Electronic Supplementary Material (ESI) for ChemComm.

Polymerase synthesis of DNA labelled with benzylidene cyanoacetamide-based fluorescent molecular rotors: fluorescent light-up probes for DNA-binding proteins

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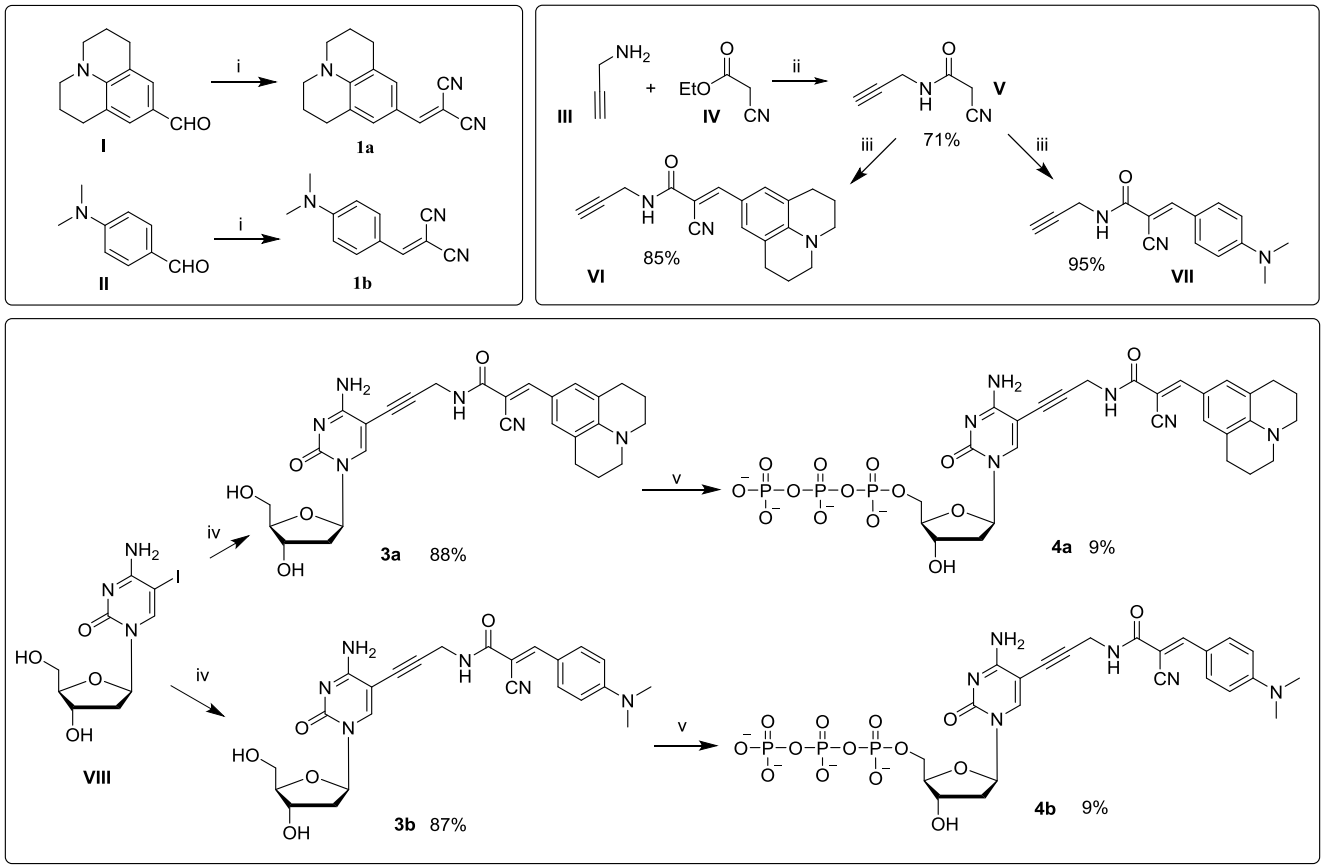
Contents

A. Additional figures and tables



Scheme S1. Chemical synthesis of the fluorescent molecular rotors. Reagents and conditions: i) malononitrile, piperidine, MeOH [ref. S1] ii) 1: 40°C, 7h; 2: 70°C, overnight. iii) DIMCARB, **I** or **II**, $CHCl_3$, r.t. 30–60 min. iv) **VI** or **VII**, $PdCl_2(PPh_3)_2$, CuI, NEt_3, DMF, 80°C, 1h. v) 1: $POCl_3$, $PO(OCH_3)_3$, 0°C, 2–4 h; 2: $(n\text{-}Bu_3NH)_2H_2P_2O_7$, NBu_3, DMF, 0°C, 1 h; 3: 1M TEAB, 5 min; 4: HPLC; 5: Dowex 50WX8 (Na^+ cycle).

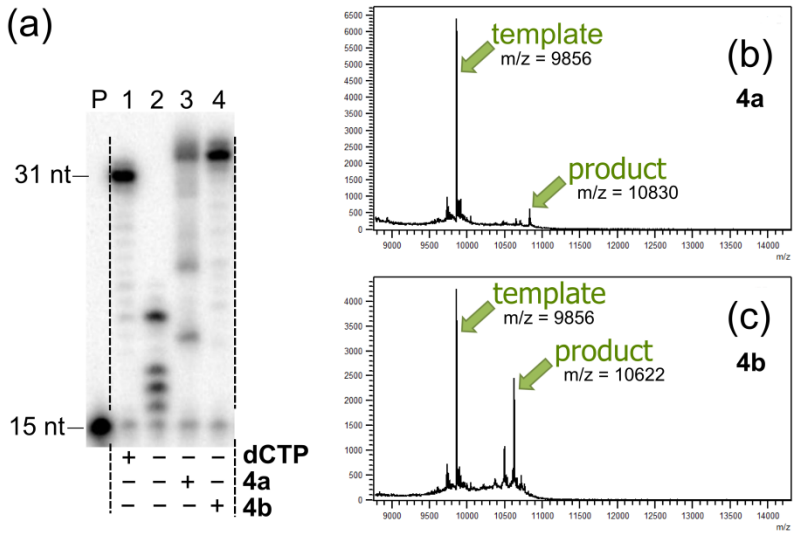


Figure S1. (a) PEX with modified dNTPs **dC^{VJ}TP** (**4a**) and **dC^{VDP}TP** (**4b**) performed by KOD XL DNA polymerase; primer **P3X** and template **T3X** were used, assuming incorporation of four modified **dC^{X}** residues (see Table S1 for sequences); P: primer; positive control: four natural dNTPs (lane 1); negative control: dATP, dGTP, dTTP (lane 2); modified dNTP **4a**: dATP, dGTP, dTTP and **4a** (lane 3); modified dNTP **4b**: dATP, dGTP, dTTP and **4b** (lane 4).
(b–c) MALDI analysis of DNA obtained by semi-preparative PEX with **4a** and **4b** followed by magnetic separation; primer **P3X** and biotinylated template **T3Xb** were used (Table S1); products incorporating **dC^{VJ}** and **dC^{VDP}** moieties are shown in figures (b) and (c), respectively; peak of the btn-template partially released from the beads during denaturation step was also observed.

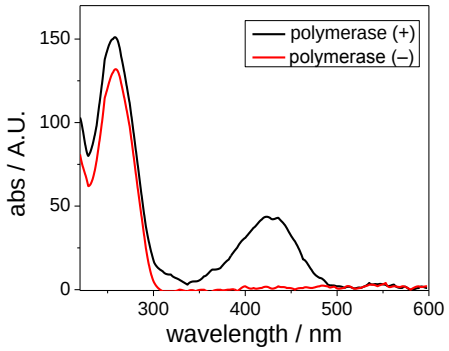


Figure S2. UV-vis spectra of purified PEX products obtained after incubation of a reaction mixture containing **dC^{VDP}TP**, dATP, dTTP, dGTP, primer **P3X** and template **T3X** either with (black line) or without (red line) KOD XL DNA polymerase at 60°C for 30 min. The mixtures were purified using QIAquick Nucleotide Removal Kit (QIAGEN). The absence of long-wavelength absorption in the spectrum of the sample incubated without polymerase indicated that **dC^{VDP}TP** does not bind unspecifically to DNA.

Table S1. Oligodeoxynucleotides used for enzymatic reactions

Name	Length (nt)	Sequence (5'→3')[a]	Used in
Primers			
P1X	25	GAA TTC GAT ATC AAG AGA CAT GCC T	PEX
P2X	15	TCA AGA GAC ATG CCT	PEX
P3X	15	CAT GGG CGG CAT GGG	PEX
P1N	19	CCG ATC TAG TGA GTC CTC G	NEAR
P1R	25	CAA GGA CAA AAT ACC TGT ATT CCT T	PCR
P2R	20	GAC ATC ATG AGA GAC ATC GC	PCR
Templates			
T1X	50	TAC CTT ATC CAT AAT AGA CAT GTC TAG GCA TGT CTC TTG ATA TCG AAT TC	PEX
T2Xb	30	btn ATA ATA GAC ATG TCT AGG CAT GTC TCT TGA	PEX
T3X	31	CTA GCA TGA GCT CAG TCC CAT GCC GCC CAT G	PEX
T3Xb	31	btn CTA GCA TGA GCT CAG TCC CAT GCC GCC CAT G	PEX
T1N	31	TCA ACT CAT GAC CGA GGA CTC ACT AGA TCG G	NEAR
T2N	31	TCA GCG TAG ACT CGA GGA CTC ACT AGA TCG G	NEAR
T1R	98	GAC ATC ATG AGA GAC ATC GCC TCT GGG CTA ATA GGA CTA CTT CTA ATC TGT AAG AGC AGA TCC CTG GAC AGG CAA GGA ATA CAG GTA TTT TGT CCT TG	PCR
Others			
L10	10	CAG TGA CTA G	ssDNA ladder[S6]
L20	20	CAG TGA CTA GCT TAC GGA CT	ssDNA ladder[S6]
L50	50	CAG TGC ATG ACT ATC GGA CCG TAT GAC TAG CTC AGG TAT CCA GTG ACT AG	ssDNA ladder[S6]

Footnotes: a) btn = 5'-biotin.

Table S2. Single-stranded modified DNA obtained by PEX and NEAR

#	Name	Sequence (5'→3') [a]	Obt. [b]	Prim/ Tmpl [c]	M_{calcd} [d]	M_{found} [e]
1	**ON1**	TCA AGA GAC ATG CCT AGA C^{VDP} AT GTC^{VDP} TAT TAT	PEX	**P2X / T2Xb**	9691	9693
2	**ON2**	P - GTC^{VJ} ATG AGT TGA	NEAR	**P1N / T1N**	4082	4083
3	**ON3**	P - GTC^{VDP} ATG AGT TGA	NEAR	**P1N / T1N**	4030	4031
4	**ON4**	P - AGT C^{VJ} TA C^{VJ} G C^{VJ} TGA	NEAR	**P1N / T2N**	4633	4634
5	**ON5**	P - AGT C^{VDP} TA C^{VDP} G C^{VDP} TGA	NEAR	**P1N / T2N**	4477	4479

Footnotes. a) P = 5' phosphate group; ODNs containing **dC^{VJ}** and **dC^{VDP}** were obtained using triphosphates **4a** and **4b**, respectively. b) Enzymatic reactions used for the synthesis. c) Primer / Template used (Table S1 for sequences). d) Molecular mass calculated using ChemBioDraw13 and OligoCalc.[S7] e) *m/z* of the major peak in the MALDI-TOF spectrum; for copies of MALDI-TOF spectra see pages S28–S30 of the Supporting information.

B. List of abbreviations

BSA – Bovine serum albumin

DIMCARB – Dimethylammonium dimethylcarbamate

DMSO – Dimethyl sulfoxide

DTT – Dithiothreitol

EA – Ethyl acetate

EDTA – Ethylenediaminetetraacetic acid

EG – Ethylene glycol

FMR – Fluorescent molecular rotor

NEAR – Nicking enzyme amplification reaction

ODNs – Oligodeoxynucleotides

PAGE – Polyacrylamide gel electrophoresis

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PEX – Primer extension

SSB – Single-strand Binding Protein from *Escherichia coli*

TBE – Tris borate – EDTA buffer

TEAA – Triethylammonium acetate buffer

TEAB – Triethylammonium bicarbonate buffer

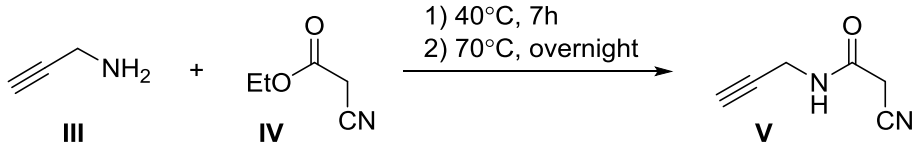
TLC – Thin-layer chromatography

C. Chemical synthesis and characterization of fluorescent nucleosides and dNTPs

Materials and methods

Reagents and solvents were purchased from Sigma-Aldrich and AlfaAesar. 5-Iodo-2'-deoxycytidine (**VIII**) was purchased from Berry & Associates. 4-formyl julolidine (**I**) was synthesized according to the literature procedure.[S2] Fluorescent molecular rotors **1a** and **1b** were synthesized as described by Theodorakis et al.[S1] Column chromatography was performed by using silica gel (40–63 μm). Semi-preparative purification of **dC^{X}TPs** was performed by HPLC (Waters modular HPLC system) on a column packed with 10 μm C18 reversed phase (Phenomenex, Luna C18 (2) 100 Å). NMR spectra were recorded on a 600 MHz (600.1 MHz for ^{1}H, 150.9 MHz for ^{13}C) or a 500 MHz (500.0 MHz for ^{1}H, 200.3 MHz for ^{31}P, 125.7 MHz for ^{13}C) spectrometer from sample solutions in $CDCl_3$, D_2O or DMSO-d6. Chemical shifts (in ppm, δ scale) were referenced as follows: $CDCl_3$ solutions, ^{1}H referenced to TMS (δ = 0 ppm), ^{13}C referenced to the solvent signal (δ = 77.0 ppm); DMSO-d6 solutions, ^{1}H referenced to the residual solvent signal (δ = 2.50 ppm), ^{13}C referenced to the solvent signal (δ = 39.7 ppm); D_2O solutions, referenced to 1,4-dioxane as an internal standard (δ(^{1}H) = 3.75 ppm, δ(^{13}C) = 69.3 ppm). ^{31}P NMR spectra were referenced to the phosphate buffer signal (δ = 2.35 ppm). Coupling constants (*J*) are given in Hz. NMR spectra of dNTPs were measured in phosphate buffer at pD = 7.1. 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 (Thermo Fisher Scientific) spectrometer using ESI ionization.

_N_-Propargyl-cyanoacetamide (V)

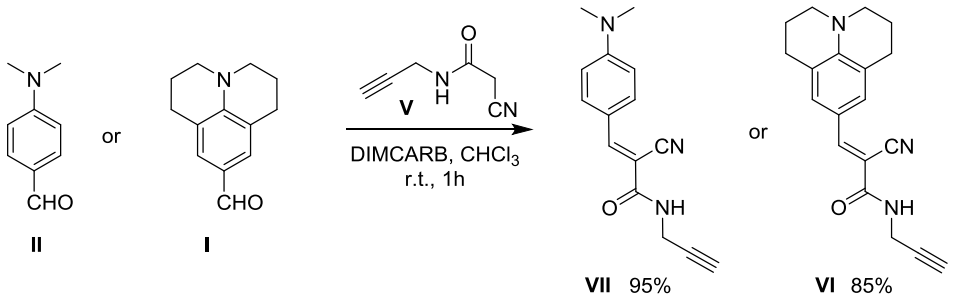


III **IV** **V**

N-Propargyl-cyanoacetamide (**V**) was synthesized using the literature procedure[S3] with minor modifications. A mixture of ethyl cyanoacetate **IV** (10 mL) and propargyl amine **III** (9 mL) was heated for 7 hours at 40 °C and then overnight at 70°C. The dark-red solid obtained was recrystallized from a minimal volume of methanol and washed with dichloromethane to give the product as pale-brown solid (8.14g, 71%). M.p. (MeOH) 107 – 108 °C (lit.[S3] 100 – 103 °C) ^{1}H NMR (500.0 MHz, DMSO-d_6): 3.18 (t, 1H, ^{4}J = 2.5, HC≡C-); 3.67 (s, 2H, CH_2CN); 3.89 (dd, 2H, ^{3}J = 5.4, ^{4}J = 2.5,

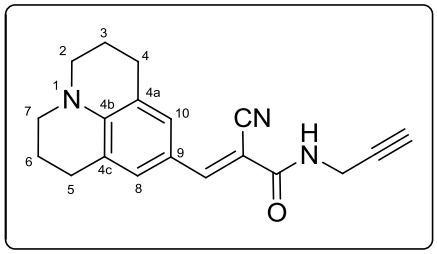
CH_2N); 8.70 (bt, 1H, ^3J = 5.4, NH). ^{13}C NMR (125.7 MHz, DMSO-d_6): 25.35 (**C**H_2CN); 28.66 (CH_2N); 73.81 (H**C**≡C-); 80.51 (-**C**≡CH); 116.17 (CN); 162.26 (CO). Anal. calcd for $C_6H_6N_2O$: C, 59.01; H, 4.95; N, 22.94. Found: C, 58.79; H, 4.88; N, 22.69.

General procedure for the Knoevenagel condensation of *N*-propargyl-cyanoacetamide (V) with *p*-dialkylaminobenzaldehydes catalyzed by DIMCARB [S4–S5]



Dimethylammonium dimethylcarbamate (DIMCARB, 3.85 mL, 30 mmol) was added to a solution of corresponding aldehyde (5 mmol) in chloroform (8 mL) and the mixture was stirred for 10 – 15 min at ambient temperature. Then, *N*-propargyl-cyanoacetamide **V** (732 mg, 6 mmol) was added at once. The mixture was stirred at room temperature for 30 – 60 minutes until the complete consumption of the starting aldehyde as detected by TLC (hexane/EA, 7/3). Then, ~2N HCl (50 mL) was slowly added to the reaction flask and the resulting mixture was stirred until the evolution of gas stopped. Organic phase was separated; the water phase was extracted with dichloromethane (3×); the combined organic layers were dried over $MgSO_4$, concentrated on a rotavap and purified by column chromatography eluted consequently with ethyl acetate in hexane (20→30%), then with hexane, and finally with acetone in dichloromethane (0→4%) to give the corresponding acetylene-containing molecular rotors **VI** or **VII**.

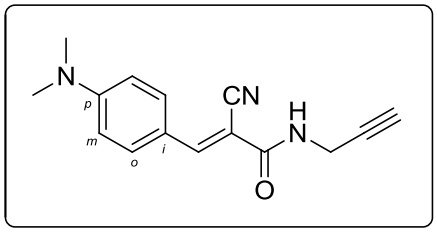
(*E*)-2-Cyano-*N*-(prop-2-yn-1-yl)-3-(julolidine-9-yl)acrylamide (VI)



Orange solid (1.29 g, 85%) was obtained following the general procedure. R_f = 0.30 (hexane/ethyl acetate, 7/3) ^{1}H NMR (500.0 MHz, $CDCl_3$): 1.96 (m, 4H, H-3,6-julolidine); 2.27 (t, 1H, ^4J = 2.5, HC≡C); 2.75 (m, 4H, H-4,5-julolidine); 3.31 (m, 4H, H-2,7-julolidine); 4.19 (dd, 2H, ^3J = 5.3, ^4J = 2.5, CH_2N); 6.37 (bt, 1H, ^3J = 5.3, NH); 7.45 (s, 2H, H-8,10-julolidine); 8.03 (t, 1H, ^4J = 0.5, HC=C). ^{13}C NMR (125.7 MHz,

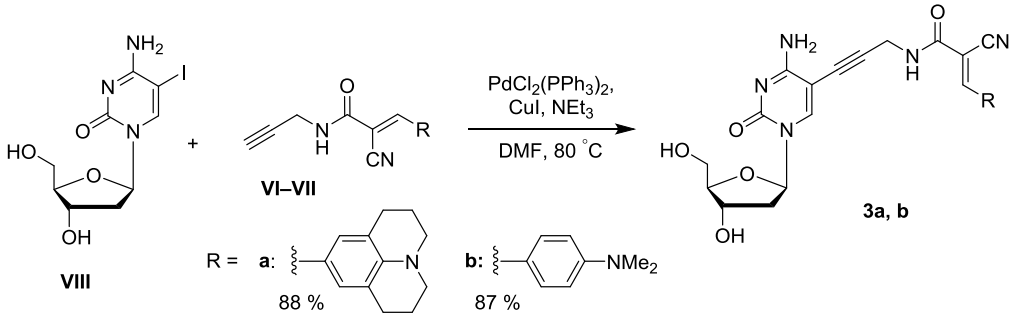
$CDCl_3$): 21.07 (CH_2-3,6-julolidine); 27.53 (CH_2-4,5-julolidine); 29.92 (CH_2N); 50.13 (CH_2-2,7-julolidine); 71.94 (H**C**≡C); 79.10 (**C**≡CH); 92.44 (HC=**C**-CN); 118.61 (C-9-julolidine); 119.26 (CN); 120.91 (C-4a,4c-julolidine); 131.19 (CH-8,10-julolidine); 147.13 (C-4b-julolidine); 152.89 (H**C**=C-CN); 162.33 (CO). HRMS: calculated for $C_{19}H_{20}ON_3$ $[M+H]^+$: 306.1601; found: 306.1602. Anal. calcd for $C_{19}H_{19}N_3O$: C, 74.73; H, 6.27; N, 13.76. Found: C, 74.54; H, 6.21; N, 13.57.

(*E*)-2-Cyano-3-[4-(dimethylamino)phenyl]-*N*-(prop-2-yn-1-yl)acrylamide (VII)



Yellow solid (1.20 g, 95%) was obtained following the general procedure. R_f = 0.26 (hexane/ethyl acetate, 7/3) ^{1}H NMR (600.1 MHz, $CDCl_3$): 2.28 (t, 1H, ^4J = 2.6, HC≡C); 3.10 (s, 6H, $(CH_3)_2N$); 4.20 (dd, 2H, ^3J = 5.3, ^4J = 2.6, CH_2N); 6.43 (bt, 1H, ^3J = 5.3, NH); 6.72 (m, 2H, H-*m*-$C_6H_4NMe_2$); 7.88 (m, 2H, H-*o*-$C_6H_4NMe_2$); 8.16 (s, 1H, HC=C). ^{13}C NMR (150.9 MHz, $CDCl_3$): 29.98 (CH_2N); 40.15 (CH_3N); 72.06 (H**C**≡C); 78.92 (**C**≡CH); 94.82 (HC=**C**-CN); 111.75 (CH-*m*-$C_6H_4NMe_2$); 118.64 (CN); 119.79 (C-*i*-$C_6H_4NMe_2$); 133.56 (CH-*o*-$C_6H_4NMe_2$); 153.01 (H**C**=C-CN); 153.11 (C-*p*-$C_6H_4NMe_2$); 161.77 (CO). HRMS for $C_{15}H_{15}N_3NaO$: $[M+Na]^+$ calculated 276.1107, found 276.1107. Anal. calcd for $C_{15}H_{15}N_3O$: C, 71.13; H, 5.97; N, 16.59. Found: C, 70.74; H, 5.92; N, 16.29.

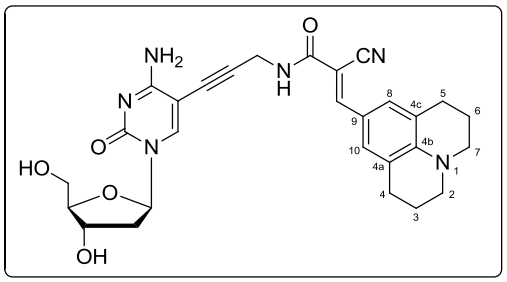
General procedure for the synthesis of the FMR-modified deoxycytidines using the Sonogashira coupling



Dry DMF (4 ml) was added to a flask containing 5-iodo-2′-deoxycytidine **VIII** (176 mg, 0.5 mmol), corresponding acetylene **VI** or **VII** (0.55 mmol), $PdCl_2(PPh_3)_2$ (18 mg, 5% mol), CuI (5 mg, 5% mol) and the mixture was purged and refilled with argon for 3−4 times. Triethylamine (0.21 ml, 1.5 mmol) was added via syringe and the mixture was stirred at 80 °C until the complete consumption of the

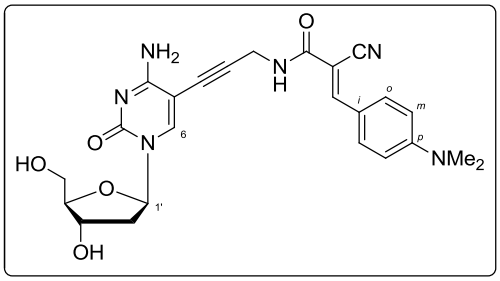
starting nucleoside as monitored by TLC (CH_2Cl_2 / MeOH, 9/1). The mixture was concentrated on a rotavap; the crude products were purified by column chromatography (MeOH in dichloromethane, 5→15%).

(*E*)-*N*-[3-(2'-Deoxycytidine-5-yl)prop-2-yn-1-yl]-2-cyano-3-(julolidine-9-yl)acrylamide (dC^{VJ}, 3a)



Yellow solid (233 mg, 88%) was obtained following the general procedure). R_f = 0.20 (dichloromethane/MeOH, 9/1). ^{1}H NMR (500.0 MHz, DMSO-d_6): 1.86 (m, 4H, H-3,6-julolidine); 1.97 (ddd, 1H, J_{gem} = 13.2, $J_{2'b,1'}$ = 7.2, $J_{2'b,3'}$ = 6.0, H-2'b); 2.13 (ddd, 1H, J_{gem} = 13.2, $J_{2'a,1'}$ = 6.0, $J_{2'a,3'}$ = 3.4, H-2'a); 2.67 (m, 4H, H-4,5-julolidine); 3.31 (m, 4H, H-2,7-julolidine); 3.54, 3.60 (2 × ddd, 2 × 1H, J_{gem} = 12.0, $J_{5',OH}$ = 5.2, $J_{5',4'}$ = 3.6, H-5'); 3.78 (q, 1H, $J_{4',3'}$ = $J_{4',5'}$ = 3.6, H-4'); 4.19 (m, 1H, H-3'); 4.22 (d, 2H, J = 5.3, CH_2N); 5.06 (t, 1H, $J_{OH,5'}$ = 5.2, OH-5'); 5.20 (d, 1H, $J_{OH,3'}$ = 4.2, OH-3'); 6.11 (dd, 1H, $J_{1',2'}$ = 7.2, 6.0, H-1'); 6.87 (bs, 1H, $NH_a\mathbf{H_b}$); 7.44 (s, 2H, H-8,10-julolidine); 7.82 (bs, 1H, $N\mathbf{H_a}H_b$); 7.84 (s, 1H, HC=C); 8.13 (s, 1H, H-6); 8.41 (t, 1H, J = 5.3, NH). ^{13}C NMR (125.7 MHz, DMSO-d_6): 21.07 (CH_2-3,6-julolidine); 27.53 (CH_2-4,5-julolidine); 30.38 (CH_2N); 40.98 (CH_2-2'); 49.54 (CH_2-2,7-julolidine); 61.23 (CH_2-5'); 70.37 (CH-3'); 74.41 (cyt-**C**≡C-CH_2-); 85.53 (CH-1'); 87.66 (CH-4'); 89.55 (C-5); 92.50 (cyt-C≡**C**-CH_2-); 93.79 (HC=**C**-CN); 117.68 (C-9-julolidine); 118.73 (CN); 120.65 (C-4a,4c-julolidine); 130.62 (CH-8,10-julolidine); 144.01 (CH-6); 147.08 (C-4b-julolidine); 151.06 (H**C**=C-CN); 153.64 (C-2); 162.69 (CONH); 164.69 (C-4). HRMS for $C_{28}H_{30}O_5N_6Na$: $[M+Na]^+$ calculated 553.2170, found 553.2170.

(*E*)-*N*-[3-(2'-Deoxycytidine-5-yl)prop-2-yn-1-yl]-2-cyano-3-[4-(dimethylamino)phenyl]acrylamide (dC^{VDP}, 3b)

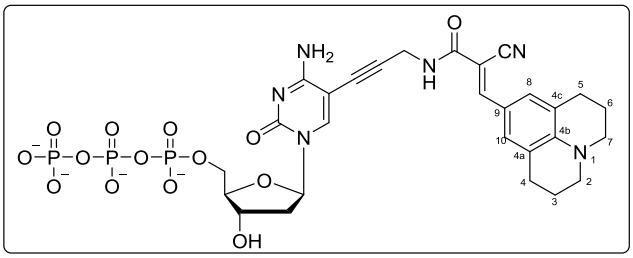


Yellow solid (209 mg, 87%) was obtained following the general procedure). R_f = 0.19 (dichloromethane / MeOH, 9/1). ^{1}H NMR (500.0 MHz, DMSO-d_6): 1.97 (ddd, 1H, J_{gem} = 13.2, $J_{2'b,1'}$ = 7.3, $J_{2'b,3'}$ = 6.0, H-2'b); 2.13 (ddd, 1H, J_{gem} = 13.2, $J_{2'a,1'}$ = 6.0, $J_{2'a,3'}$ = 3.6, H-2'a); 3.06 (s, 6H, $(CH_3)_2N$); 3.54, 3.60 (2 × ddd, 2 × 1H, J_{gem} = 11.9, $J_{5',OH}$ = 5.1, $J_{5',4'}$ = 3.6, H-5'); 3.78 (q, 1H, $J_{4',3'}$ = $J_{4',5'}$ = 3.6, H-4'); 4.19 (m, 1H, H-3'); 4.24 (d, 2H, J = 5.2, CH_2N); 5.07 (t,

1H, $J_{OH,5'}$ = 5.1, OH-5'); 5.21 (d, 1H, $J_{OH,3'}$ = 4.2, OH-3'); 6.11 (dd, 1H, $J_{1',2'}$ = 7.3, 6.0, H-1'); 6.83 (m, 2H, H-*m*-$C_6H_4NMe_2$); 6.87, 7.82 (2 × bs, 2 × 1H, NH_2); 7.88 (m, 2H, H-*o*-$C_6H_4NMe_2$); 8.00 (s, 1H, HC=C); 8.13 (s, 1H, H-6); 8.54 (t, 1H, *J* = 5.2, NH). ^{13}C NMR (125.7 MHz, DMSO-d_6): 30.42 (CH_2N); 39.78 (CH_3N); 40.99 (CH_2-2'); 61.24 (CH_2-5'); 70.38 (CH-3'); 74.50 (cyt-**C**≡C-CH_2-); 85.55 (CH-1'); 87.66 (CH-4'); 89.55 (C-5); 92.32 (cyt-C≡**C**-CH_2-); 96.18 (HC=**C**-CN); 111.88 (CH-*m*-$C_6H_4NMe_2$); 118.31 (CN); 118.80 (C-*i*-$C_6H_4NMe_2$); 133.09 (CH-*o*-$C_6H_4NMe_2$); 144.07 (CH-6); 151.23 (H**C**=C-CN); 153.31 (C-*p*-$C_6H_4NMe_2$); 153.65 (C-2); 162.46 (CONH); 164.68 (C-4). HRMS for $C_{24}H_{26}N_6O_5Na$: $[M+Na]^+$ calculated 501.1857, found 501.1857

5'-*O*-Triphosphorylation of nucleosides 3a and 3b

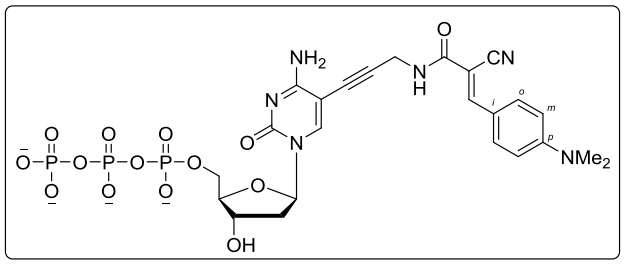
(*E*)-*N*-[3-(2'-deoxycytidine-5-yl)prop-2-yn-1-yl]-2-cyano-3-(julolidine-9-yl)acrylamide 5'-*O*-triphosphate ($dC^{VJ}TP$, 4a)



Dry trimethyl phosphate (2 ml) was added to an argon-purged flask containing nucleoside **dC^{VJ}** (112 mg, 0.21 mmol). The resulting solution was cooled down to 0 ºC and a solution of $POCl_3$ (32 µL, 0.34 mmol) in trimethyl phosphate (0.5 mL) was added dropwise. After 3 hours stirring at 0 ºC, a solution of (n-$Bu_3NH)_2H_2P_2O_7$ (522 mg, 0.95 mmol) and n-Bu_3N (0.23 mL, 0.95 mmol) in dry DMF (2.5 mL) was added. The solution was stirred for another 60 min at 0 ºC and then quenched by the addition of 1M TEAB (3 mL). The mixture was concentrated on a rotavap; the residue was co-evaporated with distilled water three times. After that the crude mixture was diluted with water to a total volume of ca. 15 mL and unreacted nucleoside was separated by filtration. 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 orange oil was co-evaporated 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 orange solid (16 mg, 9%). ^{1}H NMR (500.0 MHz, D_2O, pD = 7.1, phosphate buffer, ref(dioxane) = 3.75 ppm): 1.69 (bm, 4H, H-3,6-julolidine); 2.21 (dt, 1H, J_{gem} = 13.9, $J_{2'b,1'}$ = $J_{2'b,3'}$ = 6.4, H-2'b); 2.41 (m, 5H, H-2'a, H-4,5-julolidine); 3.08 (m, 4H, H-2,7-julolidine); 4.14-4.21 (m, 3H, H-4',5'); 4.27, 4.33 (2 × d, 2 × 1H, J_{gem} = 18.2, CH_2N); 4.55 (m, 1H, H-3'); 6.07 (t, 1H, $J_{1',2'}$ = 6.4, H-1'); 7.01 (bs, 2H, H-8,10-julolidine); 7.52 (s, 1H, HC=C); 7.99 (s, 1H, H-

6). ^{13}C NMR (125.7 MHz, D_2O, pD = 7.1, phosphate buffer, ref(dioxane) = 69.3 ppm): 23.15 (CH_2-3,6-julolidine); 29.69 (CH_2-4,5-julolidine); 33.07 (CH_2N); 41.86 (CH_2-2'); 52.42 (CH_2-2,7-julolidine); 67.89 (d, $J_{C,P}$ = 5.4, CH_2-5'); 72.92 (CH-3'); 75.68 (cyt-**C**≡C-CH_2-); 88.08 (d, $J_{C,P}$ = 8.6, CH-4'); 89.11 (CH-1'); 92.20 (C-5); 94.63 (cyt-C≡**C**-CH_2-); 95.16 (HC=**C**-CN); 120.14 (C-9-julolidine); 121.91 (CN); 123.46 (C-4a,4c-julolidine); 133.98 (CH-8,10-julolidine); 147.48 (CH-6); 150.62 (C-4b-julolidine); 155.04 (H**C**=C-CN); 158.33 (C-2); 167.63 (C-4); 168.10 (CONH). ^{31}P NMR (202.4 MHz, D_2O, pD = 7.1, ref(phosphate buffer) = 2.35 ppm): –21.04 (dd, J = 19.1, 18.9, P_β); –10.16 (d, J = 18.9, P_α); –6.86 (d, J = 19.1, P_γ). HRMS for $C_{28}H_{30}O_{14}N_6Na_2P_3$: $[M+2Na+H]^-$ calculated 813.0823; found 813.0825.

(*E*)-*N*-[3-(2'-Deoxycytidine-5-yl)prop-2-yn-1-yl]-2-cyano-3-[4-(dimethylamino)phenyl]acrylamide 5'-*O*-triphosphate (dC^{VDP}TP, 4b)



Dry trimethyl phosphate (3 ml) was added to an argon-purged flask containing nucleoside **dC^{VDP}** (165 mg, 0.345 mmol). The resulting solution was cooled down to 0 ºC and a solution of $POCl_3$ (57 μL, 0.61 mmol) in trimethyl phosphate (1 mL) was added dropwise. After 3 hours stirring at 0 ºC, a solution of (n-$Bu_3NH)_2H_2P_2O_7$ (850 mg, 1.55 mmol) and n-Bu_3N (0.37 mL, 1.55 mmol) in dry DMF (4.5 mL) was added. The solution was stirred for another 60 min at 0 ºC and then quenched by the addition of 1M TEAB (5 mL). The mixture was concentrated on a rotavap; the residue was co-evaporated with distilled water three times. After that the crude mixture was diluted with water to a total volume of ca. 20 mL and unreacted nucleoside was separated by filtration. 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 yellow oil was co-evaporated 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 yellow solid (25 mg, 9%). ^{1}H NMR (600.1 MHz, D_2O, pD = 7.1, phosphate buffer, ref(dioxane) = 3.75 ppm): 2.24 (ddd, 1H, J_{gem} = 14.0, $J_{2'b,1'}$ = 6.8, $J_{2'b,3'}$ = 6.2, H-2'b); 2.40 (ddd, 1H, J_{gem} = 14.0, $J_{2'a,1'}$ = 6.2, $J_{2'a,3'}$ = 4.6, H-2'a); 2.94 (s, 6H, $(CH_3)_2N$); 4.16-4.23 (m, 3H, H-4',5'); 4.29, 4.34 (2 × d, 2 × 1H, J_{gem} = 17.8, CH_2N); 4.56 (m, 1H, H-3'); 6.12 (dd, 1H, $J_{1',2'}$ = 6.8, 6.2, H-1'); 6.55 (m, 2H, H-m-$C_6H_4NMe_2$); 7.63 (m, 2H, H-o-$C_6H_4NMe_2$); 7.77 (s, 1H,

HC=C); 8.03 (s, 1H, H-6). ^{13}C NMR (150.9 MHz, D_2O, pD = 7.1, phosphate buffer, ref(dioxane) = 69.3 ppm): 33.15 (CH_2N); 41.83 (CH_2-2'); 41.98 (CH_3N); 67.79 (d, $J_{C,P}$ = 5.3, CH_2-5'); 72.72 (CH-3'); 75.90 (cyt-**C**≡C-CH_2-); 88.11 (d, $J_{C,P}$ = 8.7, CH-4'); 88.99 (CH-1'); 94.68 (C-5); 94.86 (cyt-C≡**C**-CH_2-); 95.34 (HC=**C**-CN); 114.18 (CH-*m*-$C_6H_4NMe_2$); 121.20 (C-*i*-$C_6H_4NMe_2$); 121.27 (CN); 136.56 (CH-*o*-$C_6H_4NMe_2$); 147.56 (CH-6); 155.75 (H**C**=C-CN); 156.50 (C-*p*-$C_6H_4NMe_2$); 158.51 (C-2); 167.71 (C-4); 167.80 (CONH). ^{31}P NMR (202.3 MHz, D_2O, pD = 7.1, ref(phosphate buffer) = 2.35 ppm): –21.62 (dd, J = 19.8, 18.6, P_β); –11.12 (d, J = 18.6, P_α); –5.96 (d, J = 19.8, P_γ). HRMS for $C_{24}H_{26}O_{14}N_6Na_2P_3$: $[M+2Na+H]^-$ calculated 761.0521 found 761.0524.

D. Enzymatic incorporation of modified dNTPs into DNA

Materials and methods

Synthetic oligonucleotides (primers and templates for PEX and NEAR, Table S1) were purchased from Generi Biotech (Czech Republic). Double-stranded 100bp DNA ladder was purchased from New England Biolabs. Single stranded DNA ladder (10, 20, 50, 98 bases) was prepared using synthetic oligonucleotides **L10**, **L20**, **L50** and **T1R** (Table S1) as described previously.[S6] DNA polymerase Vent(exo–), nicking enzyme Nt.BstNBI, corresponding reaction buffers, as well as natural nucleoside triphosphates (dATP, dCTP, dGTP, dTTP) were purchased from New England Biolabs. KOD XL DNA polymerase and corresponding reaction buffer were obtained from Merck Millipore. Streptavidin magnetic particles were obtained from Roche. All solutions for biochemical reactions were prepared using Milli-Q water. Primers for analytical primer extension experiments were labeled using T4 polynucleotide kinase (New England Biolabs) and [γ^{32}P]-ATP (Institute of Isotopes Co., Ltd.; Hungary) according to standard techniques. Radioactive gels were visualized by phosphorimaging using Storage Phosphor Screens (GE Healthcare) and Typhoon 9410 imager (Amersham Biosciences). 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.

Analytical primer extension

The reaction mixture (20 μL) contained 5'–^{32}P–labelled primer (3 μM, 1 μL), unlabelled primer (100 μM, 0.77 μL), template (100 μM, 0.8 μL), corresponding DNA polymerase (0.22–0.23 U), either natural or modified dNTPs (4 mM each, 0.4 μL) and reaction buffer (10×, 2 μL) supplied by the manufacturer with the enzyme. The reaction mixture was incubated at 60 °C for 40 min. The reaction was stopped by the addition of PAGE stop solution (40 μL; 80% [v/v] formamide, 20mM EDTA, 0.025% [w/v] bromophenol blue, 0.025% [w/v] xylene cyanol) and heated at 95 °C for 5 min. Aliquots (3 μL) were subjected to vertical electrophoresis in 12.5% denaturing polyacrylamide gel containing 1× TBE buffer (pH 8.0) and 7M urea at 42 mA for 50 min. The gel was dried in vacuo (80 °C, 70 min) and visualized by phosphor imaging autoradiography.

Semi-preparative primer extension with magnetic separation

The reaction mixture (100 μL) containing KOD XL DNA polymerase (2.5 U/μL, 0.45 μL), 10x concentrate of the KOD reaction buffer provided by the manufacturer of the enzyme (10 μL),

biotinylated template (100 μM, 4 μL), primer (100 μM, 4 μL), dNTPs (dGTP, dTTP, dATP, **dC^{X}TP**; 4 mM each, 5 μL) was incubated at 60 ºC for 40 minutes. The reaction was stoped by cooling to 4 ºC. Streptavidine magnetic particles (120 μl) were washed with binding buffer (3 × 300 μl, 10mM Tris, 1mM EDTA, 100mM NaCl, pH 7.5). The PEX solution (100 μL) and binding buffer (100 μl) were added to the magnetic beads. The mixture was incubated in a thermal mixer for 35 min at 15 °C and 1200 rpm. Then the magnetic beads were washed with wash buffer (3 × 300 μl, 10mM Tris, 1mM EDTA, 500mM NaCl, pH 7.5) and water (4 × 300 μl). Then water (100 μl) was added and the sample was denatured for 2 min at 40 °C and 900 rpm. The beads were separated and the solution containing ssDNA was transferred into a clean vial. Concentration of the resulting DNA solutions was determined on a NanoDrop.The product was analysed by MALDI-TOF mass spectrometry.

Nicking enzyme amplification reaction (NEAR) with modified dNTPs on analytical scale

The reaction mixture (50 μL) contained the template (0.125 μM), primer (0.125 μM), modified **dC^{X}TP** (187.5 μM), natural dNTPs (dGTP, dTTP, dATP; 125 μM each), 1× ThermoPol buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl/pH 8.8/, 0.1% Trit on-X-100, and 2mM $MgSO_4$), and 0.5× NEBuffer 3 (50mM NaCl, 25 mM Tris-HCl/pH 7.9/, 5 mM $MgCl_2$, and 0.5 mM DTT), Vent(exo–) DNA polymerase (7.5 U) and nickase Nt.BstNBI (30 U). The reaction mixture was incubated in a thermal mixer at 55 °C, 400 rpm for 3 h. The reaction was stopped by cooling to 4 °C. The products were analyzed by agarose gel electrophoresis using 4% agarose gels stained with GelRed™ (Biotium). Samples for electrophoresis were prepared by mixing 1.6 μL of TrackIt™ Cyan/Orange loading buffer (Invitrogen) and 8 μL of the reaction mixture or ssDNA ladder. The gel was run for 60 min at 120 V and imaged using electronic dual wave transilluminator equipped with UltraCam 8gD digital imaging system (Ultra-Lum). For MALDI-TOF mass spectrometry analysis the reaction mixtures were purified on Illustra MicroSpin G-25 columns (GE Healthcare) to remove the unincorporated nucleotides and buffer salts.

Nicking enzyme amplification reaction (NEAR) with dC^{VDP}TP on semi-preparative scale

The reaction mixture (500 μL) contained the template (0.125 μM), primer (0.125 μM), modified **dC^{VDP}TP** (187.5 μM), natural dNTPs (dGTP, dTTP, dATP; 125 μM each), 1× ThermoPol buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl/pH 8.8/, 0.1% Trit on-X-100, and 2 mM $MgSO_4$), and 0.5× NEBuffer 3 (50mM NaCl, 25 mM Tris-HCl/pH 7.9/, 5 mM $MgCl_2$, and 0.5 mM DTT), Vent(exo–) DNA polymerase (50 U) and nickase Nt.BstNBI (280 U). The reaction mixture was incubated at 55

°C, 400 rpm for 3 h. After that the reaction was stopped by cooling to 4 °C, the solution was concentrated on a vacuum concentrator to approximately 100 μL. The viscous concentrate was subjected to HPLC purification on a XBridge OST C18 Column (Waters; 2.5 μm particle size, 4.6 mm × 50mm). A linear gradient of triethylammonium acetate buffer (TEAA, pH = 7.0) and acetonitrile with flow rate 1 mL/min was used. Mobile phase A corresponds to 0.1 M TEAA in HPLC-grade water, mobile phase B to acetonitrile/0.1 M TEAA in HPLC-grade water 20/80 (v/v). The gradient started with 60% mobile phase A and 40% mobile phase B, going linearly to 80% mobile phase B in 53 min. The fractions containing the product were combined and evaporated on a vacuum concentrator. The residue was dissolved in a known volume of water and UV absorbance was measured on a Nanodrop. The products were analyzed by MALDI-TOF mass spectrometry.

Polymerase chain reaction (PCR)

The reaction mixture (10 μL) contained KOD XL DNA polymerase (2.5 U/μL, 0.1 μL), KOD XL reaction buffer supplied by the manufacturer (10×, 1 μL), primers **P1R** and **P2R** (10 μM; 1μL of each), 98-mer template **T1R** (1 μM, 0.25 μL). The amounts of dNTPs depend on whether only natural dNTPs or modified **dC^{X}TP** together with the three remaining natural dNTPs were incorporated. The following amounts were used: positive control – four natural dNTPs (2mM of each, 0.15 μL); negative control – three natural dNTPs (10 mM of dGTP, dTTP, and dATP, 0.6 μL); modified **dC^{X}TPs** – **4a** or **4b** with three natural dNTPs (10 mM of each, 0.6 μL). After the initial denaturation for 3 min at 94 °C, 40 PCR cycles were run under the following conditions: denaturation for 1 min at 94 °C, annealing for 1 min at 51°C, extension for 2 min at 72°C. These PCR process was terminated with a final extension step for 6 min at 72 °C. The reaction was stopped by cooling to 4 °C. The products were analyzed by agarose gel electrophoresis using 2% agarose gel stained with GelRed™ (Biotium). Samples for electrophoresis were prepared by mixing 1 μL of 6× DNA Loading Dye (Thermo Scientific) and 5 μL of the reaction mixture. The gel was run for 70 min at 120 V and imaged using electronic dual wave transilluminator equipped with UltraCam 8gD digital imaging system (Ultra-Lum).

E. UV-Vis and Fluorescence measurements

General notes

Spectroscopy grade solvents (DMSO, ethylene glycol, glycerol) were purchased from Sigma Aldrich and Alfa Aesar. Absorption coefficients of natural and modified ODNs (ε_{260}) were estimated using the OligoCalc.[S7] The absorption of the modified **dC^{X}** analogues at 260 nm was assumed to be identical with dC. UV-visible spectra of individual compounds were measured on a Cary 100 UV-Vis spectrometer (Agilent Technologies). UV-vis spectra of oligonucleotides were measured on a Nanodrop 1000 spectrophotometer (Thermo Scientific). Fluorescence spectra were measured on a Jasco 6600 or Fluorolog (Horiba) spectrofluorimeters.

Fluorescence of FMRs in a gradient of viscosity

For the measurements of fluorescence in the gradient of viscosity (ethylene glycol / glycerol) we used procedure described elsewhere[S1] with minor modifications. Solutions of different viscosities were prepared by mixing known volumes of pre-stained EG, EG and glycerol. Stock solutions of FMRs **1a**, **1b**, **3a** and **3b** were prepared in DMSO (1–5 mg per 0.5 mL DMSO). Pre-stained ethylene glycol was prepared by adding a small volume of the DMSO stock solution (10–50 µL) to 10 mL of ethylene glycol; absorbance of the resulting solutions was measured on a UV-Vis spectrometer. Pre-stained ethylene glycol, ethylene glycol and glycerol were mixed in proportions shown in table S3.

Table S3. Preparation of the samples for measurement of fluorescence in the gradient of viscosity

Viscosity (mPa · s)	**Components** (mL)[a]		
	pre-stained ethylene glycol [b]	ethylene glycol [b]	glycerol
74.0	x	3.0–x	2.0
112.2	x	2.5–x	2.5
170.2	x	2.0–x	3.0
258.1	x	1.5–x	3.5
391.4	x	1.0–x	4.0

Footnotes: **a**: total volume of each sample was 5 mL. **b**: x is the volume of pre-stained ethylene glycol (mL) needed to give absorbance 0.05 at λ_{max} in the final 5mL solution; x was calculated for each compound from the UV-vis absorption spectra of pre-stained EG by formula: x (mL) = (0.05 × 5 / $A_{PS\text{-}EG}$) where $A_{PS\text{-}EG}$ is the absorbance of pre-stained EG at λ_{max}.

Glycerol was heated on a water bath to 55–60 °C to allow for better pipetting. Solutions were prepared in 15 mL plastic tubes and mixed thoroughly on a tube roller for 30–60 minutes. Thorough mixing was

crucial so the tubes were watched over this time. Fluorescence spectra were recorded at room temperature (22–23 °C) in quartz cuvettes (internal volume 1.4 mL). Samples were excited at their absorption maxima. Intensity of fluorescence at the emission maximum was measured for each sample; within each series, the intensity of fluorescence was normalized to the intensity of fluorescence of the less viscous solution (I_F for 74.0 mPa·s = 1). Preparations and measurements were triplicated. Förster–Hoffmann plot was obtained by plotting log F vs logarithm of viscosity (log η).

Fluorescence titration experiments

Titrations were performed in a 100 μL quartz cuvette at room temperature (22 °C). Initial solution (120 μL) contained 1μM ssDNA probe **ON1** (Table S2) in PBS (10 mM phosphate buffer pH 7.4; 100 mM NaCl). Excitation wavelength was 430 nm. Aliquots (0.2 μL) of SSB, BSA or PBS-glycerol were added; the solution was carefully mixed with a pipette and equilibrated for 2–3 minutes before the fluorescence spectrum was recorded.
Solutions for titration were prepared as follows.

SSB

Single-strand Binding Protein from *Escherichia coli* (Sigma-Aldrich) was used as supplied (Solution in 20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 0.1 mM EDTA, 0.1 mM DTT, 50% glycerol). The concentration of the supplied solution determined on a Nanodrop (ε_{280} = 30250 M^{-1} cm^{-1} for SSB monomer[S8]) was ca. 50 μM (SSB tetramer).

BSA

Bovine serum albumin (New England Biolabs) was diluted to the same concentration as SSB (50 μM) in PBS (10 mM phosphate buffer pH = 7.4; 100 mM NaCl). ε_{280} = 43800 M^{-1} cm^{-1}.[S9]

PBS-glycerol

PBS-glycerol was prepared by mixing 100 mM phosphate buffer pH = 7.4 (100 μL), 1 M NaCl (100 μL), water (300 μL) and glycerol (500 μL).

F. References

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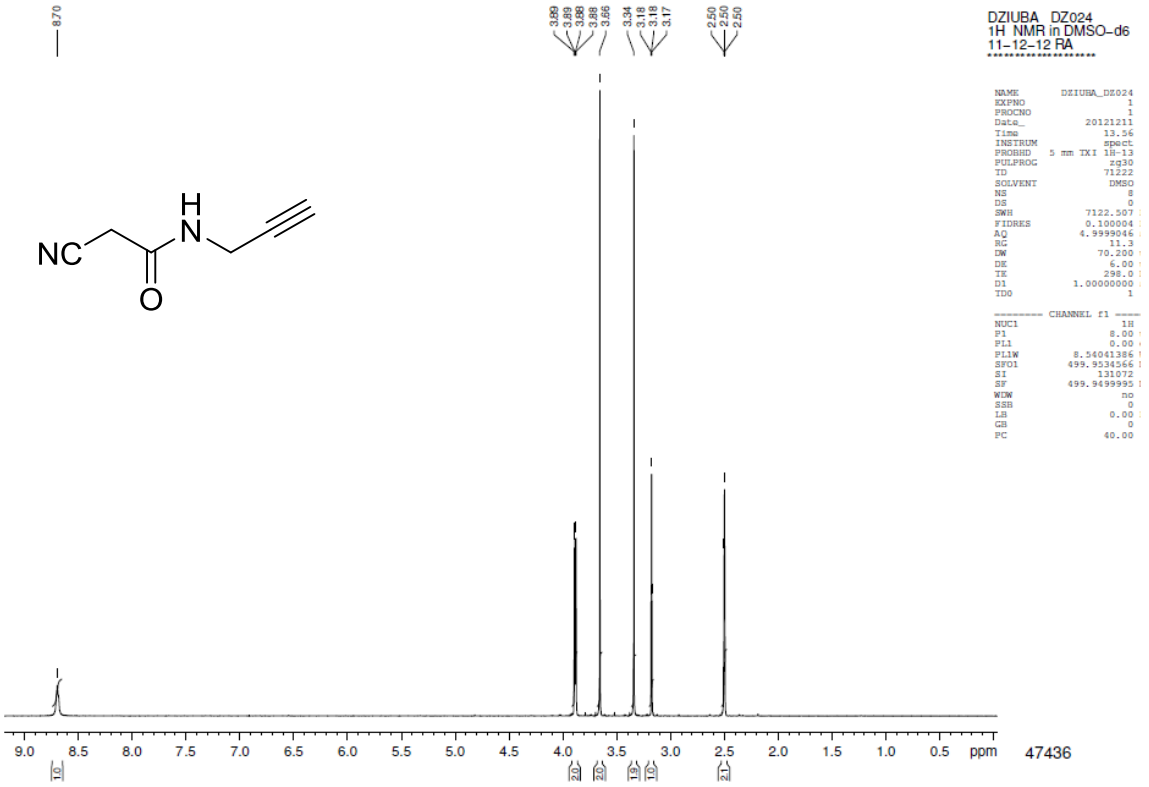
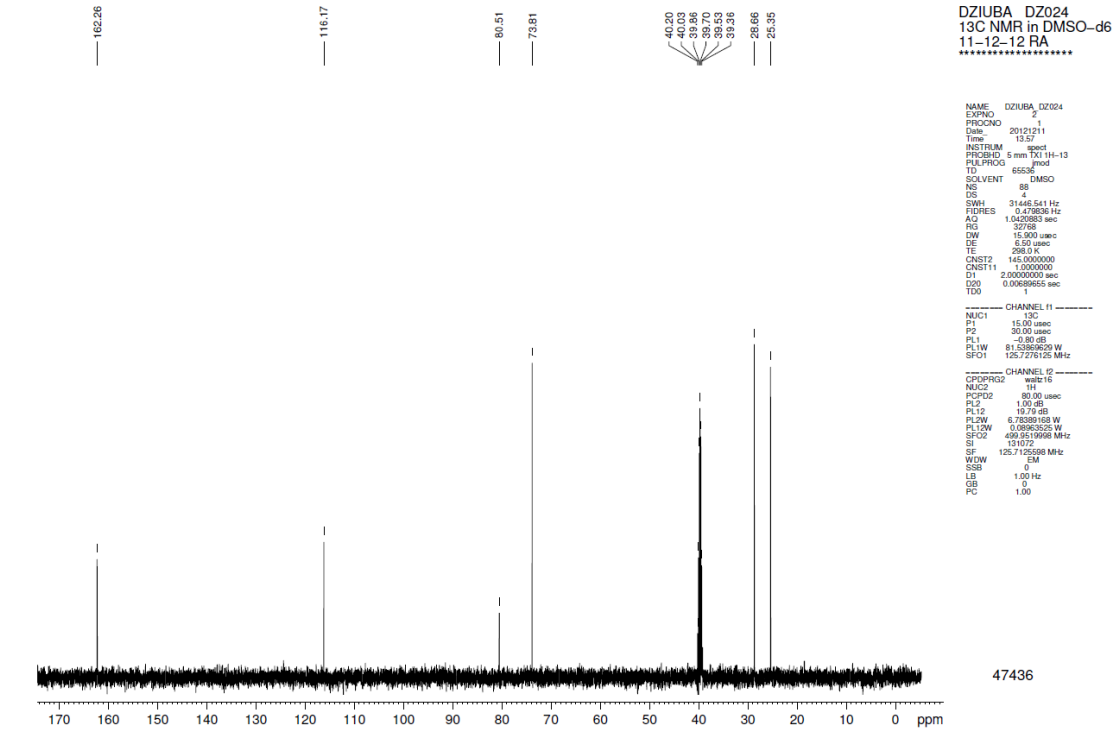
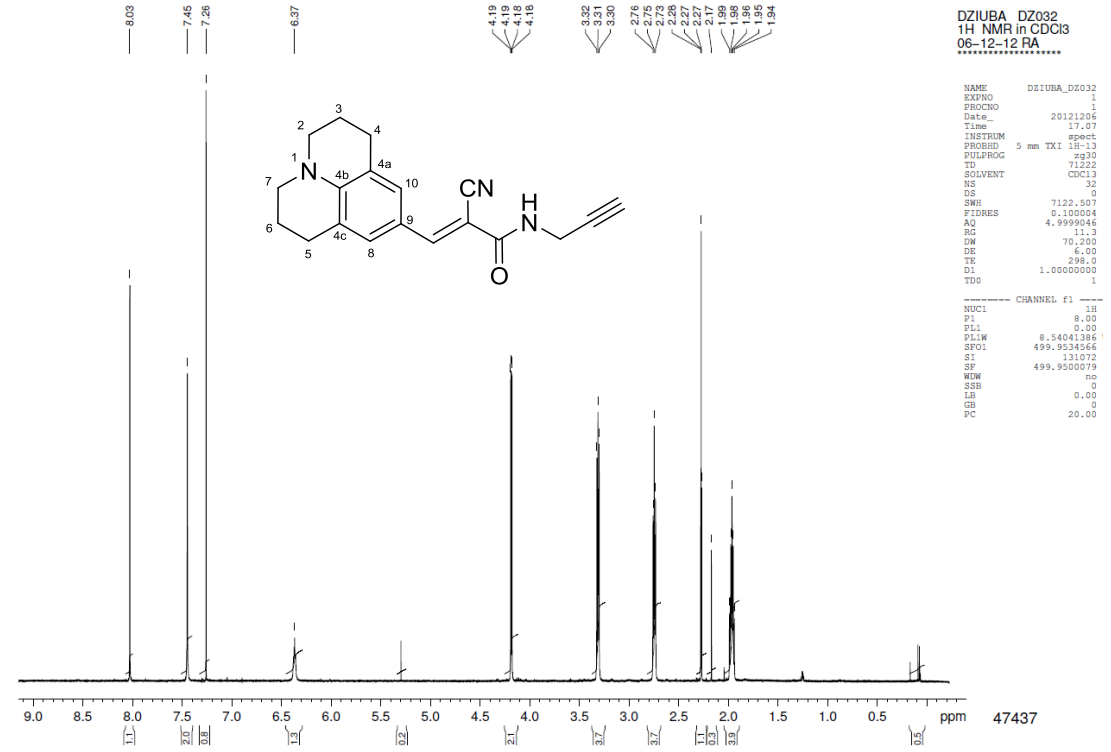
G. Copies of the NMR spectra

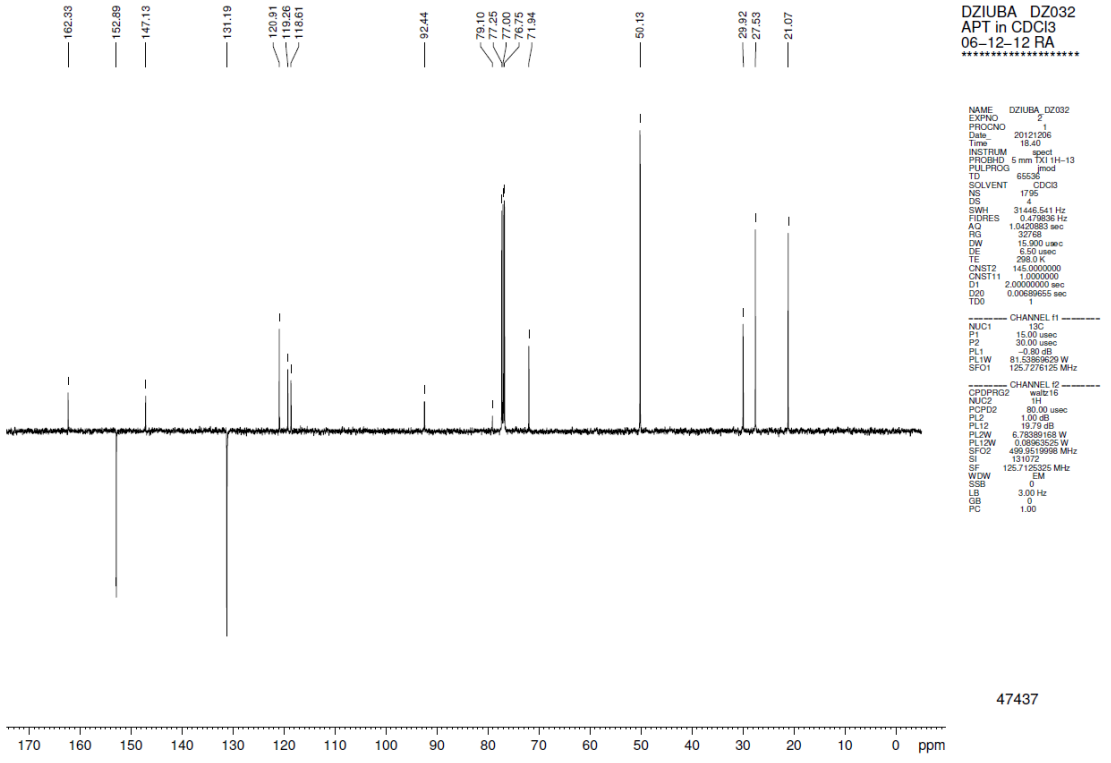
^{1}H and ^{13}C spectra of intermediate **V**





^{1}H and APT spectra of intermediate **VI**





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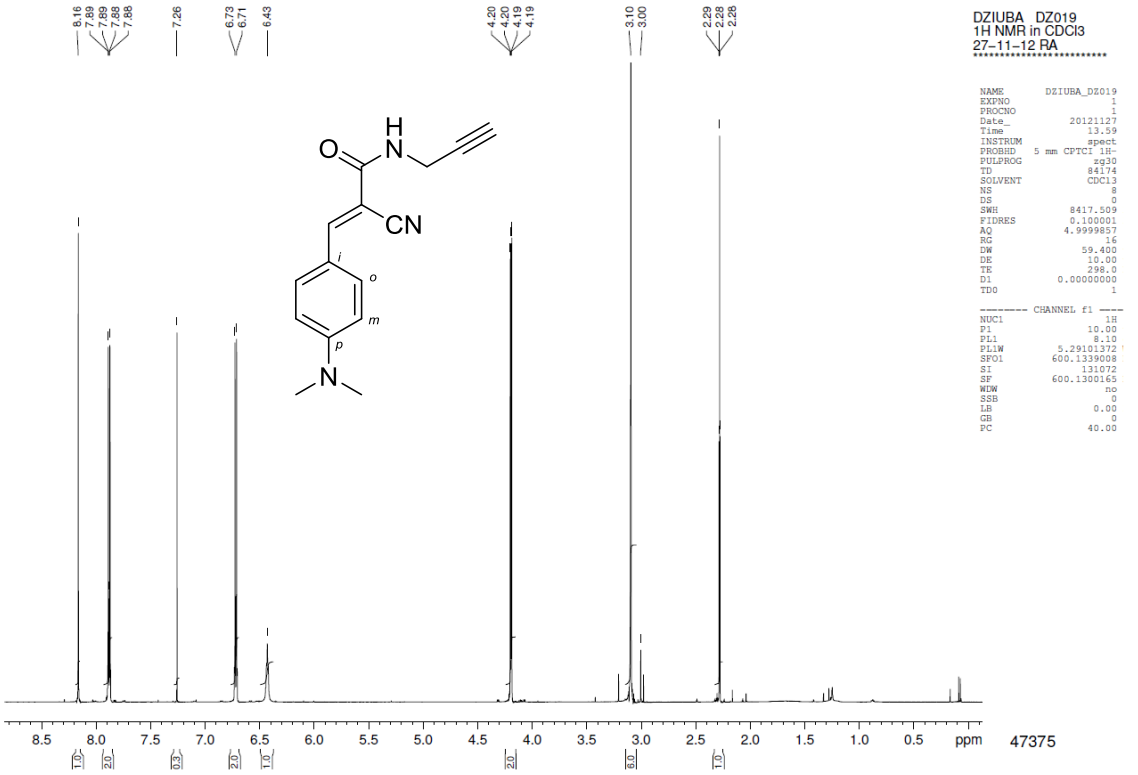
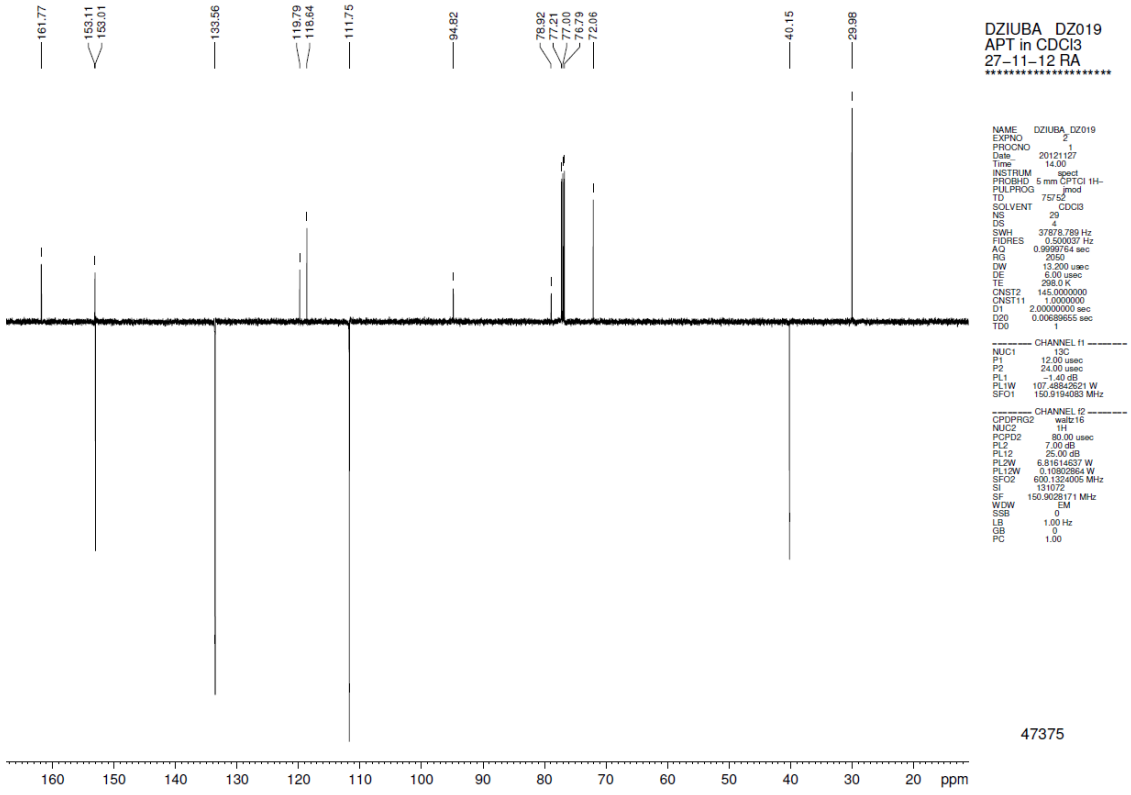
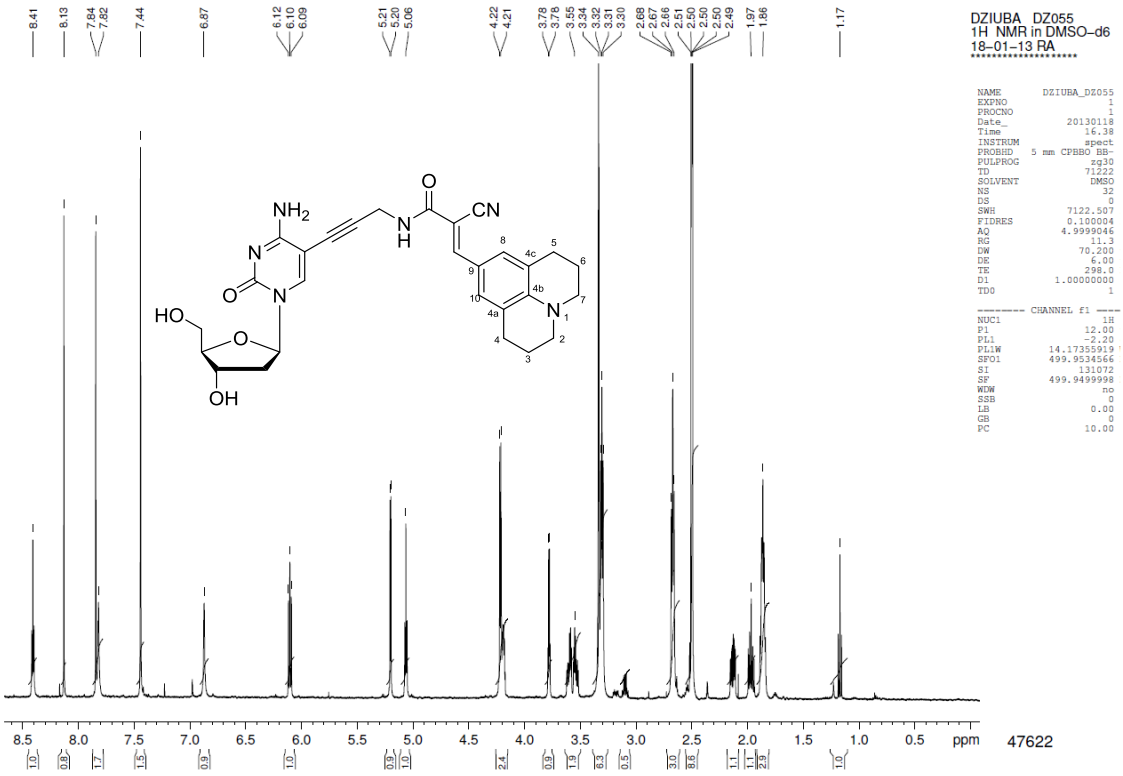
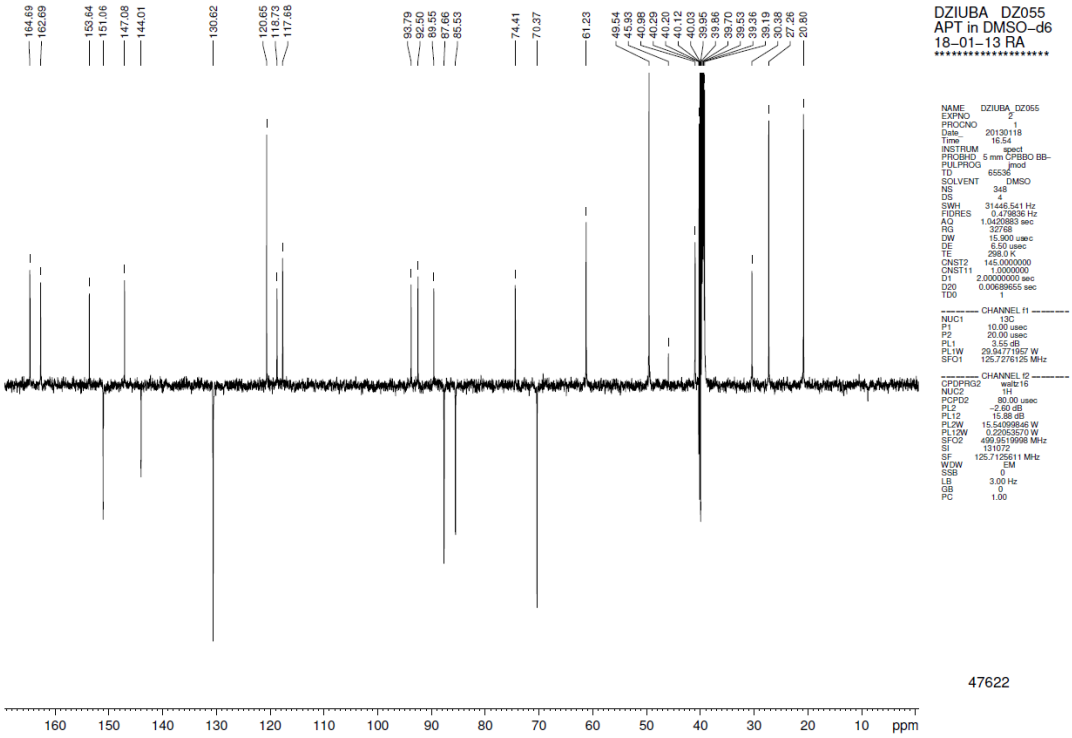


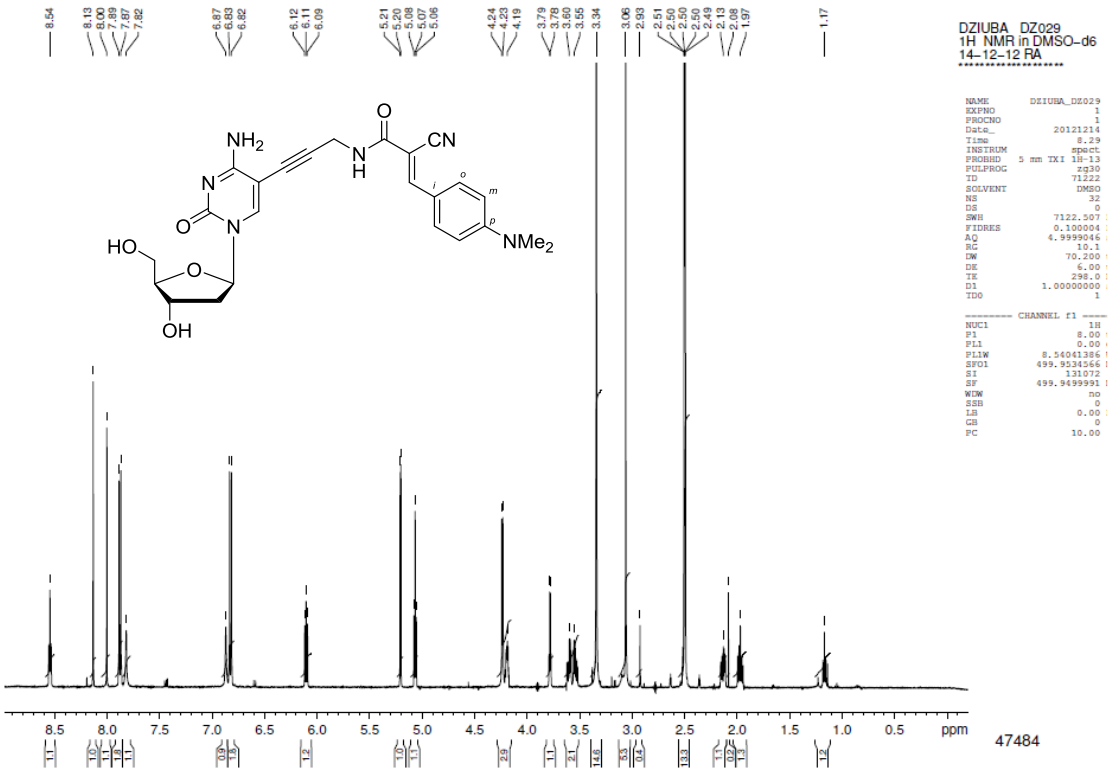


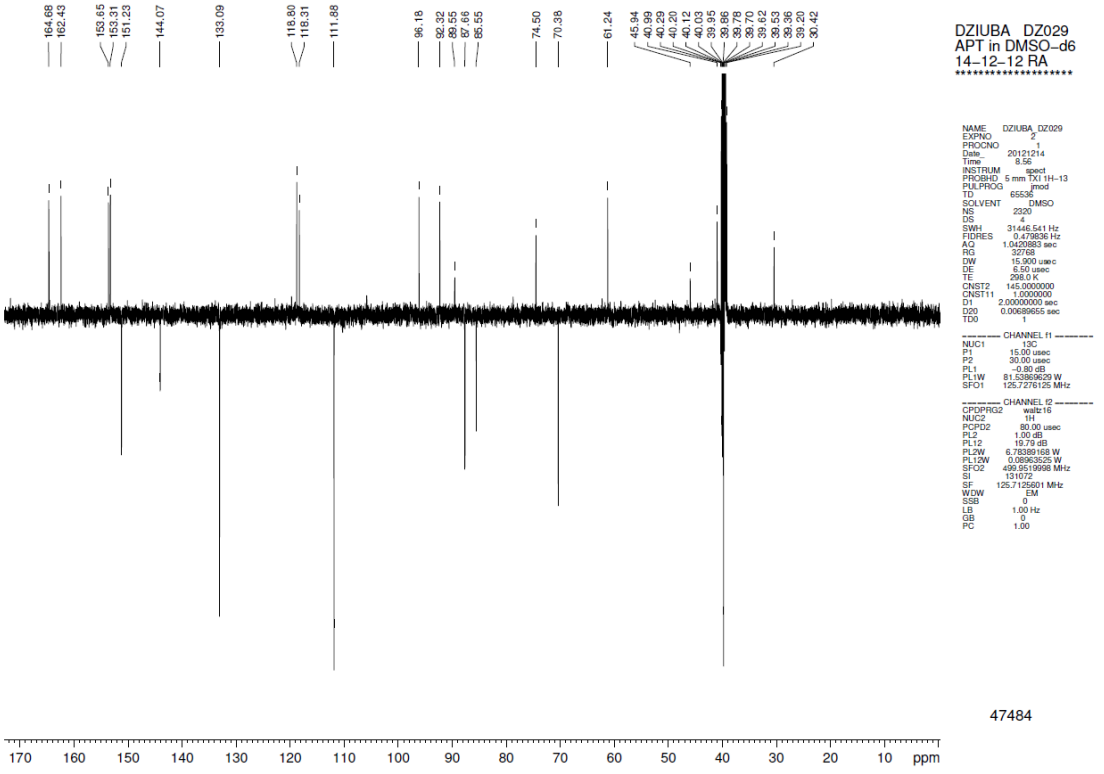
^{1}H and APT spectra of nucleoside **dC^{VJ}** (**3a**)





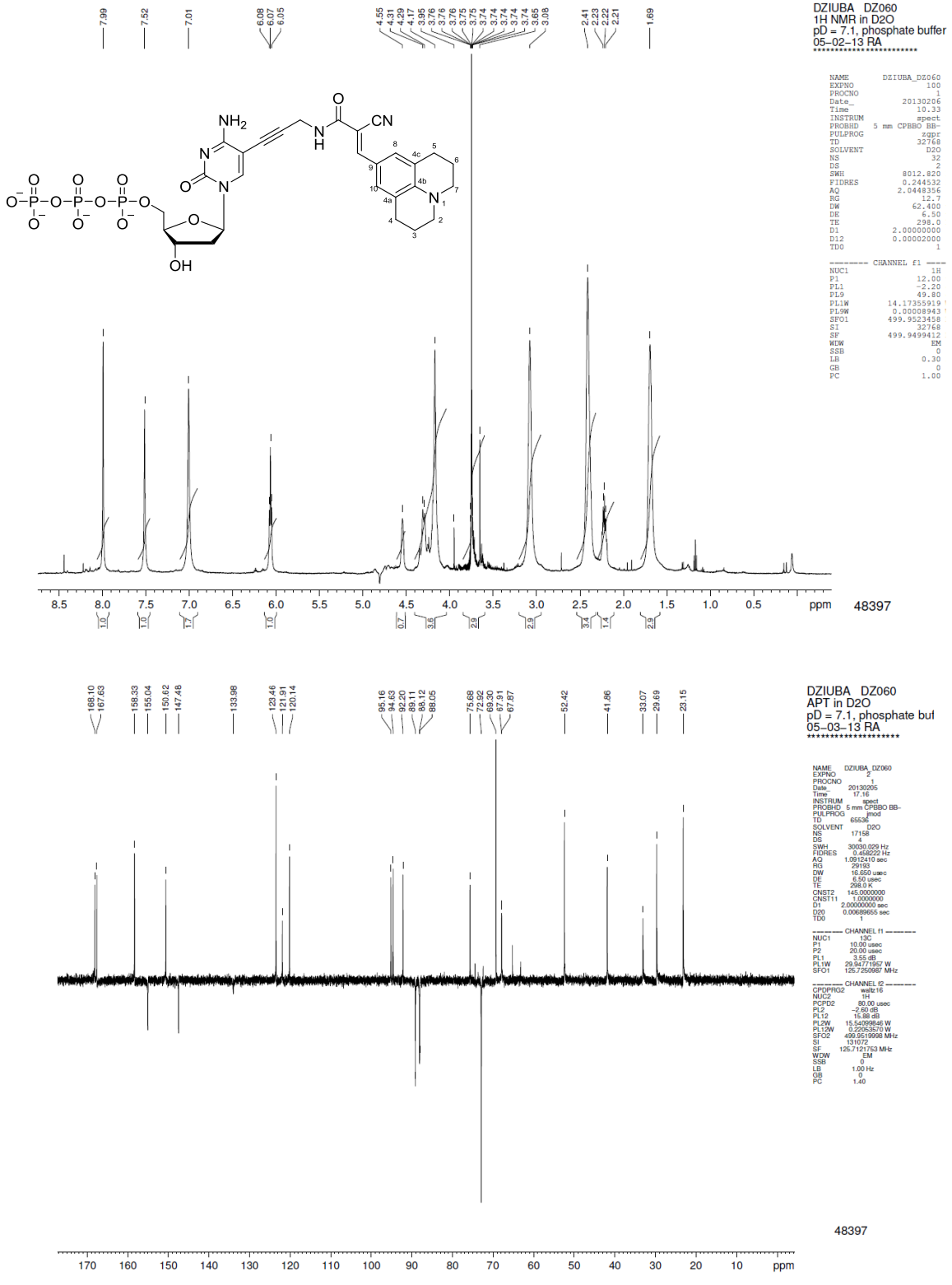
^{1}H and APT spectra of nucleoside **dC^{VDP}** (**3b**)

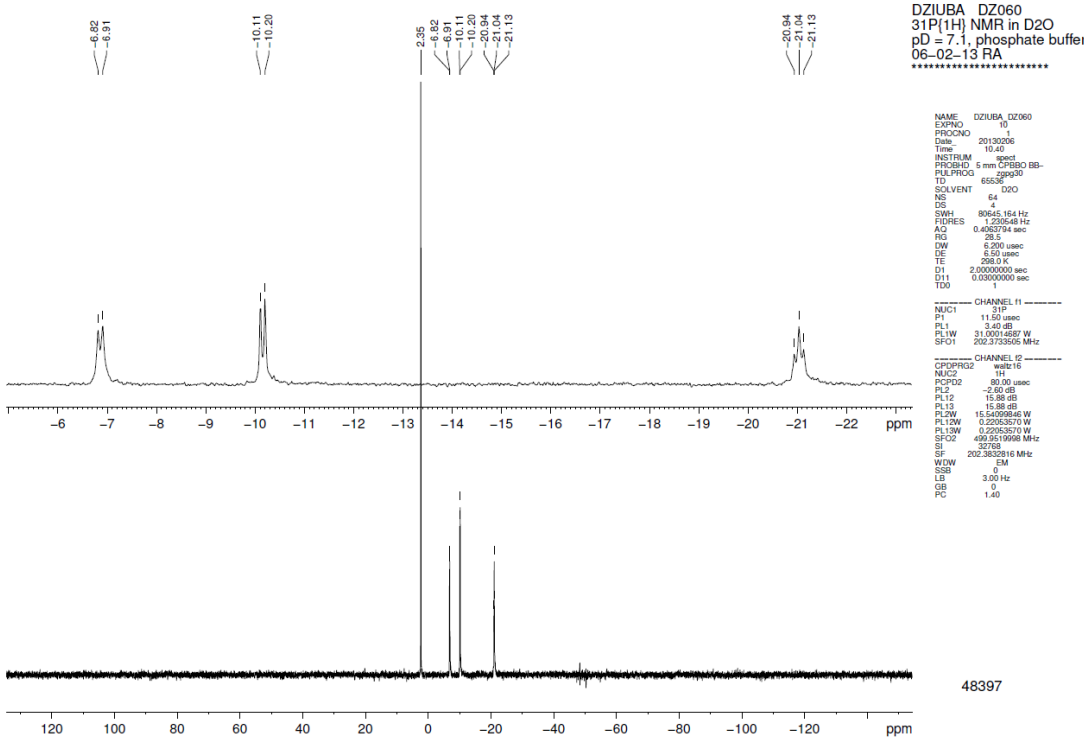




E

^{1}H, APT and ^{31}P spectra of **dC^{VJ}TP** (**4a**)

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06-02-13 RA
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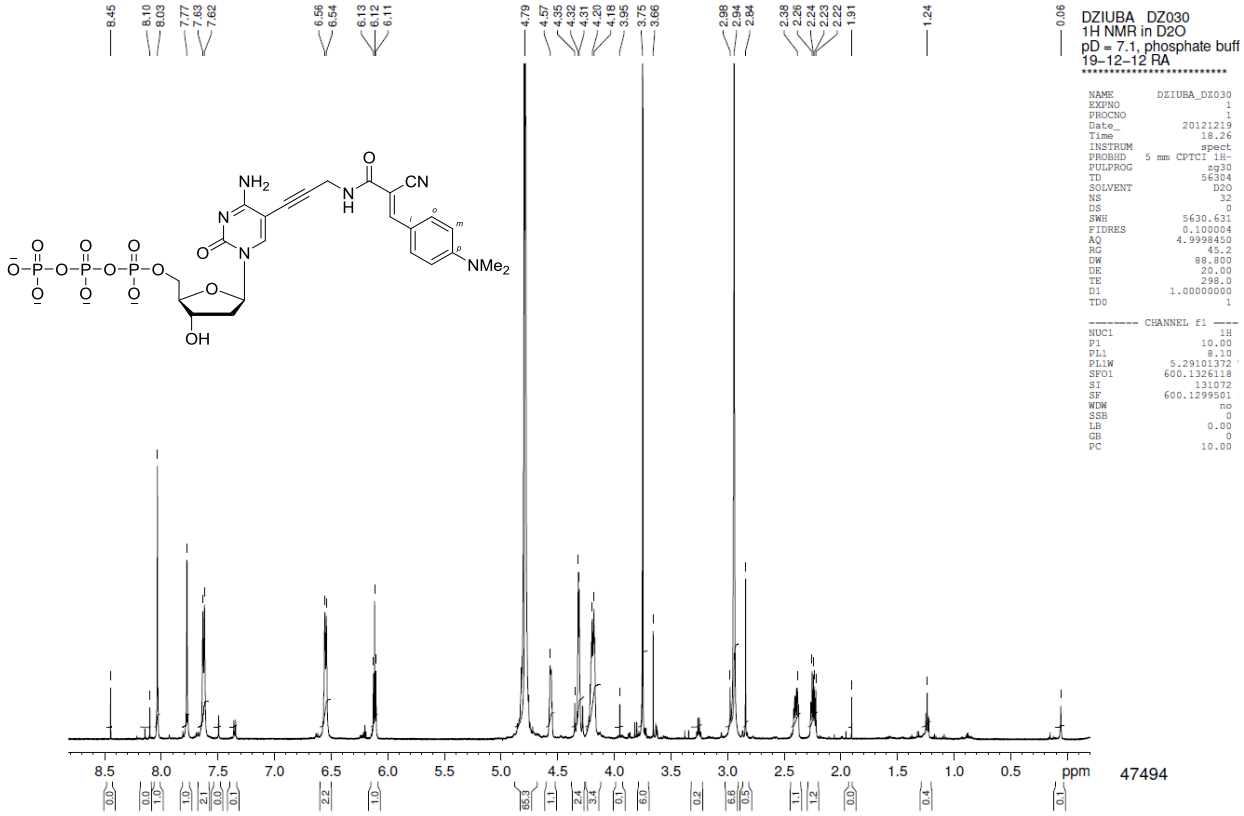
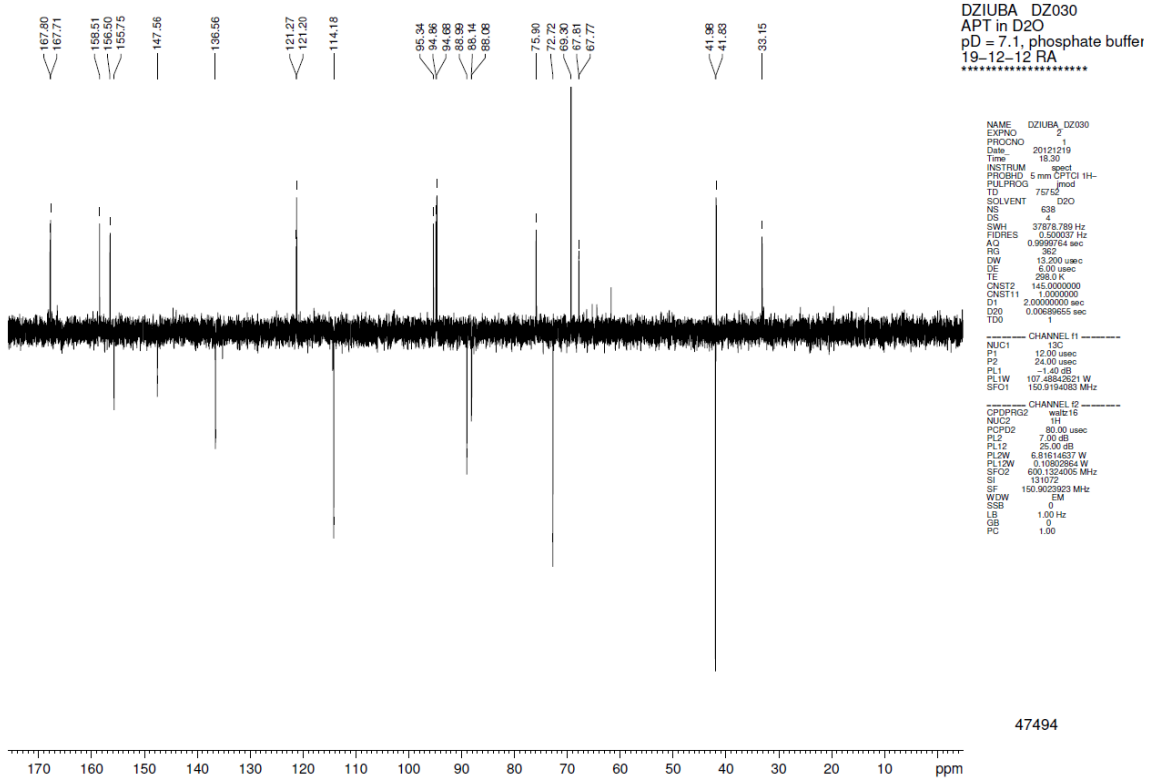
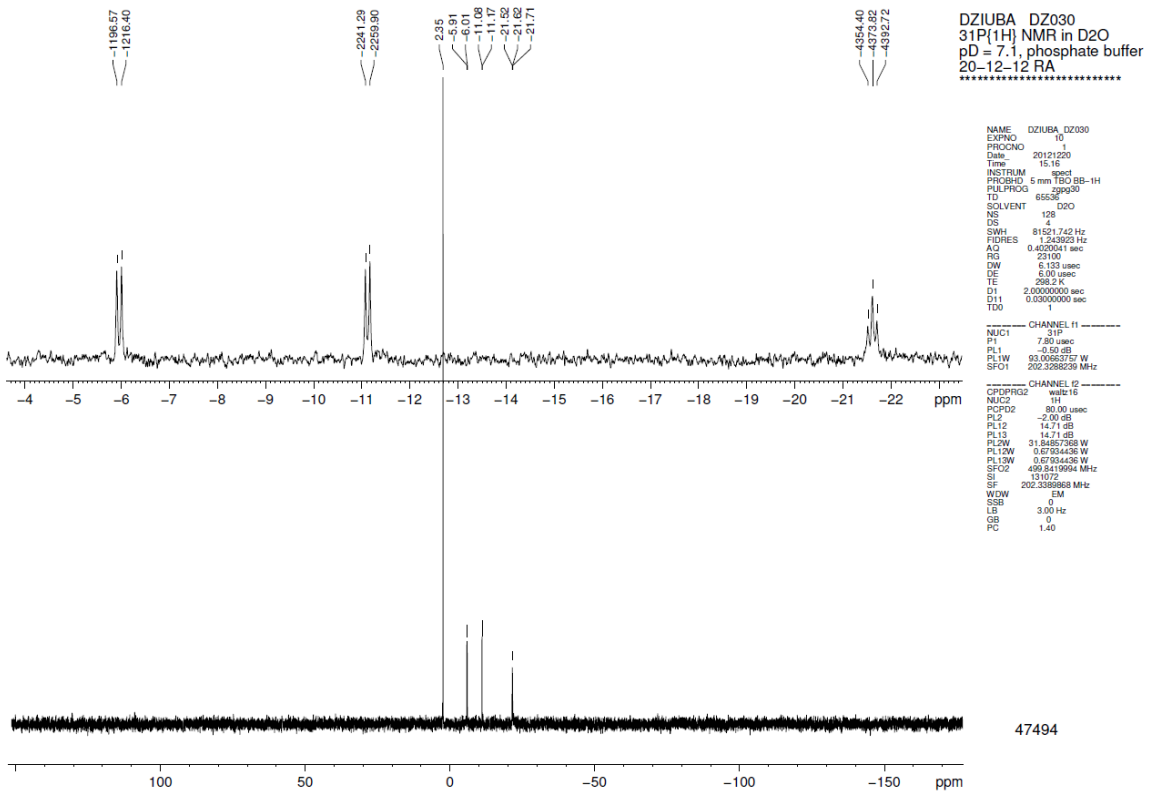
Date_ 20130206
Time 10.40
INSTRUM spect
PROBHD 5 mm CPBBO BB-
PULPROG zgpg30
TD 65536
SOLVENT D2O
NS 64
DS 4
SWH 80645.164 Hz
FIDRES 1.230548 Hz
AQ 0.4063794 sec
RG 28.5
DW 6.200 usec
DE 6.50 usec
TE 298.0 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 1
CHANNEL f1
NUC1 31P
P1 11.50 usec
PL1 3.40 dB
PL1W 31.00014687 W
SFO1 202.3733505 MHz
CHANNEL f2
CPDPRG2 waltz16
NUC2 1H
PCPD2 80.00 usec
PL2 -2.60 dB
PL12 15.88 dB
PL13 15.88 dB
PL2W 15.54099846 W
PL12W 0.22053570 W
PL13W 0.22053570 W
SFO2 499.9519998 MHz
SI 32768
SF 202.3832816 MHz
WDW EM
SSB 0
LB 3.00 Hz
GB 0
PC 1.40
48397

^{1}H, APT and ^{31}P spectra of **dC^{VDP}TP (4b)**



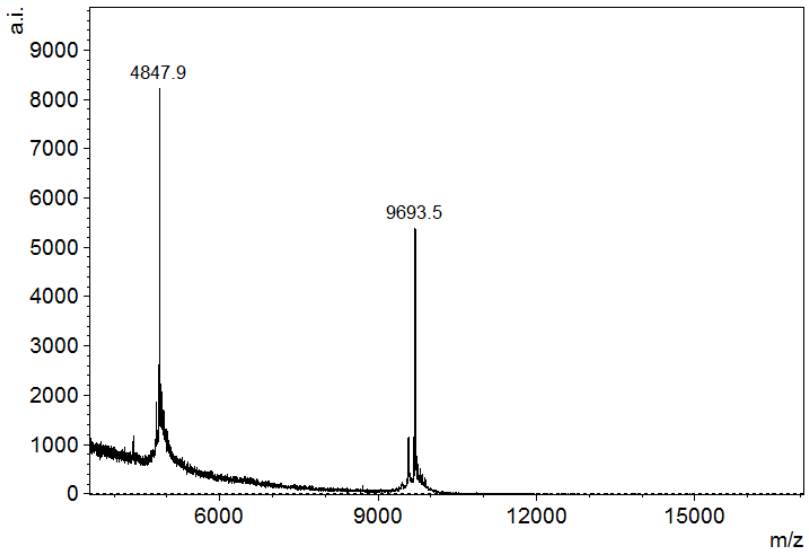



-1196.57
-1216.40
-2241.29
-2259.90
2.35
-5.91
-6.01
-11.08
-11.17
-21.52
-21.62
-21.71
-4354.40
-4373.82
-4392.72
DZIUBA DZ030
31P{1H} NMR in D2O
pD = 7.1, phosphate buffer
20-12-12 RA
****
NAME DZIUBA_DZ030
EXPNO 10
PROCNO 1
Date_ 20121220
Time 15.16
INSTRUM spect
PROBHD 5 mm TBO BB-1H
PULPROG zgpg30
TD 65536
SOLVENT D2O
NS 128
DS 4
SWH 81521.742 Hz
FIDRES 1.243923 Hz
AQ 0.4020041 sec
RG 23100
DW 6.133 usec
DE 6.00 usec
TE 298.2 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 1
CHANNEL f1
NUC1 31P
P1 7.80 usec
PL1 -0.50 dB
PL1W 93.00683757 W
SFO1 202.3288239 MHz
CHANNEL f2
CPDPRG2 waltz16
NUC2 1H
PCPD2 80.00 usec
PL2 -2.00 dB
PL12 14.71 dB
PL13 14.71 dB
PL2W 31.84857368 W
PL12W 0.67934436 W
PL13W 0.67934436 W
SFO2 499.8419994 MHz
SI 131072
SF 202.3389868 MHz
WDW EM
SSB 0
LB 3.00 Hz
GB 0
PC 1.40
-4 -5 -6 -7 -8 -9 -10 -11 -12 -13 -14 -15 -16 -17 -18 -19 -20 -21 -22 ppm
100 50 0 -50 -100 -150 ppm
47494

H. Copies of MALDI spectra

See Table S2 for sequences

ON1



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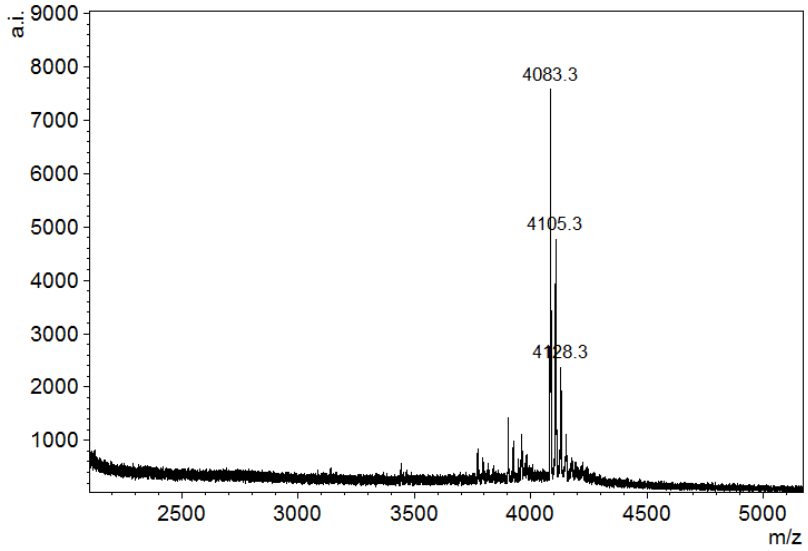
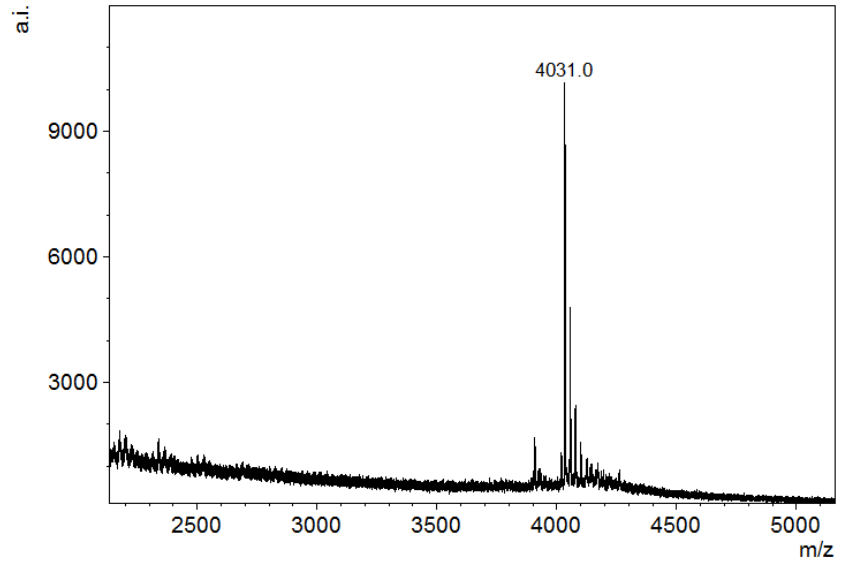


ON3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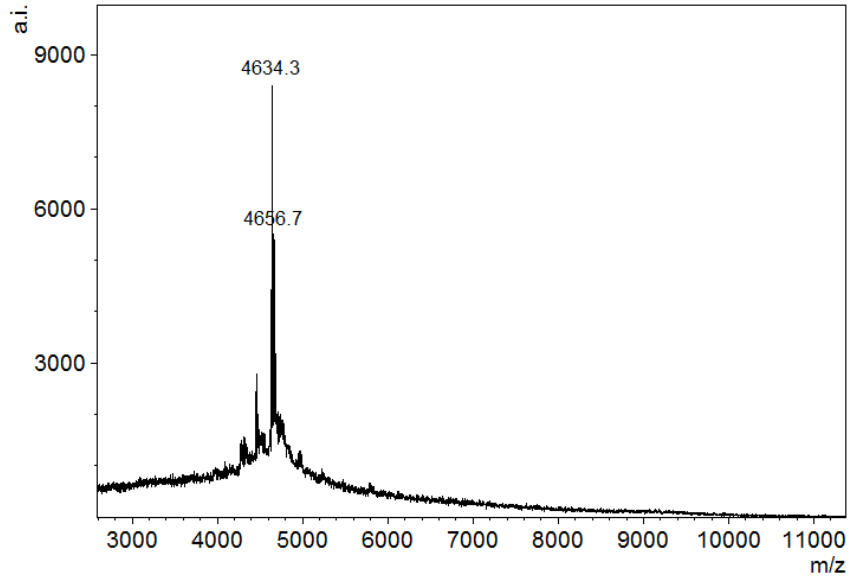
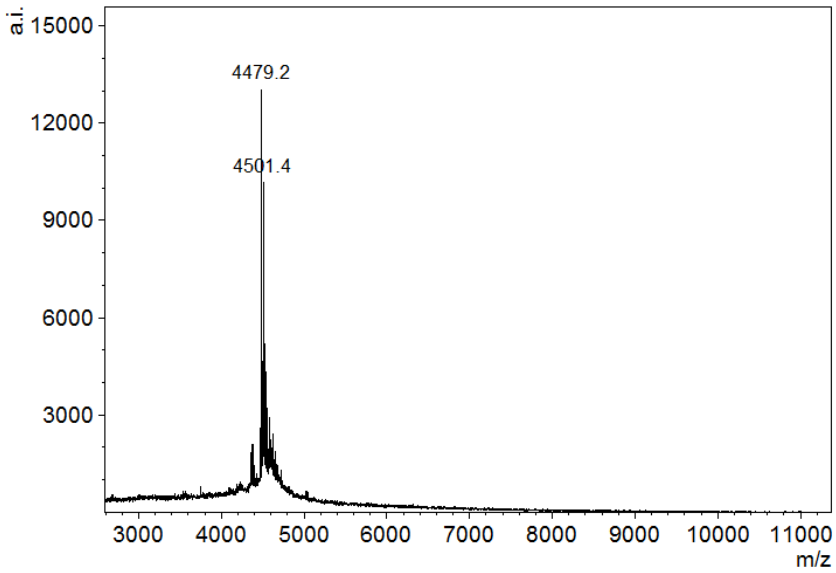
4031.0
9000
6000
3000
2500
3000
3500
4000
4500
5000
m/z

ON4


a.i.
9000
6000
3000
4634.3
4656.7
3000
4000
5000
6000
7000
8000
9000
10000
11000
m/z

ON5


a.i.
15000
12000
9000
6000
3000
4479.2
4501.4
3000
4000
5000
6000
7000
8000
9000
10000
11000
m/z