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PAPER

Synthesis of nucleoside mono- and triphosphates bearing oligopyridine ligands, their incorporation into DNA and complexation with transition metals†

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Modified nucleoside mono- (**dA^{R}MP**s and **dC^{R}MP**s) and triphosphates (**dA^{R}TP**s and **dC^{R}TP**s) bearing bipyridine or terpyridine ligands attached *via* acetylene linker were prepared by single-step aqueous-phase Sonogashira cross-coupling of 7-iodo-7-deaza-dAMP or -dATP, and 5-iodo-dCMP or -dCTP with the corresponding bipyridine- or terpyridine-linked acetylenes. The modified **dN^{R}TP**s were successfully incorporated into the oligonucleotides by primer extension experiment (PEX) using different DNA polymerases and the PEX products were used for post-synthetic complexation with Fe^{2+}.

Introduction

Nucleic acids bearing diverse functional groups are attracting growing interest due to their potential applications in chemical biology, bioanalysis, or nanotechnology.[1] Transition metal complexes within (metal-base-pairs[2] or intercalators[3]) and outside (metal complex covalently attached to a nucleobase, sugar or phosphate)[4–7] the DNA duplex have been extensively studied. Diverse oligopyridine-metal complexes attached to DNA have been prepared and applied in stabilization of duplexes,[4] redox[5] and photoredox[6] labeling or for self assembly of 2D and 3D nanostructures.[7] We[8,9] and others[10] have previously developed efficient syntheses of bipyridine-(bpy) and terpyridine-(tpy) modified nucleoside building blocks. However, despite the apparent application potential, chemical synthesis of oligopyridine-nucleobase linked oligonucleotides (ONs) and their post-synthetic complexations have been reported very scarcely.[11] In parallel with our project, the Wagenknecht group has developed[12] a chemical synthesis of tpy-linked ONs and their complexation with Ni^{2+} and Zn^{2+}.

Apart from chemical synthesis,[13] base-modified DNA can be also prepared enzymatically[14,15] by polymerase incorporation of base- modified 2′-deoxyribonucleoside triphosphates (dNTPs) bearing substituents at position 5 of pyrimidines or at position 7 of 7-deazapurines. Combination of a direct cross-coupling modification of dNTPs with enzymatic incorporation resulted in efficient and general two-step synthesis of base-modified DNA.[16] Here we report the synthesis of oligopyridine-linked nucleotides and dNTPs and enzymatic synthesis and complexation of oligopyridine-linked DNA.

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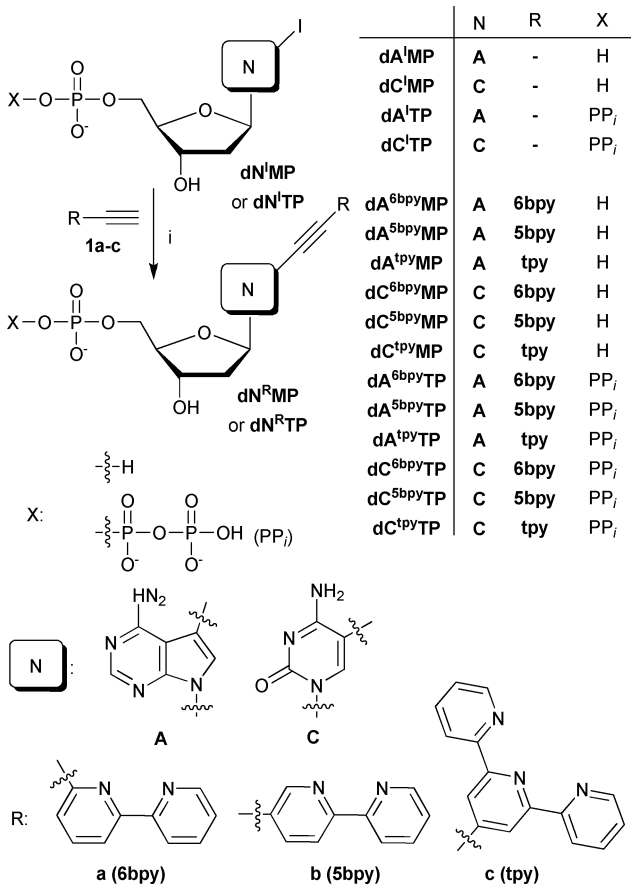
Result and discussion

Synthesis of modified nucleotides and dNTPs

In order to prepare modified oligonucleotides *via* enzymatic incorporation, oligopyridine ligands had to be attached to the nucleobase in dNTPs *via* suitable linker (in our case rigid, linear and electron-conjugate acetylene tether). To develop the synthetic methodology and complexation of ONs, the chemistry was first performed on model nucleosides monophosphates (dNMPs). The first target compounds of our choice were 7-substituted 7-deaza-2′-deoxyadenosine (**dA^{R}MP**s) and 5-substituted 2′-deoxycytidine 5′-monophosphates (**dC^{R}MP**s). They were prepared by single-step aqueous-phase Sonogashira cross-coupling reaction of halogenated nucleoside monophosphates **dA^{I}MP** and **dC^{I}MP** with terminal acetylenes **1a–c**[18] (Scheme 1, Table 1). The reactions were generally performed in the presence of $Pd(OAc)_2$, water soluble tris(3-sulfonatophenyl)phosphane (TPPTS) ligand, CuI and Hünnig base in the mixture water/acetonitrile (2 : 1) at 80 °C for 1.5 h. The desired corresponding products **dA^{6bpy}MP, dA^{5bpy}MP, dA^{tpy}MP** and **dC^{6bpy}MP, dC^{5bpy}MP, dC^{tpy}MP** were isolated after the

Table 1 Synthesis of modified dN^{R}MPs by Sonogashira cross-coupling

Entry	Monophosphate	Alkyne	Product	Yield
1	**dC^{I}MP**	**1a**	**dC^{6bpy}MP**	89%
2	**dC^{I}MP**	**1b**	**dC^{5bpy}MP**	89%
3	**dC^{I}MP**	**1c**	**dC^{tpy}MP**	47%
4	**dA^{I}MP**	**1a**	**dA^{6bpy}MP**	52%
5	**dA^{I}MP**	**1b**	**dA^{5bpy}MP**	70%
6	**dA^{I}MP**	**1c**	**dA^{tpy}MP**	57%



	N	R	X
dA^{I}MP	A	-	H
dC^{I}MP	C	-	H
dA^{I}TP	A	-	PP_i
dC^{I}TP	C	-	PP_i
dA^{6bpy}MP	A	6bpy	H
dA^{5bpy}MP	A	5bpy	H
dA^{tpy}MP	A	tpy	H
dC^{6bpy}MP	C	6bpy	H
dC^{5bpy}MP	C	5bpy	H
dC^{tpy}MP	C	tpy	H
dA^{6bpy}TP	A	6bpy	PP_i
dA^{5bpy}TP	A	5bpy	PP_i
dA^{tpy}TP	A	tpy	PP_i
dC^{6bpy}TP	C	6bpy	PP_i
dC^{5bpy}TP	C	5bpy	PP_i
dC^{tpy}TP	C	tpy	PP_i

Scheme 1 Synthesis of modified dN^{R}MPs and dN^{R}TPs. Reagents and conditions: i) $Pd(OAc)_2$ (5 mol%), TPPTS (5 equiv. to Pd), CuI (10 mol%), $Et(i\text{-}Pr)_2N$ (10 equiv.), H_2O/CH_3CN (2 : 1), 80 °C.

Table 2 Synthesis of modified dN^{R}TPs by Sonogashira cross-coupling

Entry	Triphosphate	Alkyne	Product	Yield
1	**dC^{I}TP**	**1a**	**dC^{6bpy}TP**	67%
2	**dC^{I}TP**	**1b**	**dC^{5bpy}TP**	59%
3	**dC^{I}TP**	**1c**	**dC^{tpy}TP**	69%
4	**dA^{I}TP**	**1a**	**dA^{6bpy}TP**	42%
5	**dA^{I}TP**	**1b**	**dA^{5bpy}TP**	48%
6	**dA^{I}TP**	**1c**	**dA^{tpy}TP**	40%

purification on reverse-phase HPLC in good yields (from 47% to 89%, Table 1).

Having developed the methodology for modification of nucleotides, we have proceeded with the direct functionalization of dNTPs by Sonogashira cross-coupling, in analogy to our previously developed procedures[5,16] (Scheme 1, Table 2). To avoid the hydrolysis of the starting and final triphosphates, the reaction mixture was heated at 80 °C only for 1 h.[5,16] The corresponding products **dA^{6bpy}TP**, **dA^{5bpy}TP**, and **dA^{tpy}TP**, as well as **dC^{6bpy}TP**, **dC^{5bpy}TP**, and **dC^{tpy}TP** were isolated after the purification on reverse-phase HPLC in good yields (40–48% for **dA^{R}TPs** and 59–69% for **dC^{R}TPs**, Table 2).

Incorporation of modified dNTPs by DNA polymerases

All the functionalized **dA^{R}TPs** or **dC^{R}TPs** were tested as substrates for several thermostable DNA polymerases in primer

Table 3 Primer and templates used for primer extension[a]

prim^{rnd}	5′-CATGGGCGGCATGGG-3′
temp^{rnd16}	5′-**CTAGCATGAGCTCAGT***CCCATGCCGCCCATG*-3′
temp^{C}	5′-**CCCG***CCCATGCCGCCCATG*-3′
temp^{A}	5′-**CCCT***CCCATGCCGCCCATG*-3′
temp^{A1}	5′-**T***CCCATGCCGCCCATG*-3′

[a] In the template (temp) ONs segments that form a duplex with primer are printed in *italics*, the replicated segments are printed in **bold**. For magnetic separation of the extended primer strands, the templates 5′-end biotinylated. The acronyms used in the text for primer products are analogs to those introduced for templates (*e.g.* the PEX product pex^{rnd16} was synthesized on temp^{rnd16} template.

extension experiment (PEX). Each PEX experiment, analyzed by denaturing polyacrylamide gel electrophoresis (PAGE), was compared with positive (all four natural dNTPs) and negative control experiments (absence of one natural dNTPs) in order to exclude any mis-incorporations. Single and multiple incorporation of oligopyridine-functionalized triphosphate were tested (for sequences of primer and templates see Table 3).

Single-nucleotide extension experiments were tested separately with each of the three **dA^{R}TPs** and **dC^{R}TPs** by using 19-mer templates temp^{A} and temp^{C} (Fig. 1). Experiments using Pwo polymerase were mostly successful to give fully extended products for all of the dNTPs except **dC^{tpy}TP** (lane 11), which gave the mixture of fully and partially extended ONs. Therefore the reaction condition for incorporation of **dC^{R}TPs** were further optimized and the use of DyNAzyme II enzyme leads to fully extended products. The lack of extension in negative control (A- or C-) proves that no miss-incorporation occurred. Each spot of a PEX-product (even in the positive and negative control experiments) is accompanied by a weak band of one nucleotide shorter product due to 3′-5′ exonuclease activity of the enzyme.

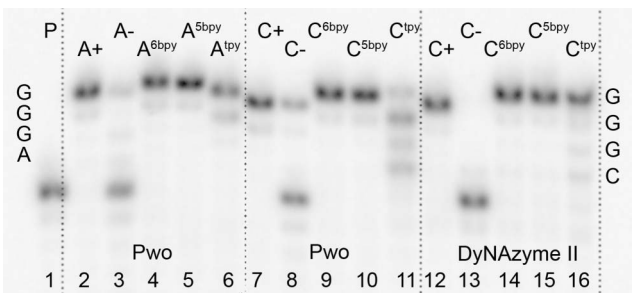


Fig. 1 Denaturing PAGE analysis of PEX experiment synthetized on temp^{A} (lines 2–6) and temp^{C} (lines 7–16) with Pwo or DyNAzyme II polymerases. 5′-^{32}P-end labelled primer-template was incubated with different combinations of natural and functionalized dNTPs. P: Primer; A+: natural dATP, dGTP; A-: dGTP; A^{6bpy}: **dA^{6bpy}TP**, dGTP; A^{5bpy}: **dA^{5bpy}TP**, dGTP; A^{tpy}: **dA^{tpy}TP**, dGTP; C+: natural dCTP, dGTP; C-: dGTP; C^{6bpy}: **dC^{6bpy}TP**, dGTP; C^{5bpy}: **dC^{5bpy}TP**, dGTP; C^{tpy}: **dC^{tpy}TP**, dGTP.

In order to compare the efficiency in incorporation of the oligopyridine-modified dNTPs in comparison with the natural ones, we have performed a simple kinetics study in single-nucleotide PEX reaction. The experiments with unmodified and modified dATPs were performed using Pwo polymerase and two modified **dA^{R}TPs** in comparison with the natural dATP (Fig. 2). The PEX with the natural dATP was finished within 1 min whereas the PEX with **dA^{5bpy}TP** took 2 min and, with the more bulky

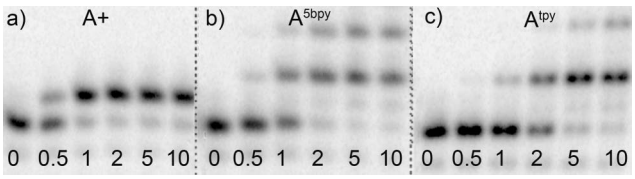


Fig. 2 Comparison of the rate of the single-nucleotide PEX with natural A+ (dATP) and modified (**dA^{5bpy}TP**, **dA^{tpy}TP**) nucleotides using Pwo polymerase with temp^{A1} without natural dGTP. The reaction mixtures were incubated for time intervals indicated (in minutes), followed by stopping the reaction by addition of PAGE loading buffer and immediate heating.

dA^{tpy}TP, even 5 min to complete. This shows that the modified **dA^{R}TP**s are worse substrates for the polymerase. Therefore, the reaction time for multiple incorporations must be prolonged to 30 min to ensure full length product formation.

Multiple incorporations were tested on 31-mer template temp^{rnd16} containing four copies of each of the four bases and requiring incorporation of four modified **dN^{R}TP**s in separate positions. Several polymerases were tested: Pwo, DyNAzyme II, Vent (*exo*$^{-}$), Deep Vent, Deep Vent (*exo*$^{-}$), Phusion, KOD XL and Therminator. Incorporation of **dA^{R}TPs** using Pwo or Deep Vent leads to fully extended ONs (Fig. 3, lanes 5–7), while incorporation of **dC^{6bpy}TP** and **dC^{tpy}TP** (lanes 8 and 10) gave a mixture of ONs of different length (for incorporation using Deep Vent and other polymerasess see ESI†). Even after the optimization of condition (testing different DNA polymerases, higher concentration of enzyme and **dC^{R}TPs**) the incorporation of **dC^{tpy}TP** was less feasible and resulted in early termination of PEX (lane 15). Five time higher concentration of DyNAzyme II DNA polymerase in combination with double concentration of **dC^{R}TP** was the most efficient in the incorporation of **dC^{R}TP**. The products of pex^{rnd16} slightly differ in electrophoretic mobilities visible on gel partly (combination of the effects of the higher molecular

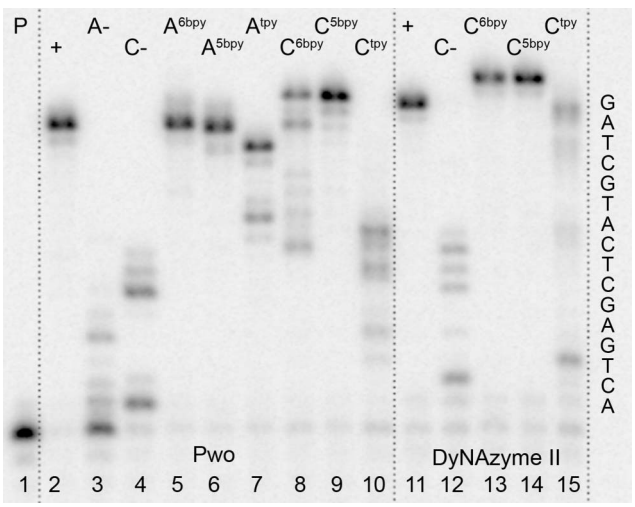


Fig. 3 Denaturing PAGE analysis of PEX experiment synthesized on temp^{rnd16} with Pwo and DyNAzyme II polymerases. 5′-^{32}P-end labelled primer-template was incubated with different combinations of natural and functionalized dNTPs. P: Primer; +: natural dNTPs; A-: dTTP, dCTP, dGTP; C-:dATP, dTTP, dGTP; A^{6bpy}: **dA^{6bpy}TP**, dTTP, dCTP, dGTP; A^{5bpy}: **dA^{5bpy}TP**, dTTP, dCTP, dGTP; A^{tpy}: **dA^{tpy}TP**, dTTP, dCTP, dGTP; C^{6bpy}: **dC^{6bpy}TP**, dATP, dTTP, dGTP; C^{5bpy}: **dC^{5bpy}TP**, dATP, dTTP, dGTP; C^{tpy}: **dC^{tpy}TP**, dATP, dTTP, dGTP.

weight and possible secondary structure formation). Therefore, the successful incorporation and full-length products were verified by measurement of MALDI mass spectra of PEX products. Single stranded DNA was prepared by PEX with biotinylated templates and then isolated by magnetoseparation.[16b] The correct masses were confirmed for the fully-extended products (see ESI†).

Complexation studies

All six nucleoside monophosphates (**dN^{R}MP**s) were tested as model compounds for further complexation studies on oligopyridine modified oligonucleotides. Aqueous solutions of **dN^{R}MP**s were mixed with 0.5 equiv. of divalent metal such as Cu^{2+}, Zn^{2+}, Ni^{2+} and Fe^{2+}. After incubation for 10 min. at room temperature, the complex formation was detected by UV/Vis spectroscopy. The spectra were recorded for the non-metalated and metalated monophospate. While MLCT bands of bpy- or tpy-modified monophosphates with Cu^{2+}, Zn^{2+}, Ni^{2+} are partially overlapping with dominating absorbance at 350 nm due to bpy and tpy ligands (see ESI†), the complex formed by mixing **dN^{tpy}MP** with Fe^{2+} can be easily detected due to characteristic absorbance at 580 nm[17] (Fig. 4, magenta line).

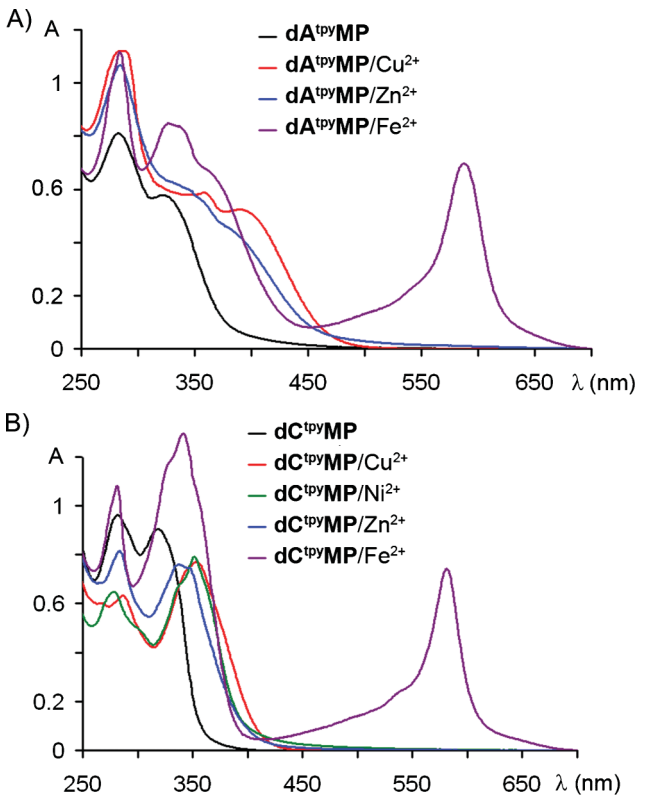


Fig. 4 UV/Vis spectra of: A) **dA^{tpy}MP**, B) **dC^{tpy}MP** with divalent metals.

After successful complexation of nucleoside monophosphates, we proceeded with complexation of tpy-modified oligonucleotides with Fe^{2+}, due to this easy detectable MLCT band. For the complexation studies detected by UV/Vis spectroscopy, we have chosen ON prepared by PEX on larger scale using Deep Vent polymerase and template temp^{rnd16}, due to the higher concentration of tpy-modification in product. Natural DNA was prepared by incorporaion of dCTP, dGTP, dTTP and dATP, while the modified

ON was prepared by using **dA^{tpy}TP** as surrogates of natural dATP. Oligonucleotides had to be purified to remove unincorporated **dA^{tpy}TP**. After addition of 0.5 equiv. of $Fe(BF_4)_2{\cdot}6H_2O$ per each modification and incubation for several hours at room temperature, the UV/Vis spectra were recorded for non-metalated and metalated DNA duplexes, either natural or modified one (Fig. 5). The dominant absorbance at *ca.* 260 nm originates from the absorbance of natural nucleotides (black line), small absorbance band at *ca.* 350 nm is due to the presence of tpy-modification (green line) while the band at *ca.* 590 is the MLCT band[7c] of complex formed by tpy-modified ON with Fe^{2+} (blue line). Similar MLCT band is not observed for the natural DNA mixed with Fe^{2+} (red line).

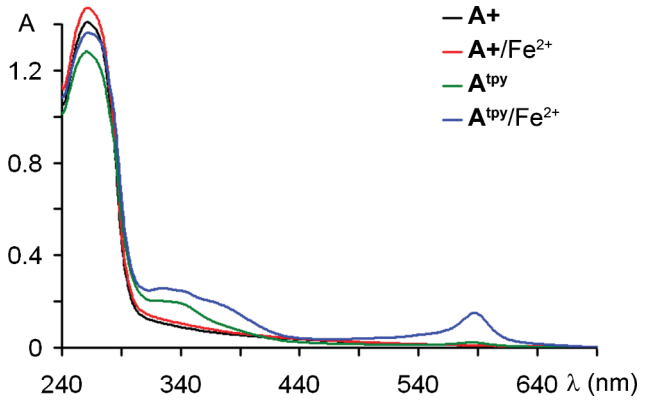


Fig. 5 UV/Vis spectra of natural and modified DNA duplexes with $Fe(BF_4)_2{\cdot}6H_2O$.

The complex formation was also detected by native polyacrylamide gel electrophoresis. For the first experiments (monoincorporations), ONs were prepared by the PEX experiment using temp^A and dATP (natural DNA) or **dA^{tpy}TP**, or temp^C and dCTPs (natural DNA) or **dC^{tpy}TP**. The PEX products were directly, without previous purification, mixed with 1 eqiv. of $Fe(BF_4)_2{\cdot}6H_2O$ or $FeCl_2$ (calculated to the amount of modified **dN^{tpy}TP** in PEX experiment) and incubated at room temperature for 3 h. Successful complex formation of pex^A and pex^C, containing one tpy-modification with Fe^{2+} ions is clearly shown by bands with slower mobility (Fig. 6, lanes 4 and 8), while no change in mobility was not observed for natural DNA mixed with Fe^{2+} ions (lanes 2 and 6).

Similar results were shown also for pex^{rnd16} containing four tpy-modifications (Fig. 7). Since the **dC^{tpy}TP** was not a good substrate in multiple incorporations, this experiment was only performed with **A^{tpy}**. Here the bands of the complexes are more smeared since mixtures of possible products are formed.

Conclusions

Novel **dN^{R}MP**s and **dN^{R}TP**s bearing oligopyridine ligands attached *via* acetylene linker were prepared in single-step aqueous-phase Sonogashira cross-coupling reaction of iodinated **dN^{I}MP**s or **dN^{I}TP**s with corresponding terminal acetylene. Functionalized **dN^{R}TP**s were shown to be good substrates for DNA polymerases and were incorporated into the DNA by primer extension. Pwo polymerase, which was successfully used for incorporation of modified **dA^{R}TP**s, did not incorporate **dC^{R}TP**s with the same

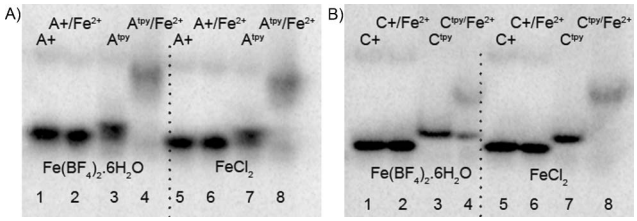


Fig. 6 Non-denaturing gel electrophoresis (8% SB_PAGE) of DNA duplexes in the absence and in the presence of Fe^{2+} for pex^A (A) or pex^C (B). 5′-^{32}P-end labelled primer-template was incubated with different combinations of natural and functionalized dNTPs: A+: unmodified DNA (dATP, dGTP); A+/Fe^{2+}: unmodified DNA (dATP, dGTP) mixed with Fe^{2+}; A^{tpy}: tpy-modified DNA (**dA^{tpy}TP**, dGTP); A^{tpy}/Fe^{2+}: tpy-modified DNA (**dA^{tpy}TP**, dGTP) mixed with Fe^{2+}; C+: unmodified DNA (dCTP, dGTP); C+/Fe^{2+}: unmodified DNA (dCTP, dGTP) mixed with Fe^{2+}; C^{tpy}: tpy-modified DNA (**dC^{tpy}TP**, dGTP); C^{tpy}/Fe^{2+}: tpy-modified DNA (**dC^{tpy}TP**, dGTP) mixed with Fe^{2+}.

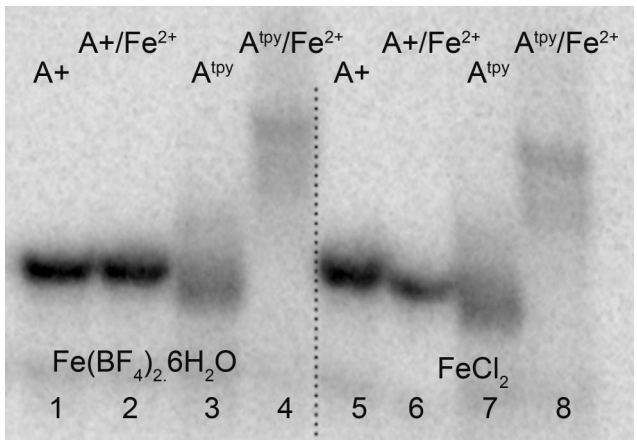


Fig. 7 Non-denaturing gel electrophoresis (8% SB_PAGE) of DNA duplexes in the absence and in the presence of Fe^{2+} pex^{rnd16}. 5′-^{32}P-end labelled primer-template was incubated with different combinations of natural and functionalized dNTPs: A+: unmodified DNA (dATP, dTTP, dCTP, dGTP); A+/Fe^{2+}: unmodified DNA (dATP, dTTP, dCTP, dGTP) mixed with Fe^{2+}; A^{tpy}: tpy-modified DNA (**dA^{tpy}TP**, dTTP, dCTP, dGTP); A^{tpy}/Fe^{2+}: tpy-modified DNA (**dA^{tpy}TP**, dTTP, dCTP, dGTP) mixed with Fe^{2+}.

efficiency. For incorporation of modified **dC^{R}TP**s, DyNAzyme II was identified as the most suitable enzyme, although the multiple incorporation of **dC^{tpy}TP** was still less successful, resulting early termination of PEX experiment. Oligopyridine functionalized ONs, containing either one or four modifications, were successfully used for post-synthetic complexation with Fe^{2+} metals ions. Therefore, the **A^{tpy}**-containing DNA has potential for self-assembly studies.

Experimental

Sonogashira cross-coupling reaction were performed under argon atmosphere. Oligopyridinyl acetylenes,[18] halogenated monophosphates[16g,19] and halogenated triphosphates[16a,c] were prepared according to the literature procedures. Other chemicals were purchased from commercial suppliers and were used as received. Preparative HPLC separations were performed on column packed with 10 μm C18 reversed phase (Phenomenex, Luna C18(2)).

NMR spectra were measured on a Bruker 500 or Bruker 600 (500 or 600 MHz for ^{1}H, 125.7 or 150.9 MHz for ^{13}C and 202.3 for ^{31}P) in D_2O (referenced to dioxane as internal standard, $_{\delta}$H = 3.75 ppm, $_{\delta}$C = 69.3 ppm, standard for ^{31}P NMR was external H_3PO_4) or in CD_3OD (referenced to TMS as an internal standard). Chemical shifts are given in ppm (δ scale), coupling constants (J) in Hz. Complete assignment of all NMR signals was achieved by use of a combination of H,H-COSY, H,C-HSQC, and H,C-HMBC experiments. NMR spectra of dNTPs were measured in phosphate buffer at pH 7.1. Mass spectra were measured on LCQ classic (Thermo-Finnigan) spectrometer using ESI or Q-Tof Micro (Waters, ESI source, internal calibration with lockspray). Mass spectra of functionalized DNA were measured by Maldi-TOF, Reflex IV (Bruker) with nitrogen laser. UV/Vis spectra were measured on Varian CARY 100 Bio spectrophotometer at room temperature.

General procedure for Sonogashira cross-coupling – synthesis of modified dN^{R}MPs

Mixture CH_3CN/H_2O (1 : 2) (1.5 ml) and Et(i-Pr)$_2$N (10 equiv.) were added to an argon-purged flask containing halogenated nucleoside monophosphate **dC^{I}MP** or **dA^{I}MP** (60 mg), an alkyne **1a–c** (1.5 equiv.) and CuI (10 mol%). In a separate flask, Pd(OAc)$_2$ (5 mol%) and TPPTS (5 equiv. to Pd) were combined, evacuated and purged with argon followed by addition of CH_3CN/H_2O (1 : 2) (0.5 ml). The mixture of catalyst was then injected into the reaction mixture and the reaction mixture was stirred at 80 °C for 1.5 h. The solvent was evaporated in vacuo. Products were purified by semi-preparative HPLC on C18 column using linear gradient of 0.1 M TEAB (triethylamonium bicarbonate) in H_2O to 0.1 M TEAB in H_2O/MeOH (1 : 1) as an eluent. Several co-distillations with water and conversion to sodium salt form (Dowex 50 in Na^+ cycle) followed by freeze-drying from water, gave the products as brownish or yellowish powder.

5-[(2″,2‴-bipyridin-6″-yl)ethynyl]-2′-deoxycytidine 5′-*O*-monophosphate (dC^{6bpy}MP)

This compound was prepared according to the general procedure from 5-iodo-2′-deoxycytidine monophosphate **dC^{I}MP** and **1a** in the yield 89%.

^{1}H NMR (600.1 MHz, CD_3OD): 2.26 (ddd, 1H, J_{gem} = 13.7, $J_{2'b,1'}$ = 7.1, $J_{2'b,3'}$ = 6.2, H-2′b); 2.41 (ddd, 1H, J_{gem} = 13.7, $J_{2'a,1'}$ = 6.0, $J_{2'a,3'}$ = 3.6, H-2′a); 4.05 (dt, 1H, J_{gem} = 10.4, $J_{H,P}$ = $J_{5'b,4'}$ = 4.4, H-5′b); 4.10 (m, 1H, H-4′); 4.12 (ddd, 1H, J_{gem} = 10.4, $J_{H,P}$ = 6.0, $J_{5'a,4'}$ = 3.6, H-5′a); 4.56 (dt, 1H, $J_{3',2'}$ = 6.2, 3.6, $J_{3',4'}$ = 3.6, H-3′); 6.27 (dd, 1H, $J_{1',2'}$ = 7.1, 6.0, H-1′); 7.49 (dd, 1H, $J_{5''',4'''}$ = 7.3, $J_{5''',6'''}$ = 4.8, H-5‴); 7.88 (d, 1H, $J_{5'',4''}$ = 7.7, H-5″); 7.99 (m, 2H, H-4″,4‴); 8.23 (d, 1H, $J_{3'',4''}$ = 7.9, H-3″); 8.27 (d, 1H, $J_{3''',4'''}$ = 7.9, H-3‴); 8.45 (s, 1H, H-6); 8.70 (d, 1H, $J_{6''',5'''}$ = 4.8, H-6‴); ^{13}C NMR (150.9 MHz, CD_3OD): 41.66 (CH_2-2′); 65.10 (d, $J_{C,P}$ = 4.5, CH_2-5′); 72.41 (CH-3′); 82.78 (bpy-C≡**C**); 87.88 (CH-1′); 88.36 (d, $J_{C,P}$ = 8.4, CH-4′); 91.71 (C-5); 94.66 (bpy-**C**≡C); 122.26 (CH-3″); 122.90 (CH-3‴); 125.64 (CH-5‴); 128.81 (CH-5″); 138.95 (CH-4‴); 139.44 (CH-4″); 143.86 (C-6″); 147.22 (CH-6); 150.57 (CH-6‴); 156.50 (C-2‴); 156.68 (C-2); 157.58 (C-2″); 166.26 (C-4); ^{31}P NMR (202.3 MHz, CD_3OD): 5.06; MS (ES$^-$): found m/z: 484.2 (M), 485.2 (M+H), 486.2 (M+2H); HRMS (ES$^-$): m/z calcd for $C_{21}H_{19}O_7N_5P$: 484.1028; found: 484.1028.

General procedure for Sonogashira cross-coupling – synthesis of modified dN^{R}TPs

Mixture CH_3CN/H_2O (1 : 2) (1.5 ml) and Et(i-Pr)$_2$N (10 equiv.) were added to an argon-purged flask containing halogenated nucleoside triphosphate **dC^{I}TP** or **dA^{I}TP** (60 mg), an alkyne **1a–c** (1.5 equiv. for **dC^{I}TP** and 2 equiv. for **dA^{I}TP**) and CuI (10 mol%). In a separate flask, Pd(OAc)$_2$ (5 mol%) and TPPTS (5 equiv. to Pd) were combined, evacuated and purged with argon followed by addition of CH_3CN/H_2O (1 : 2) (0.5 ml). The mixture of catalyst was then injected into the reaction mixture and the reaction mixture was stirred at 80 °C for 1 h. The solvent was evaporated in vacuo. Products were purified by semi-preparative HPLC on C18 column using linear gradient of 0.1 M TEAB (triethylamonium bicarbonate) in H_2O to 0.1 M TEAB in H_2O/MeOH (1 : 1) as an eluent. Several co-distillations with water and conversion to sodium salt form (Dowex 50 in Na^+ cycle) followed by freeze-drying from water, gave the products as white or yellow powder.

5-[(2″,2‴-bipyridin-6″-yl)ethynyl]-2′-deoxycytidine 5′-*O*-triphosphate (dC^{6bpy}TP)

This compound was prepared according to the general procedure from 5-iodo-2′-deoxycytidine monophosphate **dC^{I}TP** and **1a** in the yield 67%.

^{1}H NMR (499.8 MHz, D_2O, ref$_{dioxane}$ = 3.75 ppm, pD = 7.1, phosphate buffer): 2.22 (dt, 1H, J_{gem} = 14.1, $J_{2'b,1'}$ = $J_{2'b,3'}$ = 6.8, H-2′b); 2.42 (ddd, 1H, J_{gem} = 14.1, $J_{2'a,1'}$ = 6.3, $J_{2'a,3'}$ = 4.1, H-2′a); 4.23 (m, 3H, H-4′,5′); 4.57 (dt, 1H, $J_{3',2'}$ = 6.8, 4.1, $J_{3',4'}$ = 4.1, H-3′); 6.11 (dd, 1H, $J_{1',2'}$ = 6.8, 6.3, H-1′); 7.45 (bdd, 1H, $J_{5''',4'''}$ = 7.8, $J_{5''',6'''}$ = 4.2, H-5‴); 7.57 (bd, 1H, $J_{5'',4''}$ = 7.8, H-5″); 7.92 (bt, 1H, $J_{4'',3''}$ = $J_{4'',5''}$ = 7.8, H-4″); 7.95 (m, 2H, H-3″, H-4‴); 8.02 (bd, 1H, $J_{3''',4'''}$ = 7.8, H-3‴); 8.03 (s, 1H, H-6); 8.54 (bd, 1H, $J_{6''',5'''}$ = 4.2, H-6‴); ^{13}C NMR (125.7 MHz, D_2O, ref$_{dioxane}$ = 69.3 ppm, pD = 7.1, phosphate buffer): 41.93 (CH_2-2′); 67.92 (d, $J_{C,P}$ = 5.6, CH_2-5′); 72.90 (CH-3′); 83.58 (bpy-C≡**C**); 88.16 (d, $J_{C,P}$ = 8.7, CH-4′); 89.11 (CH-1′); 94.06 (C-5); 96.48 (bpy-**C**≡C); 124.25 (CH-3″); 124.88 (CH-3‴); 127.45 (CH-5‴); 130.46 (CH-5″); 141.35, 141.49 (CH-4″, CH-4‴); 144.23 (C-6″); 148.11 (CH-6); 151.53 (CH-6‴); 156.84 (C-2‴); 158.03 (C-2″); 158.21 (C-2); 167.00 (C-4); ^{31}P NMR (202.3 MHz, D_2O, ref$_{phosphate\ buffer}$ = 2.35 ppm, pD = 7.1): –21.06 (t, J = 19.7, P_β); –10.10 (d, J = 19.6, P_α); –6.74 (bd, J = 19.6, P_γ); MS (ES$^-$): found m/z: 644.0 (M-1), 564.1 (M–PO_3H_2–1); HRMS (ES$^-$): m/z calcd for $C_{21}H_{21}O_{13}N_5P_3$: 644.0354; found: 644.0348.

Primer extension, purification and analysis of the PEX products

Synthetic ONs were purchased from Sigma Aldrich (USA). Primer: 5′-CAT GGG CGG CAT GGG-3′; templates: 5′-**CTA GCA TGA GCT CAG T***CC CAT GCC GCC CAT G*-3′(temp^{rndl6}), 5′-**CCC G***CC CAT GCC GCC CAT G*-3′ (temp^{C}), 5′-**CCC T***CC CAT GCC GCC CAT G*-3′ (temp^{4}), **T***CC CAT GCC GCC CAT G*-3′ (temp^{41}) (segments forming duplex with the primer are in italics, the replicated segments are in bold). Templates used in experiment involving the DBstv magnetoseparation procedure were biotinylated at their 5′ ends. Streptavidine magnetic beads were obtained from Sigma Aldrich (MagSelect, USA) or

Novagen (MagPrep, USA), Pwo DNA polymerase from PeqLab (Germany), DyNAzyme II and Phusion DNA polymerases from Finnzymes (Finland), KOD XL DNA polymerase from Novagen, Vent (*exo*$^-$), Deep Vent, Deep Vent (*exo*$^-$) and Therminator DNA polymerases as well as T4 polynukleotide kinase and natural nucleoside triphosphate (dATP, dCTP, dGTP and dCTP) from New England Biolabs (Great Britain) and γ-^{32}P-ATP from Izotop, Institute of isotopes Co, Ltd. (Hungary).

Primer extension experiment for single incorporation by using Pwo polymerase. The reaction mixture (20 μl) contained Pwo polymerase (0.1 U/μl, 2 μl), dNTPs (either natural or modified, 4 mM, 1 μl), ^{32}P-prelabelled primer at 5′-end (3 μM, 1 μl) and template temp^{A} (3 μM, 1.5 μl) in 2 μl of corresponding buffer supplied by manufacturer. Reaction mixture was incubated for 30 min at 60 °C.

Primer extension experiment for single incorporation by using DyNAzyme II. The reaction mixture (20 μl) contained DyNAzyme II polymerase (0.1 U/μl, 1 μl), natural dNTPs (4 mM, 0.5 μl) and modified **dN^{R}TPs** (4 mM, 1 μl), ^{32}P-prelabelled primer at 5′-end (3 μM, 1 μl) and template temp^{C} (3 μM, 1.5 μl) in 2 μl of corresponding buffer supplied by manufacturer. Reaction mixture was incubated for 10 min at 60 °C.

Primer extension for multiple incorporation by using Pwo. The reaction mixture (20 μl) contained Pwo polymerase (0.1 U/μl, 2 μl), dNTPs (either natural or modified, 4 mM, 1 μl), ^{32}P-prelabelled primer at 5′-end (3 μM, 1 μl) and template temp^{rnd16} (3 μM, 1.5 μl) in 2 μl of corresponding buffer supplied by manufacturer. Reaction mixture was incubated for 30 min at 60 °C.

Primer extension for multiple incorporation by using DyNAzyme II. The reaction mixture (20 μl) contained DyNAzyme II polymerase (1U/μl, 1 μl), natural dNTPs (4 mM, 1 μl) and modified **dN^{R}TPs** (4 mM, 2 μl), ^{32}P-prelabelled primer at 5′-end (3 μM, 1 μl) and template temp^{rnd16} (3 μM, 1.5 μl) in 2 μl of corresponding buffer supplied by manufacturer. Reaction mixture was incubated for 30 min at 60 °C.

For magnetoseparation unlabelled primers and biotinylated templates were used.

Primer extension for kinetics study. The reaction mixture (20 μl) contained DNA polymerase: Pwo (0.1 U/μl, 2 μl), dATP/**dA^{R}TP** (4 mM, 1 μl), ^{32}P-prelabelled primer at 5′-end (3 μM, 1 μl) and template temp^{A1} (3 μM, 1.5 μl) in 2 μl of corresponding buffer supplied by manufacturer. Reaction mixture was incubated at 60 °C for required time followed by stopping the reaction by addition of PAGE loading buffer and immediate heating.

Denaturating polyacrylamide gel electrophoresis. The products of the primer extension reaction were mixed with loading buffer (40 μl, 80% [w/v] formamide, 20 mM EDTA, 0.025% [w/v] bromphenole blue, 0.025% [w/v] xylene cyanol), heated 5 min at 95 °C and subjected to gel electrophoresis in 12.5% denaturating polyacrylamide gel containing 1xTBE buffer (pH 8) and 7% urea at 60 W for ~60 min. Gel was dried and visualized by phosphoimager.

General procedure for complexation

Complexation of dN^{R}MPs. Complexes of modified nucleoside monophosphates **dN^{R}MPs** with diverse transition metals were prepared by mixing 100 μl of aqueous solution corresponding monophosphate (100 μM) with 100 μl of divalent metal ions M^{2+} (50 μM, $Cu(BF_4)_2{\cdot}6H_2O$, $Ni(BF_4)_2{\cdot}6H_2O$, $Zn(BF_4)_2{\cdot}H_2O$, $Fe(BF_4)_2{\cdot}6H_2O$) at room temperature for 10 min.

Complexation of ON for recording UV-spectra. Double stranded ONs were prepared by PEX-experiment on larger scale. The reaction mixture (200 μl) contained Deep Vent polymerase (2 U/μl, 15 μl), dNTP (either natural or modified, 4 mM, 30 μl), unlabeled primer (100 μM, 12 μl), and temp^{rnd16} (100 μM, 12 μl) in 20 μl of corresponding buffer supplied by manufacturer. Reaction mixture was incubated for 30 min. at 60 °C. PEX-products were purified by NucAway Spin Columns (Ambion), where 50 μl portions of each sample were applied on the top of the column. After collecting all the portions 0.5 equiv. of $Fe(BF_4)_2{\cdot}6H_2O$ to number of modification (0.24 μl, 10 mM) was added and the solution was mixed for 3 h (25 °C, 550 rpm).

Complexation of ON for gel electrophoresis. Double stranded ONs were prepared by PEX-experiment. The reaction mixture (20 μl) contained DNA polymerase: Pwo (0.1 U/μl, 2 μl), DyNAzyme II (0.1 U/μl, 1 μl), dNTP (either natural or modified, 4 mM, 1 μl), ^{32}P-prelabelled primer at 5′-end primer (3 μM, 1 μl), and temp (3 μM, 1.5 μl) in 2 μl of corresponding buffer supplied by manufacturer. Reaction mixture was incubated for 30 min. at 60 °C. For incorporation of **dC^{tpy}TP** using Dynazyme II polymerase and temp^{C} was used 0.5 μl of natural dNTPs (4 mM) and the mixture was incubated only for 15 min. After addition of 1 μl of Fe^{2+} (4 mM, $Fe(BF_4)_2{\cdot}6H_2O$ or $FeCl_2$) the solution was mixed for 3 h (25 °C, 550 rpm).

Non-denaturating SB polyacrylamide gel electrophoresis. The products of the primer extension reaction were mixed with loading buffer (4 μl, 40% [w/v] sacchrose, 0.2% [w/v] bromphenole blue, 0.2% [w/v] xylene cyanol) subjected to gel electrophoresis in 8% non-denaturating polyacrylamide gel containing 1xSB buffer (pH 8) and at 500 V for ~3 h at room temperature. Gel was dried and visualized by phosphoimager.

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