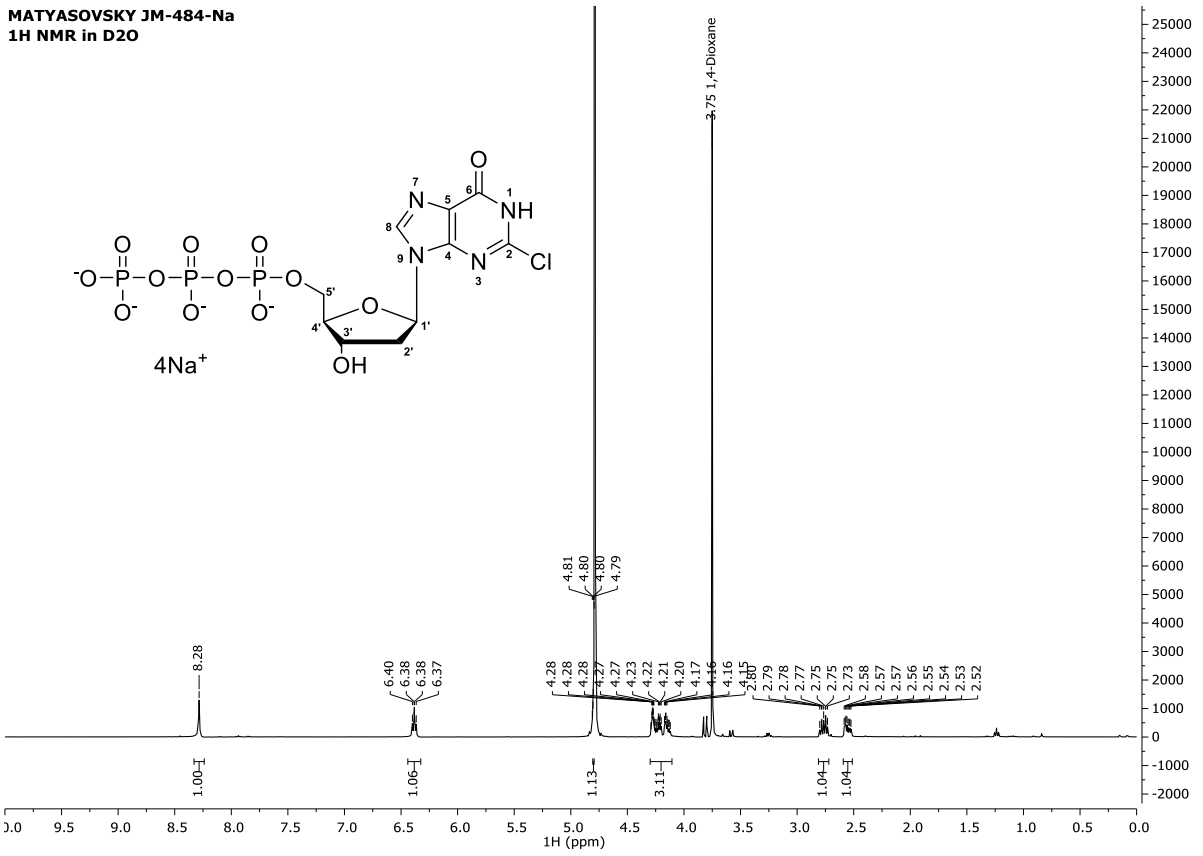

MATYASOVSKY JM-487
APT in DMSO-d6
13C (ppm)

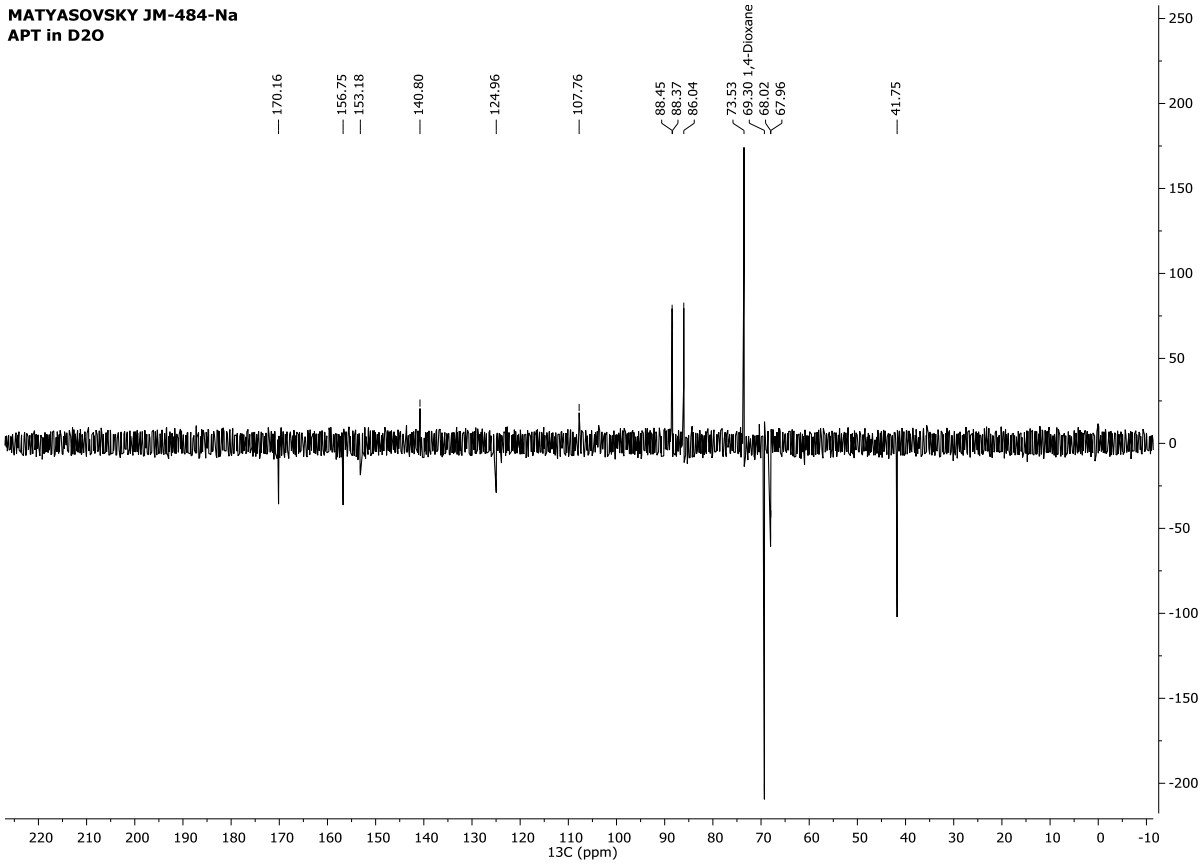

MATYASOVSKY JM-477
1H NMR in DMSO-d6
HO
OH
NH
8.32
8.13
8.13
8.11
8.11
7.59
7.59
7.58
7.57
7.56
7.56
7.55
7.54
7.53
7.52
7.52
7.51
6.42
6.40
6.40
6.38
12.53
5.36
4.94
4.44
4.43
3.88
3.87
3.59
3.55
2.75
2.73
2.73
2.71
2.70
2.70
2.68
2.36
2.35
2.35
2.34
2.33
2.32
2.31
2.31
0.80
1.01
2.07
3.12
0.98
0.73
0.79
1.07
1.09
2.23
0.99
1.13
1H (ppm)

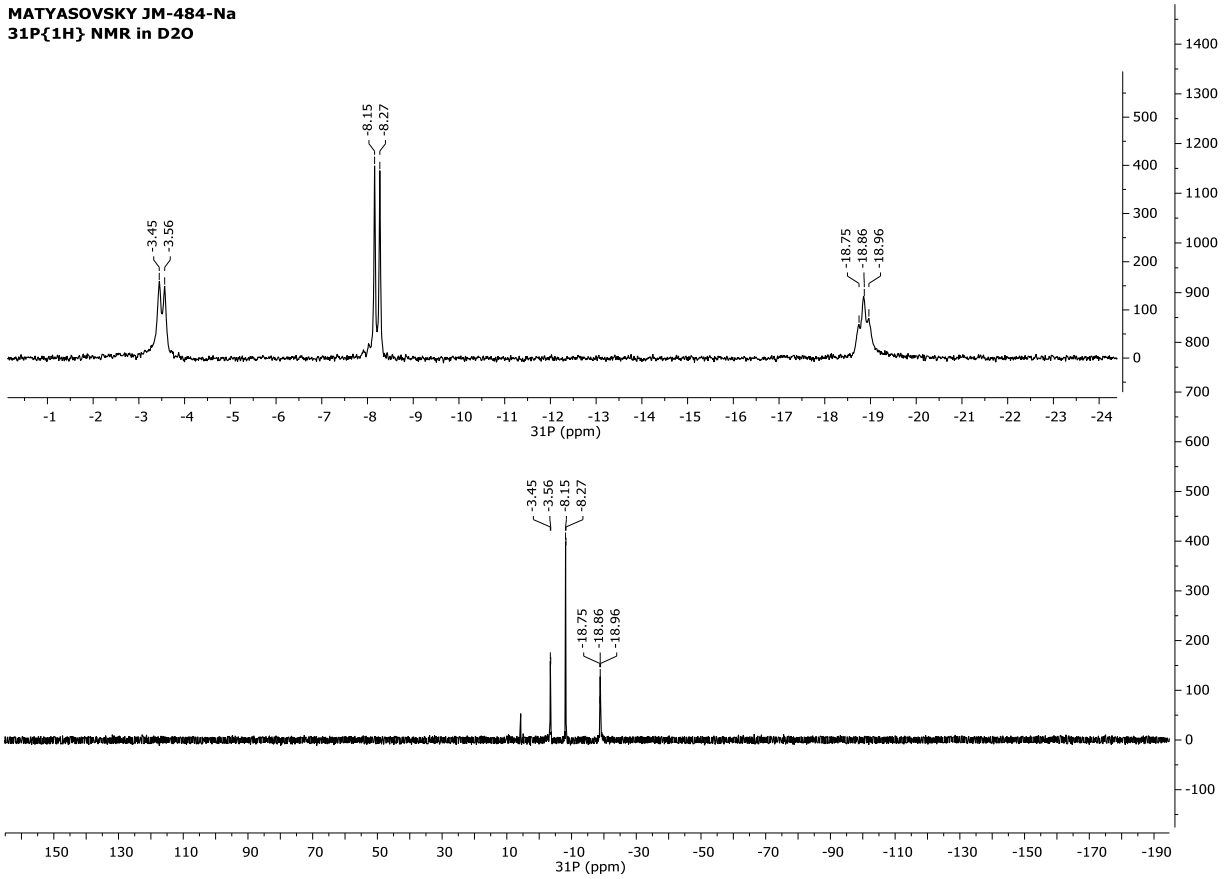

MATYASOVSKY JM-477
APT in DMSO-d6
157.72
153.55
148.36
139.06
132.50
131.25
128.64
127.85
122.99
87.92
83.41
70.77
61.67
39.59
39.52 DMSO-d6
13C (ppm)

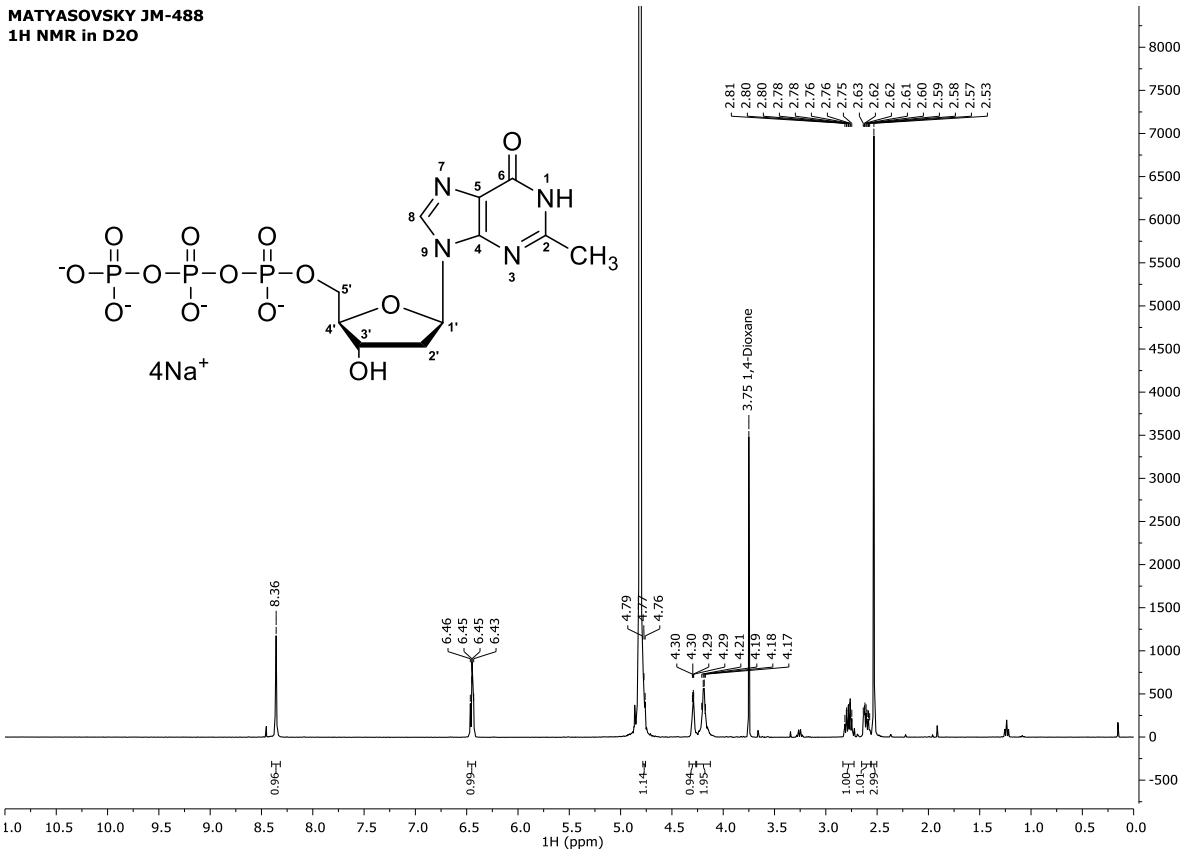

MATYASOVSKY JM-482
1H NMR in DMSO-d6
O
NH
Cl
HO
OH
7.85
6.17
6.15
6.14
6.13
5.29
4.37
4.36
4.35
4.35
4.34
3.85
3.84
3.84
3.83
3.58
3.57
3.51
3.50
2.65
2.64
2.63
2.62
2.60
2.60
2.58
2.50 DMSO-d6
2.18
2.17
2.16
2.16
2.14
2.14
2.13
2.12
0.98
1.05
1.85
0.99
1.01
1.02
1.08
0.97
1.06
1H (ppm)

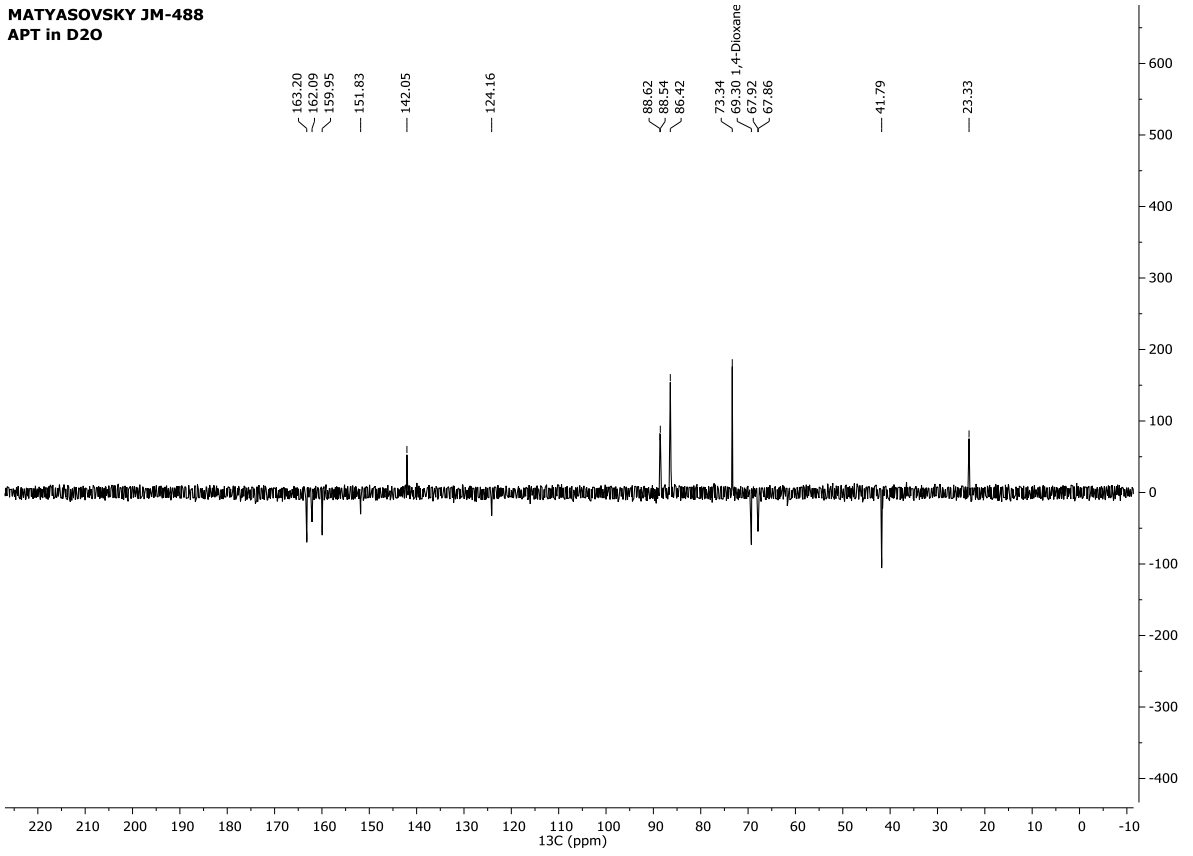

MATYASOVSKY JM-482
APT in DMSO-d6
166.17
153.74
149.76
135.70
123.94
87.89
83.82
71.18
62.15
39.52 DMSO-d6
39.38
13C (ppm)

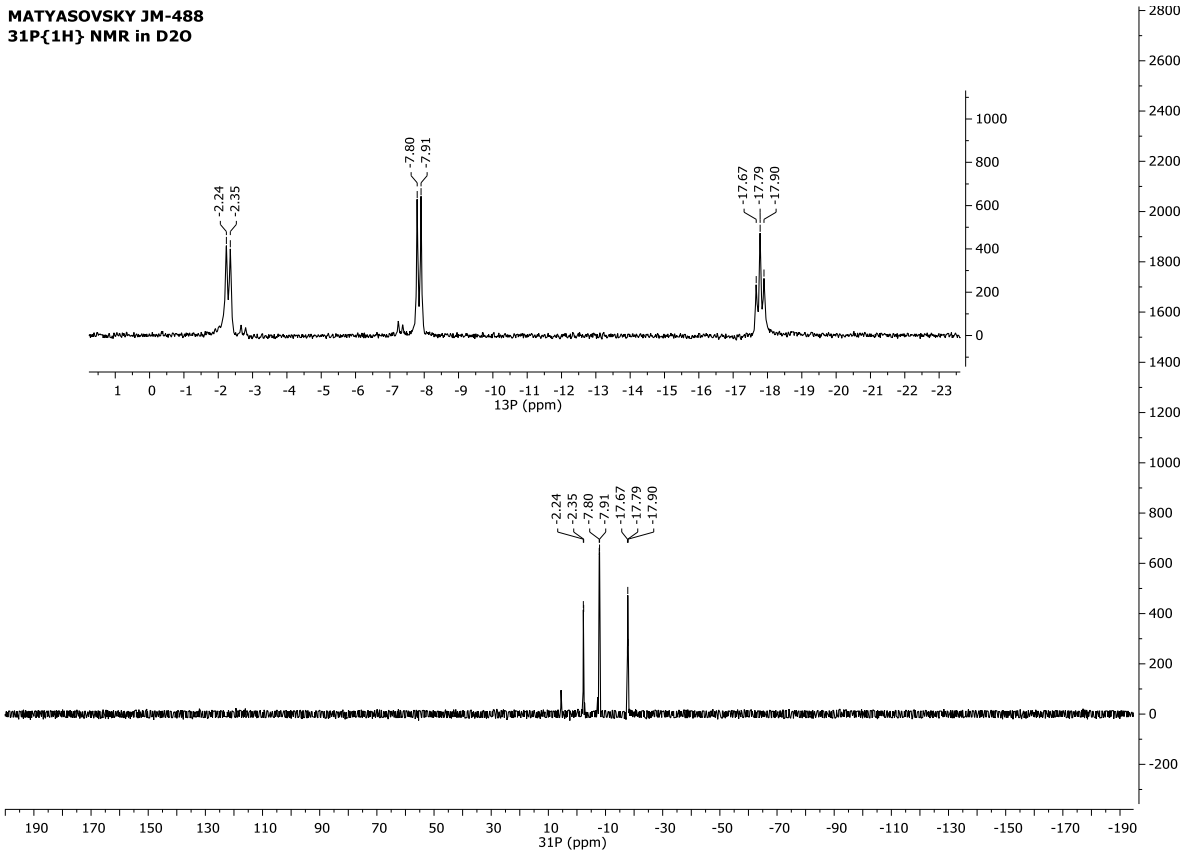

MATYASOVSKY JM-484-Na
1H NMR in D2O
3.75 1,4-Dioxane
8.28
6.40
6.38
6.38
6.37
4.81
4.80
4.80
4.79
1.00
1.06
1.13
3.11
1.04
1.04
1H (ppm)

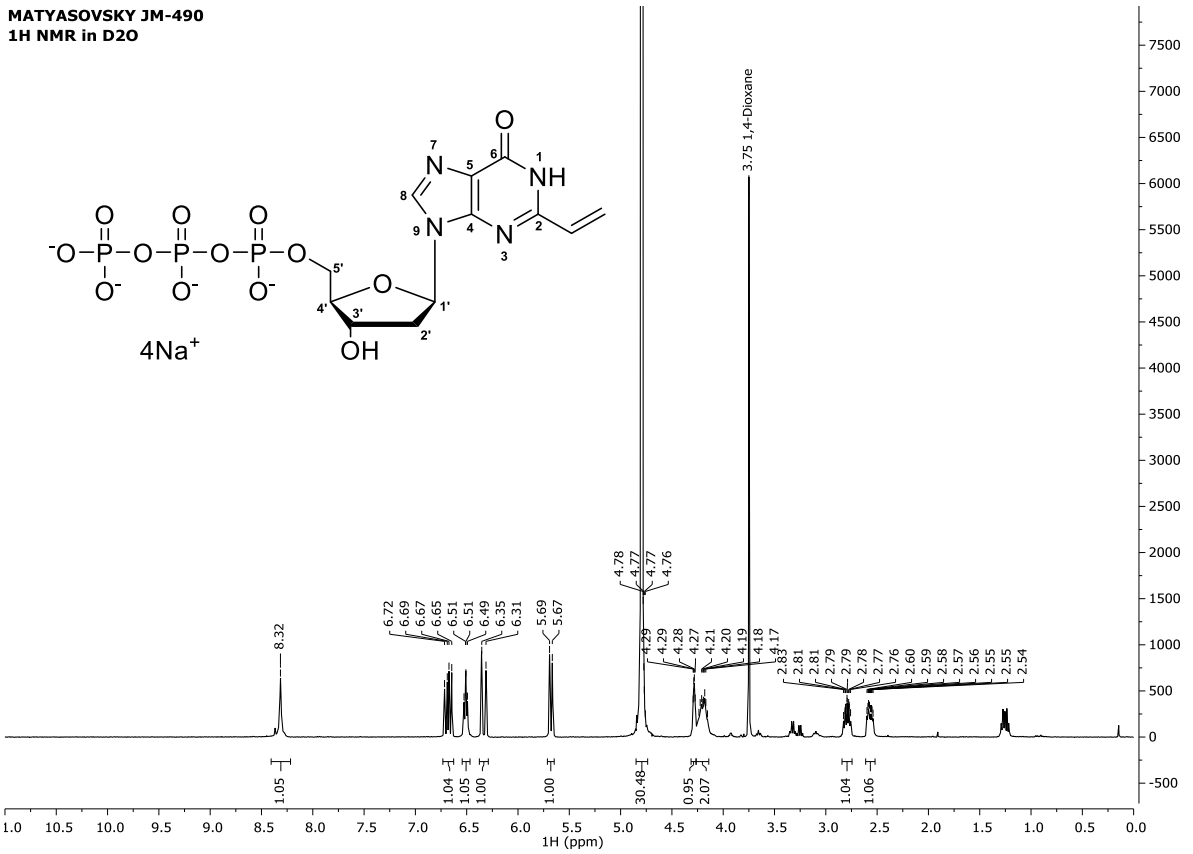

MATYASOVSKY JM-484-Na
APT in D2O
170.16
156.75
153.18
140.80
124.96
107.76
88.45
88.37
86.04
73.53
69.30 1,4-Dioxane
68.02
67.96
41.75
13C (ppm)

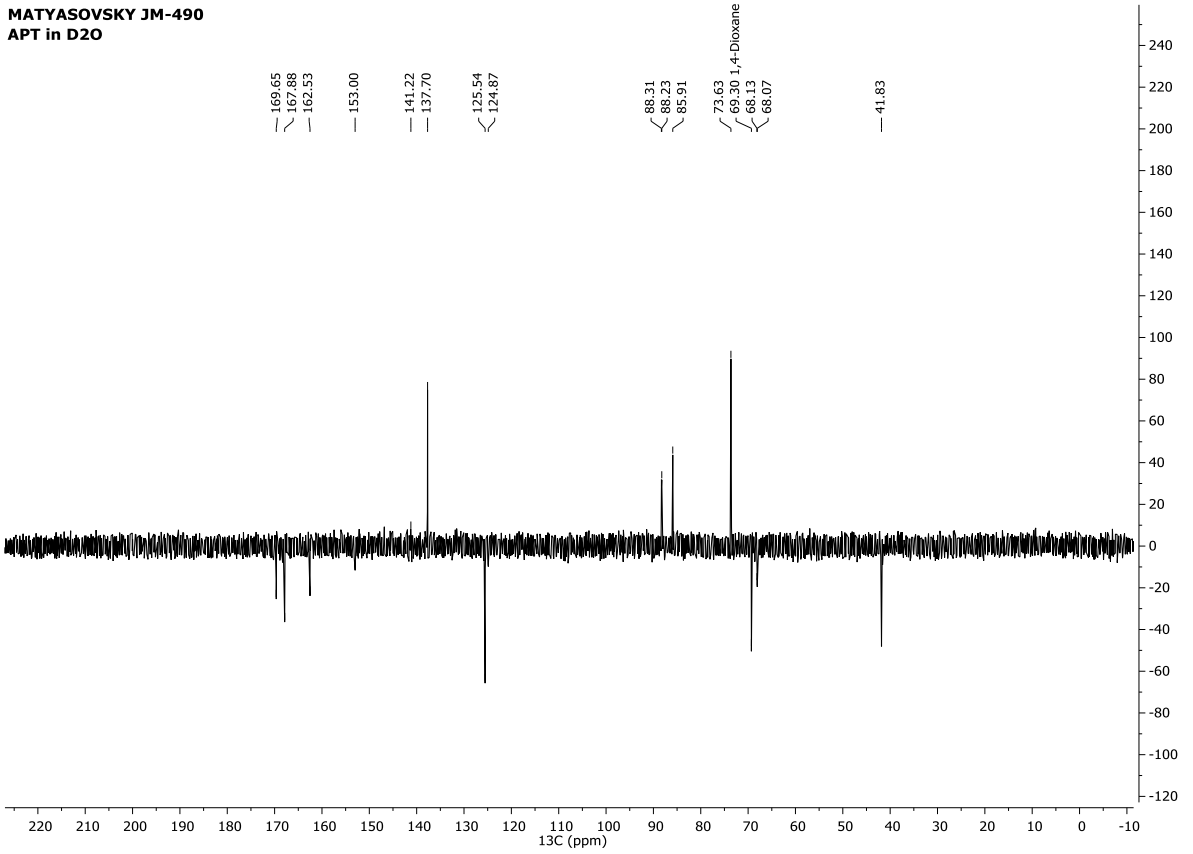

MATYASOVSKY JM-484-Na
31P{1H} NMR in D2O
-3.45
-3.56
-8.15
-8.27
-18.75
-18.86
-18.96
31P (ppm)
-3.45
-3.56
-8.15
-8.27
-18.75
-18.86
-18.96
31P (ppm)

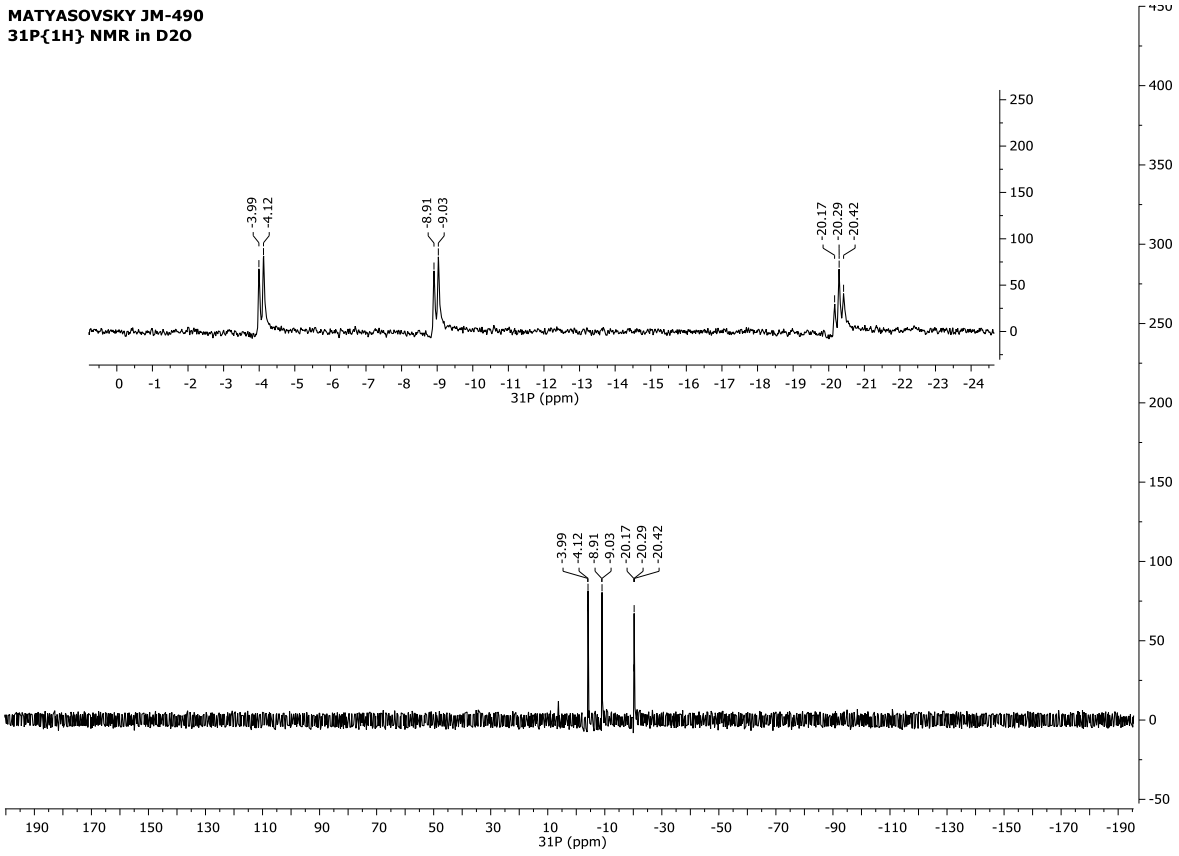

MATYASOVSKY JM-488
1H NMR in D2O
4Na+
3.75 1,4-Dioxane
1H (ppm)

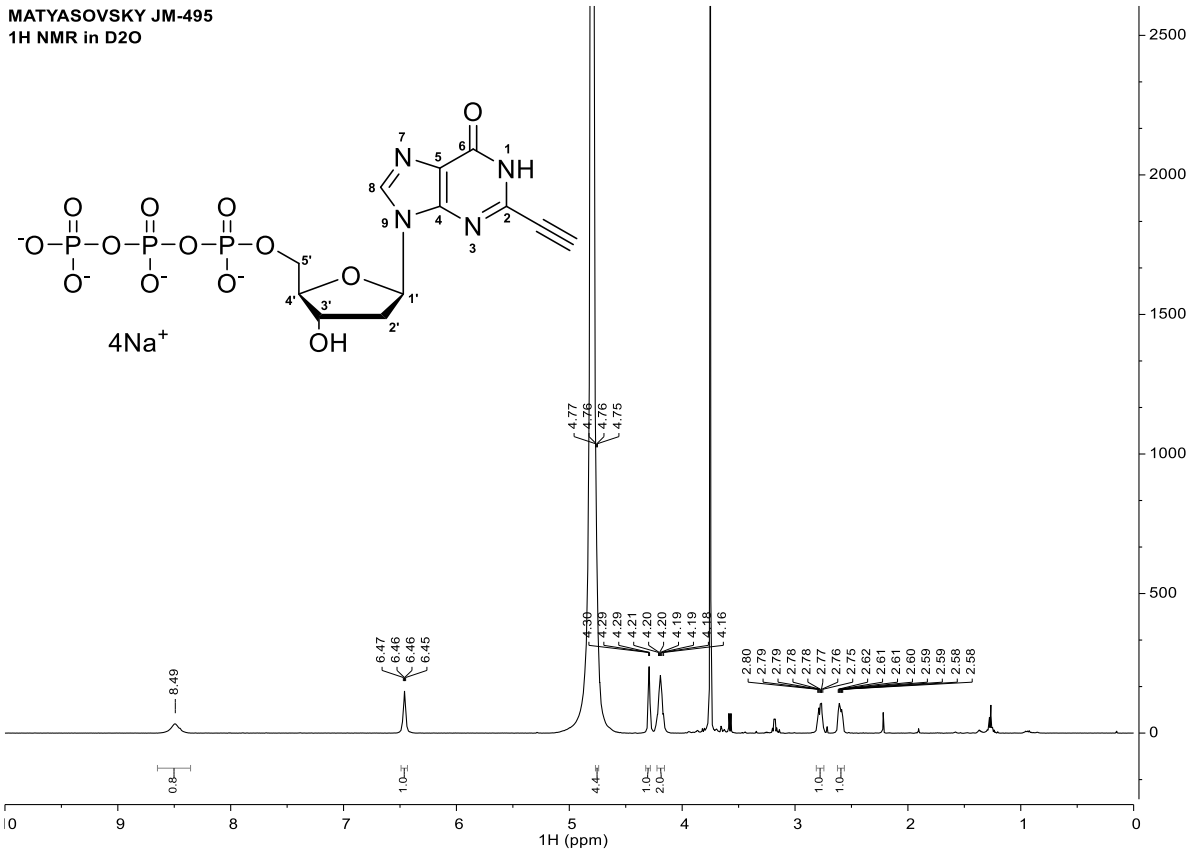

MATYASOVSKY JM-488
APT in D2O
3.75 1,4-Dioxane
13C (ppm)

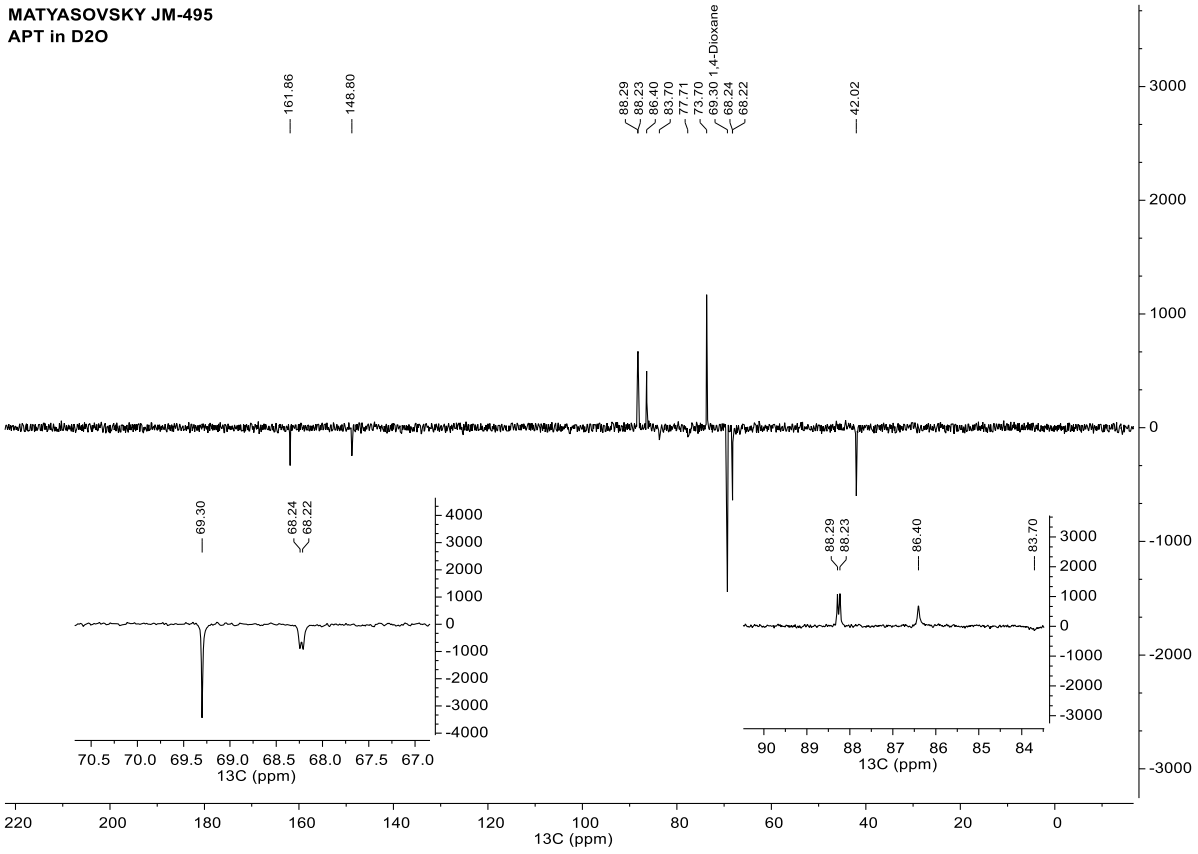

MATYASOVSKY JM-488
31P{1H} NMR in D2O
-2.24
-2.35
-7.80
-7.91
-17.67
-17.79
-17.90
13P (ppm)
31P (ppm)

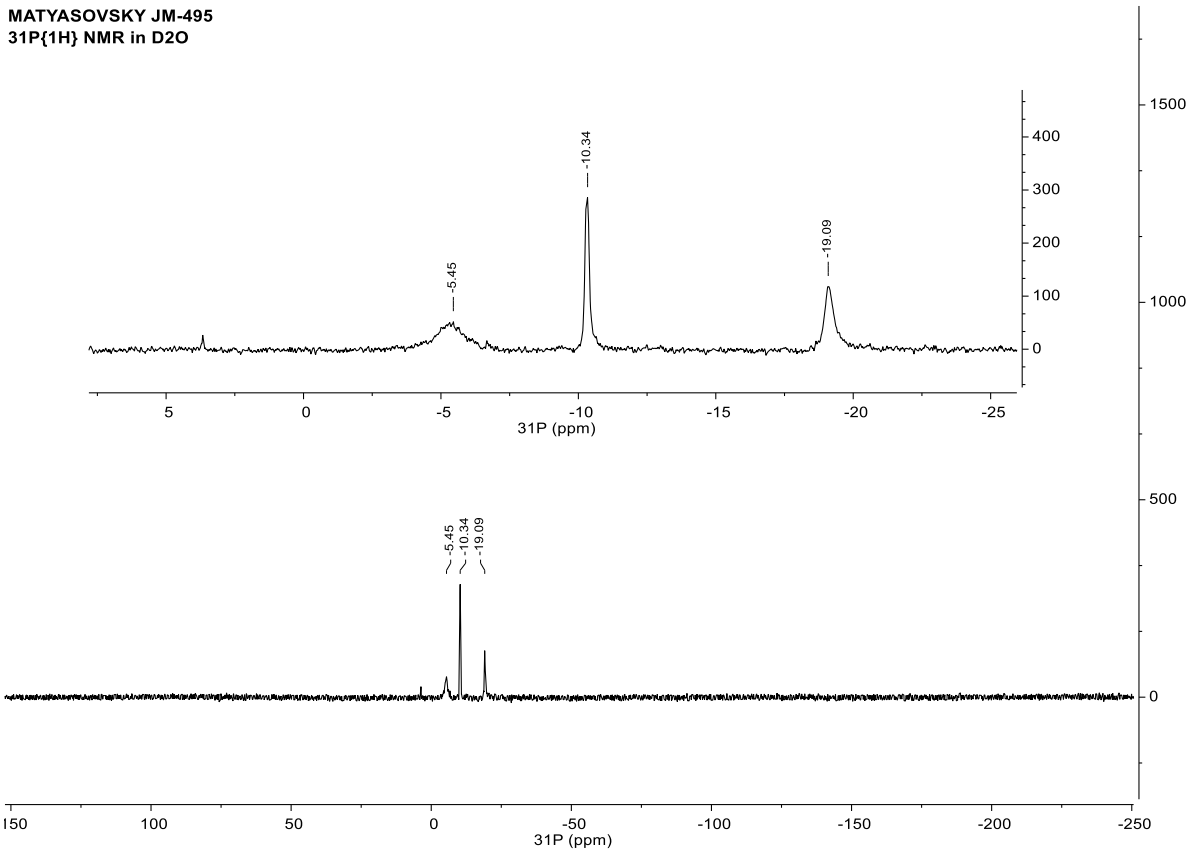

MATYASOVSKY JM-490
1H NMR in D2O
4Na+
OH
3.75 1,4-Dioxane
8.32
6.72
6.69
6.67
6.65
6.51
6.51
6.49
6.35
6.31
5.69
5.67
4.78
4.77
4.77
4.76
4.29
4.29
4.28
4.27
4.21
4.20
4.19
4.18
4.17
2.83
2.81
2.81
2.79
2.79
2.78
2.77
2.76
2.60
2.59
2.58
2.57
2.56
2.55
2.55
2.54
1.05
1.04
1.05
1.00
1.00
30.48
0.95
2.07
1.04
1.06
1H (ppm)

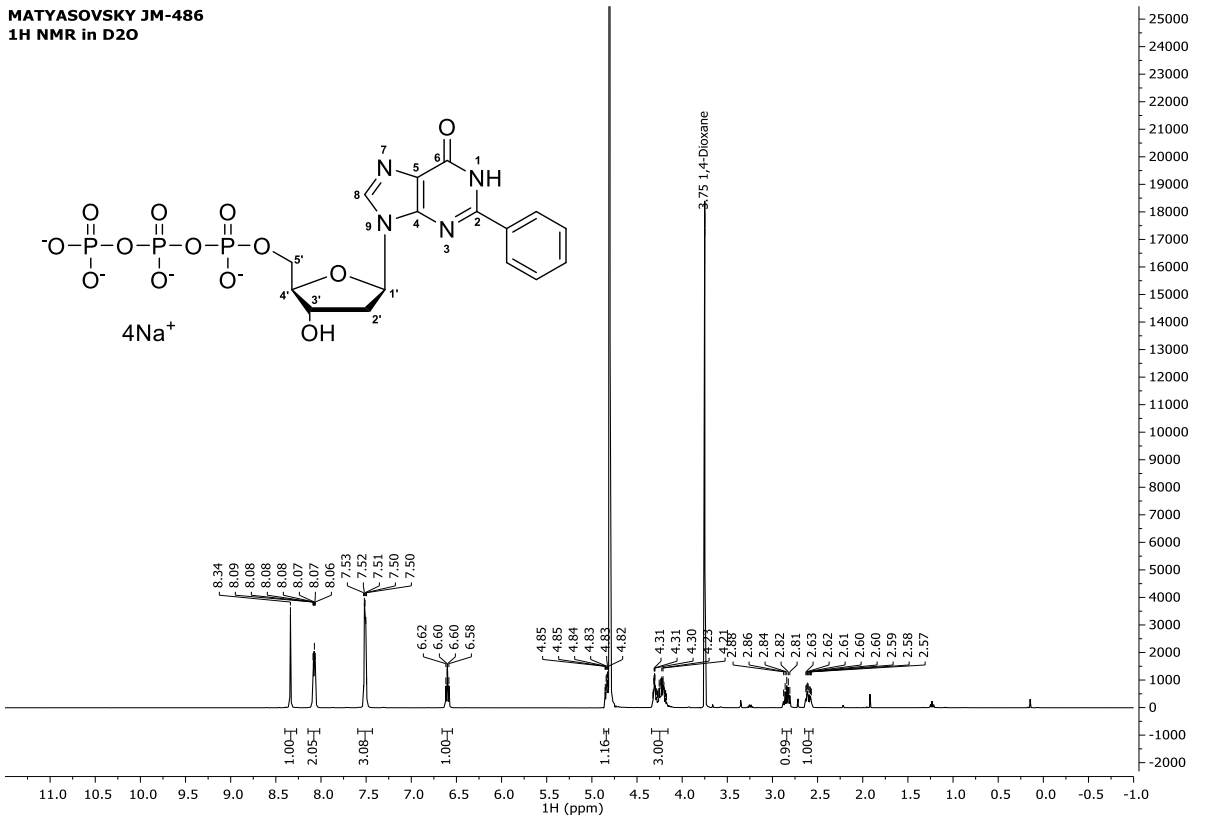

MATYASOVSKY JM-490
APT in D2O
169.65
167.88
162.53
153.00
141.22
137.70
125.54
124.87
88.31
88.23
85.91
73.63
69.30 1,4-Dioxane
68.13
68.07
41.83
13C (ppm)

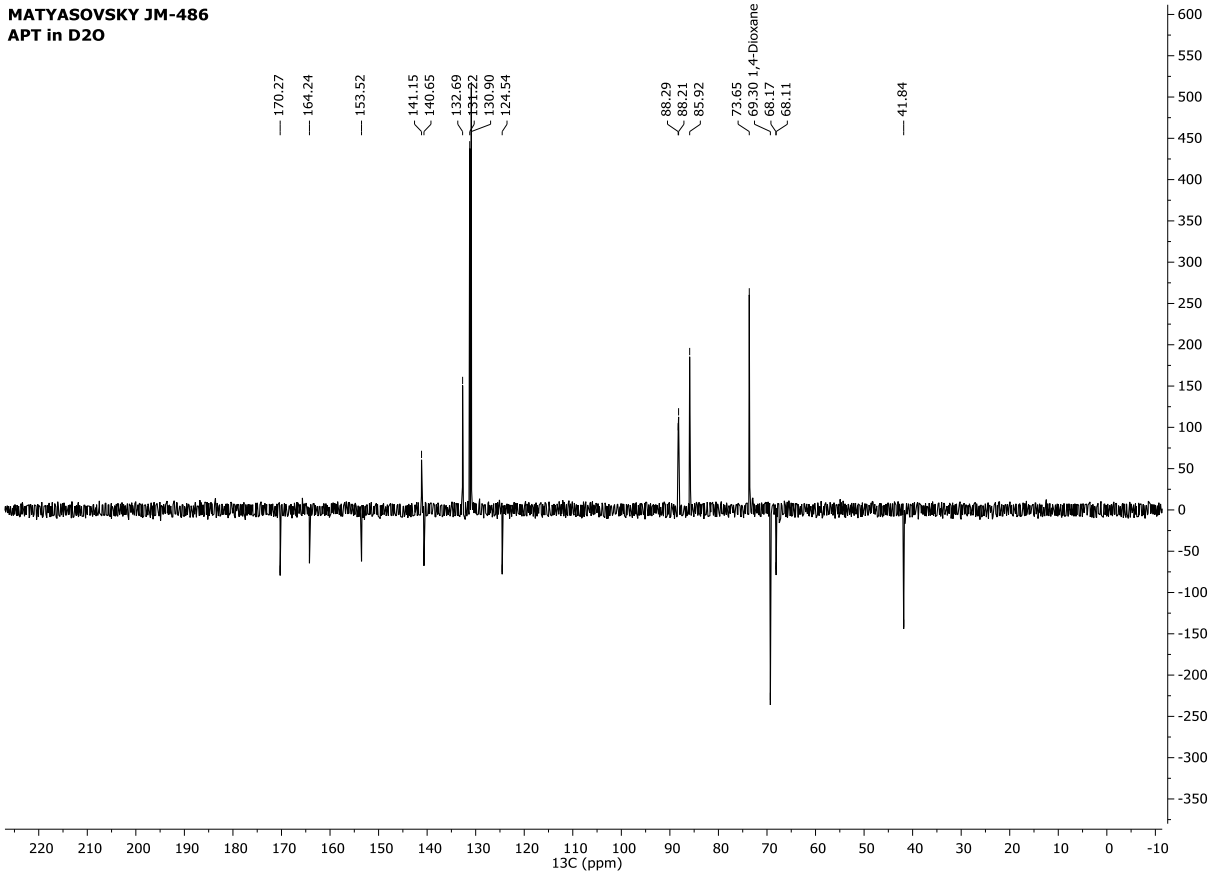

MATYASOVSKY JM-490
31P{1H} NMR in D2O
-3.99
-4.12
-8.91
-9.03
-20.17
-20.29
-20.42
31P (ppm)
-3.99
-4.12
-8.91
-9.03
-20.17
-20.29
-20.42
31P (ppm)

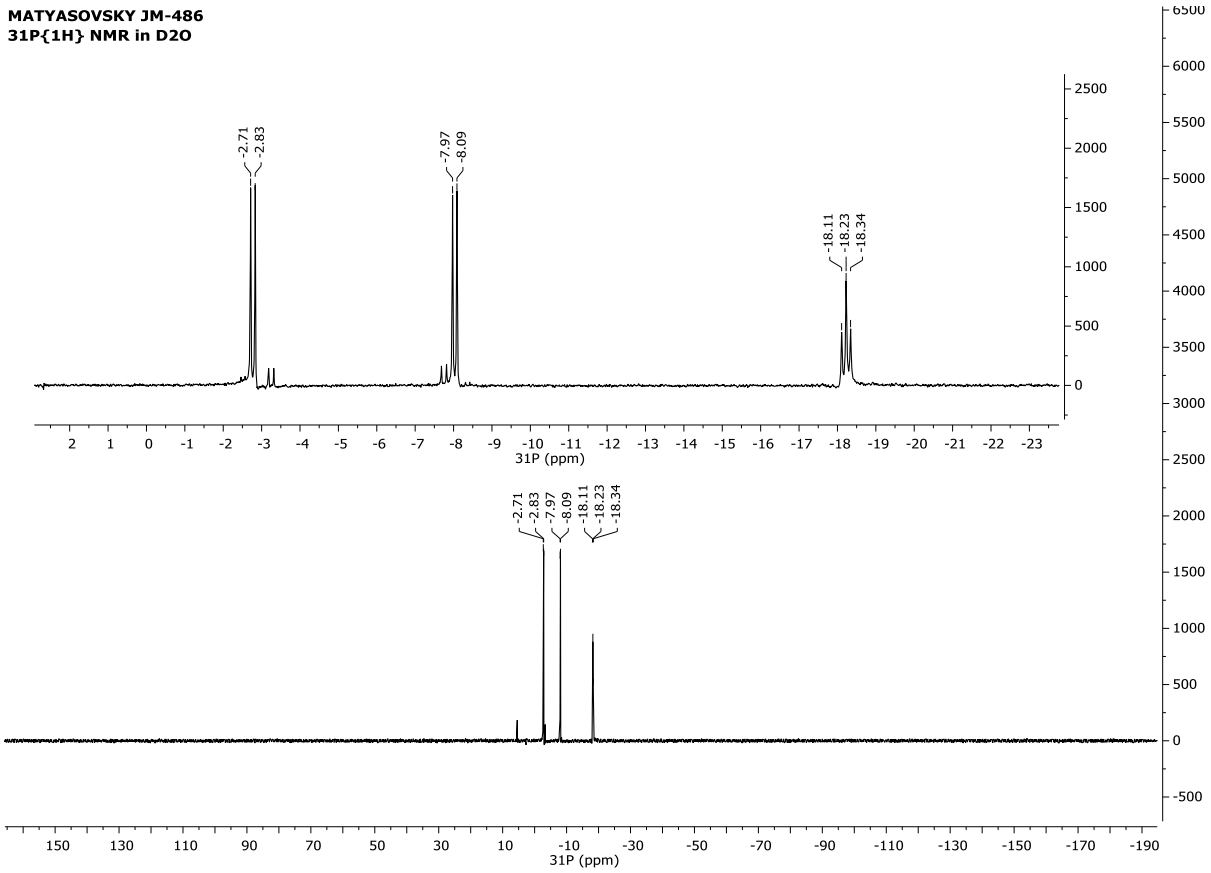

MATYASOVSKY JM-495
1H NMR in D2O
4Na+
8.49
6.47
6.46
6.46
6.45
4.77
4.78
4.76
4.75
4.30
4.29
4.29
4.21
4.20
4.20
4.19
4.19
4.18
4.16
2.80
2.79
2.79
2.78
2.78
2.77
2.76
2.75
2.62
2.61
2.61
2.60
2.59
2.59
2.58
2.58
0.8
1.0
4.4
1.0
2.0
1.0
1.0
1H (ppm)


MATYASOVSKY JM-495
APT in D2O
161.86
148.80
88.29
88.23
86.40
83.70
77.71
73.70
69.30
1,4-Dioxane
68.24
68.22
42.02
69.30
68.24
68.22
88.29
88.23
86.40
83.70
13C (ppm)
13C (ppm)


MATYASOVSKY JM-495
31P{1H} NMR in D2O
-5.45
-10.34
-19.09
31P (ppm)
-5.45
-10.34
-19.09
31P (ppm)


MATYASOVSKY JM-486
1H NMR in D2O
4Na+
OH
3.75 1,4-Dioxane
1H (ppm)


MATYASOVSKY JM-486
APT in D2O
170.27
164.24
153.52
141.15
140.65
132.69
131.22
130.90
124.54
88.29
88.21
85.92
73.65
69.30 1,4-Dioxane
68.17
68.11
41.84
13C (ppm)


MATYASOVSKY JM-486
31P{1H} NMR in D2O
-2.71
-2.83
-7.97
-8.09
-18.11
-18.23
-18.34
31P (ppm)
31P (ppm)