Univerzita Karlova v Praze

Přírodovědecká fakulta

Studijní program: Organická chemie

Studijní obor: Organická chemie

Anna Simonova

Nové redoxní značky pro DNA

New redox labels for DNA

Disertační práce

Školitel: prof. Ing. Michal Hocek, CSc., DSc.

Praha, 2018

Disertační práce byla vypracována na Ústavu organické chemie a biochemie, Akademie věd České Republiky v Praze v období říjen 2012 –říjen 2018.

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 8.10.2018

Podpis

ACKNOWLEDGEMENT

I would like to thank my supervisor Prof. Michal Hocek for an interesting research project, his leadership, excellent guidance and support. I would also like to acknowledge Doc. Miroslav Fojta, Dr. Ludek Havran and their co-workers for collaboration with electrochemical measurements, Dr. Radek Pohl for measurement and interpretation of NMR spectra and Ing. Kateřina Nováková for measuring MALDI-TOF mass spectra. I would like to thank the members of our research group for their advices and suggestions as well as for friendly and inspiring working atmosphere. I am grateful to Dr. Michael A. Downey , Nemanja Milisavljevic and Alessandro Panattoni for inspiration, motivation and lots of fun. Finally, my great thanks belong to my family for their understanding, tolerance and constant support.

My PhD work was a part of multidisciplinary project performed by Prof. Hocek research group at the Institute of Organic Chemistry and Biochemistry AS CR in collaboration with Doc. Fojta research group (Institute of Biophysics in Brno). All the synthetic work and enzymatic incorporations in this thesis were performed by me in person. Measurement and interpretation of complex NMR spectra were done by Dr. Radek Pohl. Most of the electrochemical measurements were performed by me under supervision of Doc. Fojta research group (Sections 3.1.4, 3.1.5, 3.2.5, 3.2.6, 3.3.5). The parts of this thesis, which were performed by collaborating researchers are distinctly denoted in the thesis.

This work was supported by the Academy of Sciences of the Czech Republic (RVO 61388963 and RVO 68081707), by the Czech Science Foundation (P206/12/G151) and by Gilead Sciences, Inc. (Foster City, CA, USA).

Abstract

The aim of my thesis was the synthesis of the modified 2'-deoxyribonucleoside triphosphates (dNTPs) bearing electrochemically oxidizable labels and their incorporation into DNA for the application in bioanalysis.

In the first part of my thesis, I developed the synthesis of modified dNTPs bearing 2,3-dihydrobenzofuran (DHB) or 2-methoxyphenol (MOP) labels at 5-position of 2'-deoxycytidine 5′-*O*-triphosphate and at the 7-position of 7-deaza-2'-deoxyadenosine 5′-*O*-triphosphate by Suzuki-Miyaura cross-coupling reactions. Then modified dNTPs were used as substrates for DNA polymerases in enzymatic synthesis of modified DNA by PCR and primer extension. Electrochemical properties of the DHB and MOP-labeled nucleosides, dNTPs and DNA were studied by using of a square-wave voltammetry (SWV) at the pyrolytic graphite electrode (PGE) giving signals of MOP oxidation around 0.5 V and DHB oxidation around 0.85 V. The use of DHB group in combination with other electrochemical active labels was limited by close position of its oxidation peak to the signals of oxidation of natural nucleobases, whereas MOP moiety was successfully used for redox coding of nucleobases in combination with aminophenyl or benzofurazane label giving two independently readable redox signals in each case.

In the second part of this thesis, phenothiazine (PT) was tested as a new redox label. Synthesis of PT-modified nucleosides and dNTPs were performed by Suzuki-Miyaura and Sonogashira cross-coupling reactions. Modified DNA bearing PT labels were enzymatically synthesized by primer extension, PCR and nicking enzyme amplification reactions. Modified nucleosides and dNTPs containing PT-group through the acetylene linker also displayed fluorescence properties. Electrochemical properties of the PT-modified nucleosides, nucleotides and DNA were studied by cyclic (CV) and square-wave voltammetry at the pyrolytic graphite electrode showing anodic peaks in the region 0.66 V and 0.86 V. PT moiety was also studied as a label for multipotential coding of DNA bases in combination with benzofurazane or nitrophenyl moiety.

Finally, new substituted ferrocene (Fc) derivatives with electron donating and electron withdrawing groups were studied as new redox labels with tunable redox potential. I designed and prepared 1-(N,N-dimethylaminocarbonyl)-1'-ethynylferrocene and 1-fluoro-1'-ethynylferrocene as building blocks for the synthesis of modified nucleosides and nucleoside triphosphates. These new derivatives, as well as known 1-ethynyl-1',2,2',3,3',4,4',5-octamethylferrocene and propargylamidoferrocene, were used for Sonogashira cross-coupling reactions with halogenated dNTPs. The obtained nucleotides bearing modified Fc labels were used as substrates for enzymatic synthesis of modified DNA. So far, electrochemical properties of modified nucleosides were studied by SWV, whereas further electrochemical studies and

applications of Fc-modified dNTPs and DNA will be examined in near future in collaboration with Fojta group.

Abstrakt

Cílem mé disertace byla syntéza modifikovaných 2'-deoxyribonukleosid trifosfátů (dNTP) nesoucích elektrochemicky oxidovatelné značky a jejich inkorporace do DNA pro aplikace v bioanalýze.

V první části disertace jsem vyvinula syntézu modifikovaných dNTP nesoucích 2,3-dihydrobenzofuran (DHB) nebo 2-methoxyphenol (MOP) v poloze 5 u 2'-deoxycytidin-5′-*O*-trifosfátu a v poloze 7 u 7-deaza-2'-deoxyadenosin-5′-*O*-trifosfátu pomocí Suzukiho-Miyaurových cross-coupling reakcí. Poté byly modifikované dNTP využity jako substráty pro DNA polymerasy při enzymové syntéze modifikovaných DNA pomocí PCR nebo prodlužování primeru. Studovali jsme elektrochemické vlastnosti nukleosidů, dNTP a DNA značených DHB nebo MOP skupinami s použitím metod square-wave voltametrie (SWV) na pyrolytické grafitové elektrodě (PGE) a pozorovali signály oxidace MOP při cca. 0.5 V a oxidaci DHB při 0.85V. Využití DHB skupiny v kombinaci s dalšími redoxními značkami bylo limitováno blízkostí pozice jejího oxidačního píku k signálům přirozených nukleobází, zatímco MOP skupina byla úspěšně použita k redoxnímu kódování nukleobází v kombinaci s aminofenylovou nebo benzofurazanovou značkou, kdy každá značka poskytla odlišný a nezávisle detekovatelný redoxní signál.

V druhé části disertace jsem studovala fenothiazin (PT) jako novou redoxní značku. Syntéza nukleosidů a dNTP modifikovaných PT byla prováděna pomocí Suzukiho-Miyaurových nebo Sonogashirových cross-coupling reakcí. Modifikované DNA nesoucí PT značky byly syntetizovány pomocí enzymových reakcí: prodlužování primeru, PCR nebo nicking enzyme amplifikační reakce. Modifikovaná nukleosidy a dNTPs nesoucí PT skupinu vázanou přes acetylenový můstek vykazovaly fluorescenční vlastnosti. Elektrochemické vlastnosti nukleosidů, dNTPs a DNA modifikovaných PT byly studovány cyklickou voltametrií (CV) a SWV na pyrolytické grafitové elektrodě a vykazovaly anodické signály v oblasti 0.66 V a 0.86 V. PT skupina byla rovněž studována pro multipotenciálové kódování DNA bázi v kombinaci s benzofuranovou nebo nitrofenylovou skupinou.

V poslední části byly studovány ferrocenové (Fc) deriváty s elektron-donorními a elektron-akceptorními skupinami jako nové redoxní značky s laditelným redoxním potenciálem. Navrhla a syntetizovala jsem 1-(*N,N*-dimethylaminocarbonyl)-1'-ethynylferrocen a 1-fluoro-1'-ethynylferrocen jako stavební bloky pro syntézu modifikovaných nukleosidů a nukleotidů. Tyto nové deriváty, a také známé 1-ethynyl-1',2,2',3,3',4,4',5-octamethylferrocen a propargylamidoferrocen, byly použity pro Sonogashirovy cross-coupling reakce s halogenovanými dNTPs. Získané značené nukleotidy nesoucí modifikované Fc skupiny byly

použity jako substráty pro enzymovou syntézu modifikované DNA. Dosud byly prostudovány elektrochemické vlastnosti modifikovaných nukleosidů pomocí SWV, zatímco další elektrochemické studie a aplikace dNTP a DNA značených modifikovanými Fc skupinami bude studována v blízké budoucnosti ve spolupráci s týmem Doc. Fojty.

List of abbreviations

AdTS	adsorptive transfer stripping
BF	benzo[c][1,2,5]oxadiazole
bp	base pair
Cp	cyclopentadienyl
CV	cyclic voltammetry
dATP	2'-deoxyadenosine 5'-*O*-triphosphate
dCTP	2'-deoxycytidine 5'-*O*-triphosphate
dGTP	2'-deoxyguanosine 5'-*O*-triphosphate
DHB	2,3-dihydrobenzofuran
DCC	dicyclohexylcarbodiimide
DMAP	dimethylaminopyridine
DMF	*N,N*-dimethylformamide
DMSO	dimethylsulfoxide
DMT	dimethoxytrityl
dN	2'-deoxyribonucleoside
DNA	deoxyribonucleic acid
dNTP	2'-deoxyribonucleoside 5'-*O*-triphosphate
dppf	1,1'-bis(diphenylphosphino)ferrocene
dsDNA	double-stranded DNA
dTTP	2'-deoxythymidine 5'-*O*-triphosphate
E	ethynyl
equiv.	equivalents
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
FAM	fluorescein
Fc	ferrocene
HMDE	hanging mercury drop electrode
HPLC	high performance liquid chromatography
MALDI	matrix-assisted laser desorption/ionization
MOP	2-methoxyphenol
m.p.	melting point
NEAR	nicking enzyme amplification reaction
NFSI	N-Fluorobenzenesulfonimide

nt	nucleotide
ON	oligonucleotide
RNA	ribonucleic acid
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEX	primer extension experiment
PGE	pyrolytic graphite electrode
Ph	phenyl
pin	pinacol ester
PT	phenothiazine
RP-HPLC	reverse phase high performance liquid chromatography
SCE	saturated calomel electrode
SNI	single nucleotide incorporation
SNP	single-nucleotide polymorphism
ssDNA	single-stranded DNA
SWV	square wave voltammetry
TdT	terminal deoxynucleotidyl transferase
TEAB	triethylammonium bicarbonate
THF	tetrahydrofurane
TMEDA	N,N,N′,N′-tetramethylethylenediamine
TMS	trimethylsilyl
TMSA	trimelthylsilylacetylene
TPPTS	tris(3-sulfophenyl)phosphine trisodium salt
Tris	tris(hydroxymethyl)aminomethane
UV-Vis	ultraviolet-visible spectroscopy
X	modification

Contents

1 Introduction

1.1 DNA structure

"DNA is like a computer program but far, far more advanced than any software ever created." (Bill Gates, The Road Ahead).

DNA is a macromolecule carrying the genetic information used for the growth, development, functioning, and reproduction of all known living organisms and many viruses. Nucleic acids were discovered in 1869 by Friedrich Miescher and in 1953 James D. Watson and Francis H.C.Crick determined the structure of DNA by x-ray diffraction analysis[1,2].

There are three major forms of double helical DNA, B-DNA(the most common), A-DNA and Z-DNA. All three forms consist of two antiparallel strands coiled around one another to form a double helix. Chains are built by the sugar-phosphate backbone and two chains are held together by hydrogen bonding between pairs of complementary nucleobases. In each case a bulkier two-ring base (a purine) is paired with a single-ring base (a pyrimidine), adenine with thymine and guanine with cytosine[3].

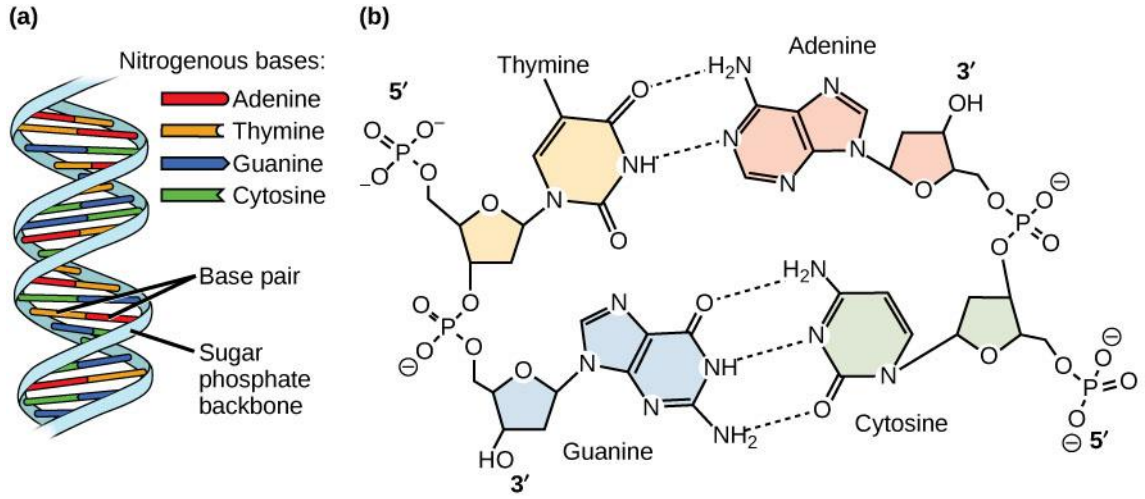


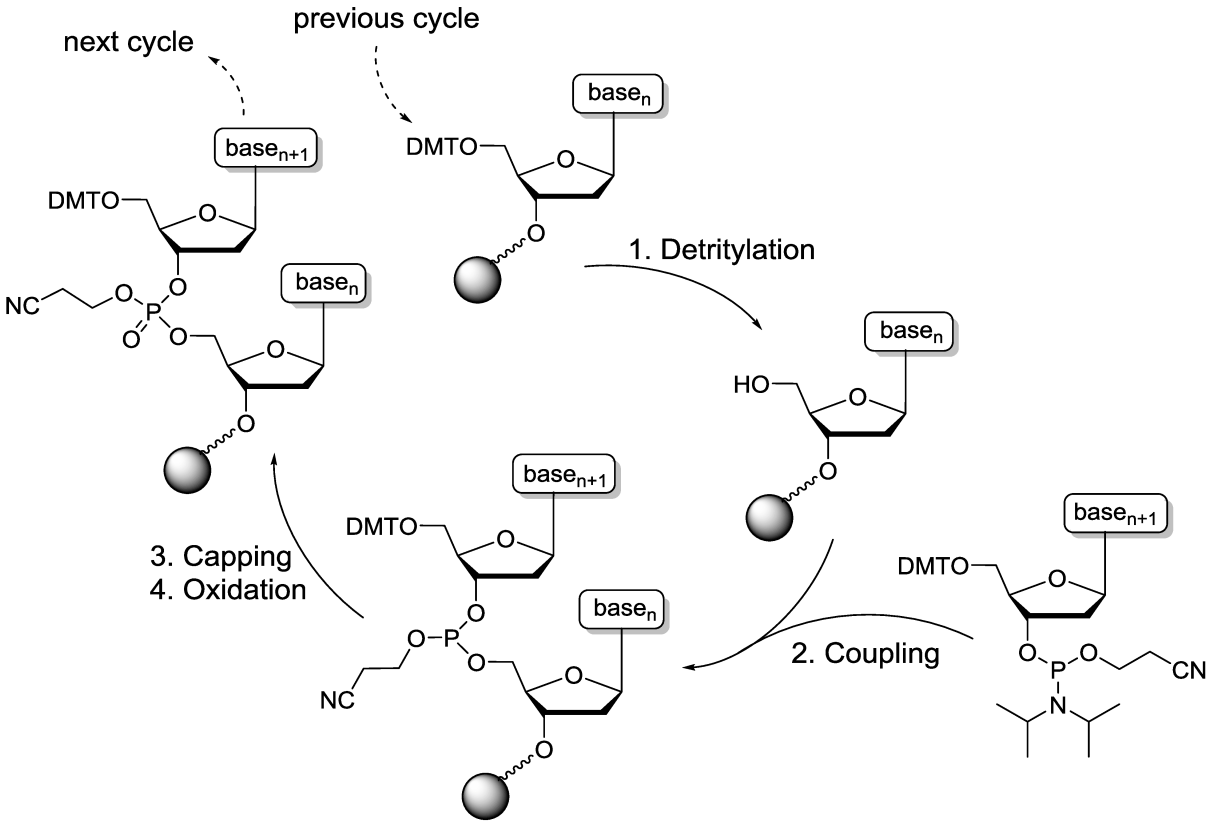
Figure 1. a) The DNA double helix. b) The primary structure of the DNA molecule. (Figure was taken from http://www.alyvea.com/)

The biological importance of DNA as genetic material explains the importance of studying its structure and functions in various scientific fields.

1.2 Chemical and enzymatic synthesis of functionalized DNA

1.2.1 Chemical synthesis

Chemical synthesis is a simple, rapid, versatile and inexpensive method to produce short DNAs or RNAs (up to 150-mers), which are widely used in nearly every molecular biology technique including single-nucleotide polymorphism (SNP) assays[4], DNA sequencing and amplification[5,6], synthesis of artificial genes[7,8]. Many different techniques for DNA synthesis were developed such as H-phosphonate synthesis[9,10], phosphodiester synthesis[11], phosphotriester synthesis[12,13], phosphite triester synthesis[14]. Nowadays, the solid-phase phosphoramidite synthesis is the most popular method for producing short strand DNAs on a large scale and high yields[15–19]. The synthesis proceeds in the 3′ to 5′ direction on a solid support, where each nucleotide addition requires a synthetic cycle of four chemical reactions (Scheme 1). This method was developed by Caruthers lab and was fully automated in the late 1980s.



Scheme 1. The phosphoramidite oligonucleotide synthesis cycle.

The synthetic cycle is initiated by detritylation step (de-blocking), where 5'-dimethoxytrityl (DMT) protective group is cleaved by a solution of an acid from the support-bound 3'-nucleoside. Next step is coupling, the nucleoside 3'-phosphoramidite which serves as a building block in DNA synthesis is activated by tetrazole to react with deprotected nucleoside in order to

form a phosphite triester linkage. The capping step is performed to prevent side reactions by treating the unreacted solid support-bound 5'-OH groups with a mixture of acetic anhydride and 1-methylimidazole. In the final step called oxidation newly formed phosphite triester linkage is transferred to phosphate triester by the oxidation reaction of iodine and water in the presence of pyridine. The synthetic cycle should be repeated several times to get the desired oligonucleotide with the required length and sequence.

1.2.2 Enzymatic synthesis

The enzymatic synthesis is an alternative approach for incorporation of nucleoside triphosphates into DNA by DNA polymerases[20,21]. This method allows the incorporation of functionalized 2′-deoxynucleoside 5′-*O*-triphosphates (dNTPs) to synthesize long DNA strands with high efficiency. Functionalized nucleic acids are receiving growing interest for their potential use in biological assays and clinical diagnostics[22–24]. While phosphoramidite method limits use of functional groups, which are not compatible with acidic conditions or easily react with nucleophiles during the phosphoramidite oligonucleotide synthesis cycle, the enzymatic synthesis proceeds under very mild conditions and tolerates many functional groups without the need to use protecting groups.

Most DNA polymerases used for enzymatic synthesis are not able to start a new chain *de novo*, therefore they move along the template strand in a 3′-5′ direction forming the daughter strand in 5′-3′ direction. The resulting DNA is made of two DNA strands that are antiparallel to each other (Figure 2A).

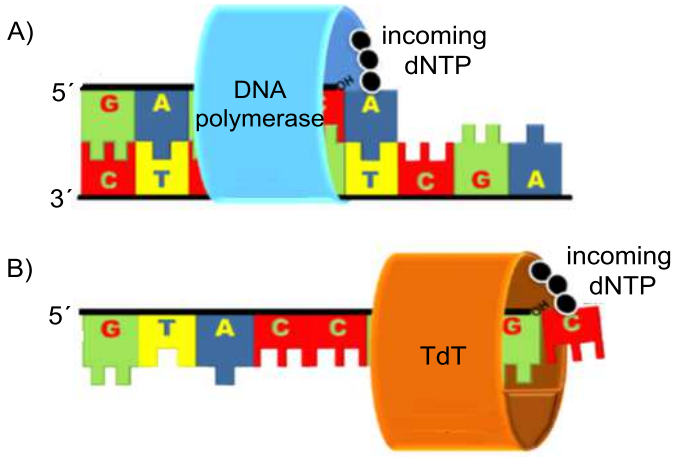
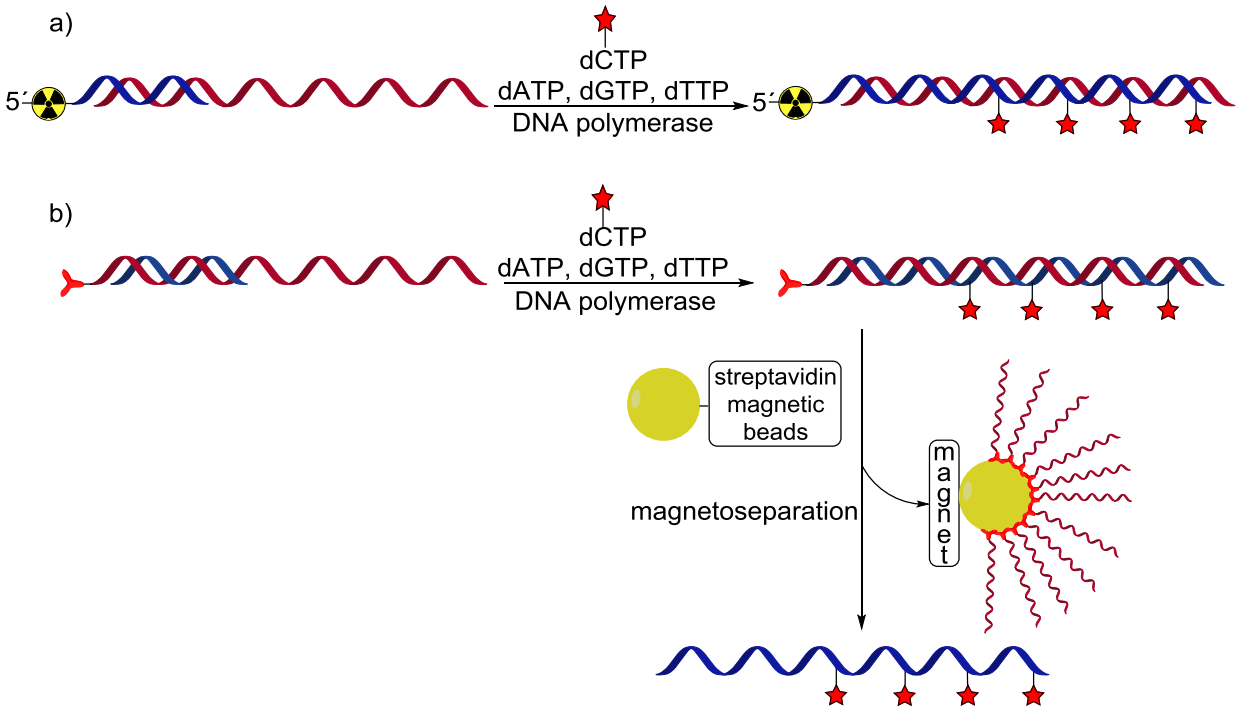


Figure 2. Simplified models for template-dependent (A) and template-independent (B) DNA polymerase activity. (Figure was taken from the reference 24).

The unique DNA polymerase that incorporates nucleotides in a template-independent manner is a terminal deoxynucleotidyl transferase (TdT)[25,26]. The ability of TdT to create genomic material

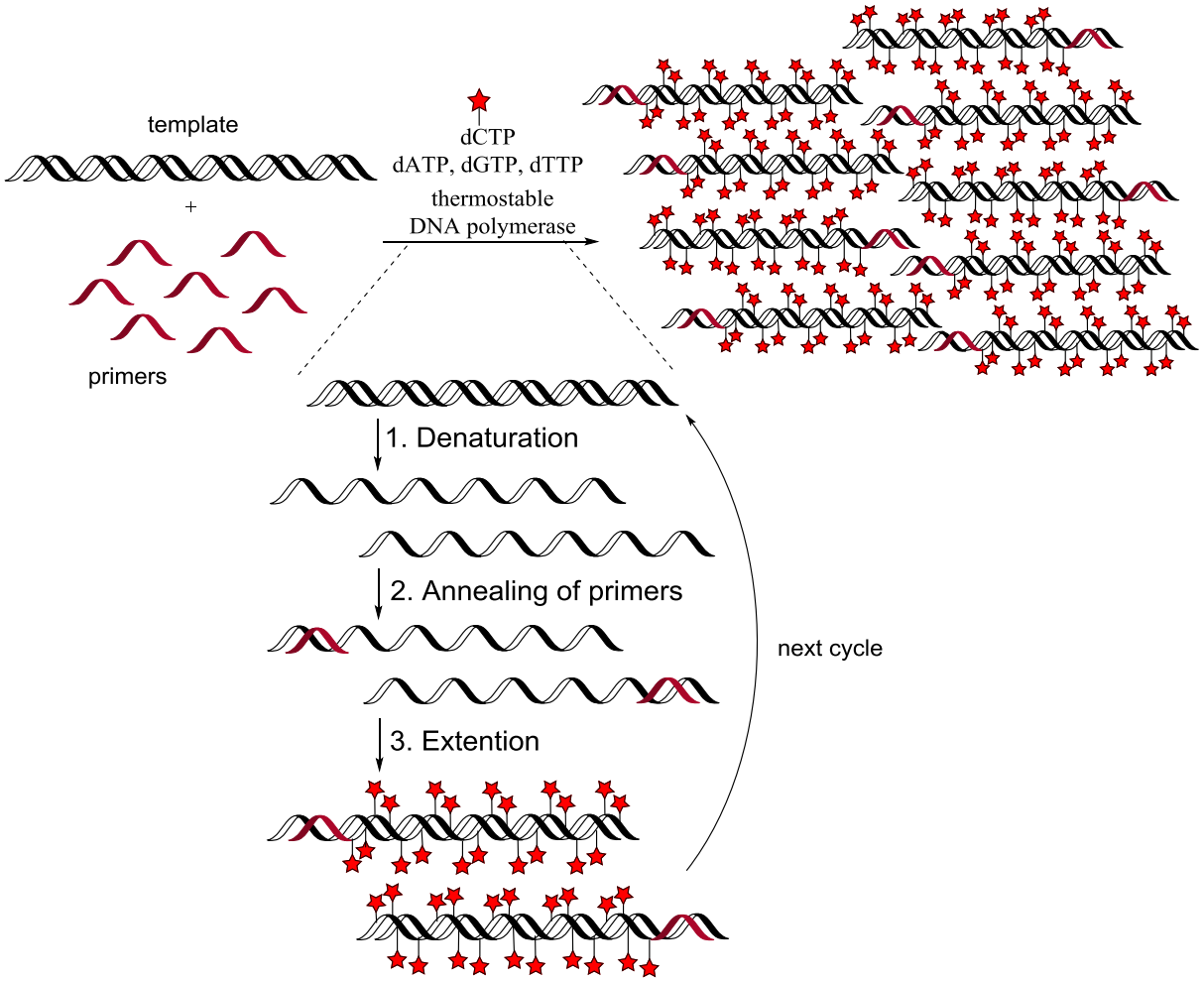
de novo makes it an effective biochemical tool for labeling the 3´-termini of synthetic oligonucleotides with radioactive or fluorescent probes or different functionalized groups for diagnostics (Figure 2B).

The primer extension (PEX) is a common method for the synthesis of short DNAs bearing modifications in one strand. The primer (typically 15- to 25-mer) is usually labeled on its 5'-end by ^{32}P-phosphate or fluorescent label for visualization of the reaction by gel analysis (Scheme 2a). In order to obtain a modified single-stranded DNA template is functionalized by biotin moiety at its 5'-end. After PEX reaction the interaction between streptavidin and biotin is used to release modified single-stranded oligonucleotide. The biotinylated double-stranded product is immobilized onto the streptavidin magnetic beads and the modified single-strand is released then by denaturation conditions (Scheme 2b).



Scheme 2. a) PEX reaction with radioactively labeled primer; b) Enzymatic method for the synthesis of modified single-stranded oligonucleotides.

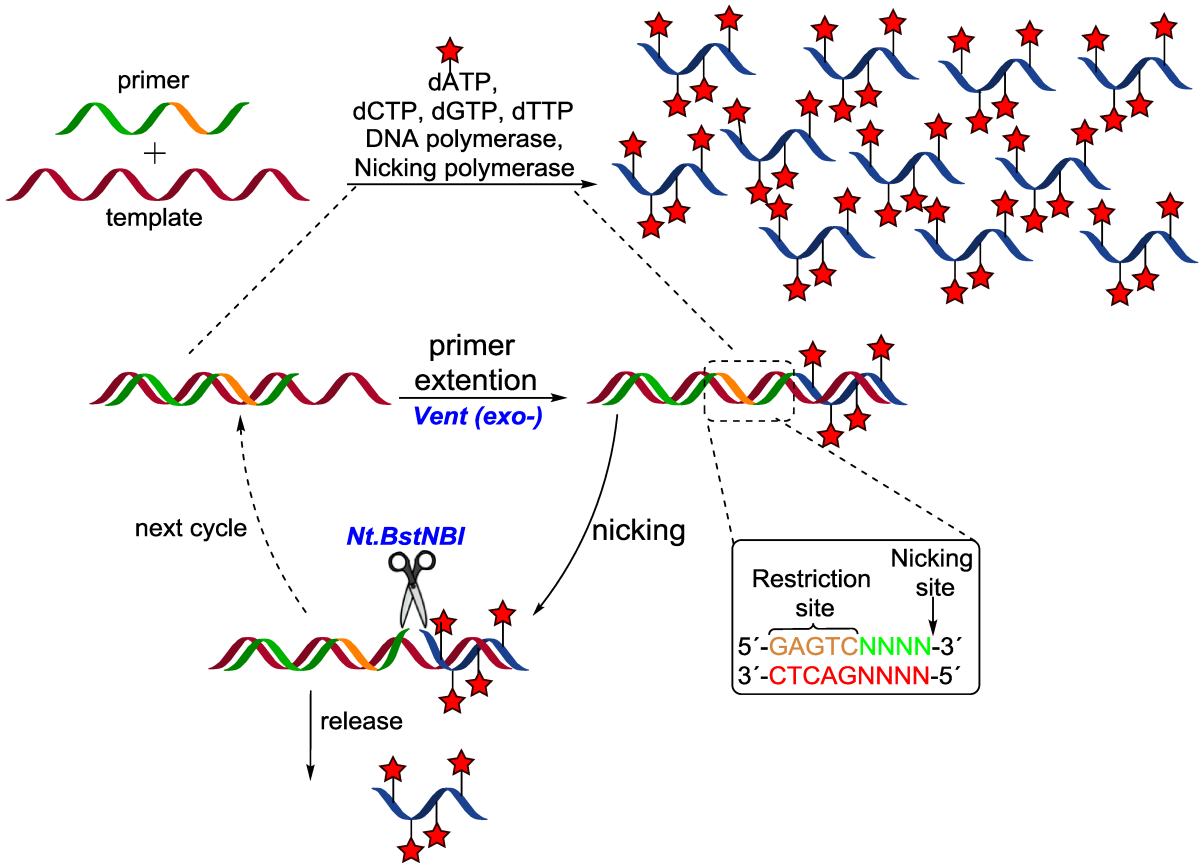
Polymerase chain reaction (PCR) is mainly used method for the synthesis and amplification of long DNA, resulting in functionalization of both DNA strands if modified nucleoside triphosphates are used[27–29]. It is an easy, cheap and reliable method developed by Kary Mullis in 1983 for generating thousands to millions of copies of DNA, making this technique applicable to various fields in medicine and biology. The amplification process relies on thermal cycling and each cycle consists of three steps: denaturation of a double-stranded template, annealing of primers and primer extension (Scheme 3).



Scheme 3. Polymerase chain reaction.

In the past three decades, a number of isothermal amplification methods[30,31] have been developed based on mimicking *in vivo* replication mechanisms of DNA with the aid of various accessory proteins as a promising alternative to PCR. New amplification protocols, not requiring a thermocycling apparatus, are easier to operate and less energy-consuming, and therefore isothermal methods are useful in bioanalysis, diagnostics, and device integration.

Nicking enzyme amplification reaction (NEAR) is one of the isothermal amplification methods, combining polymerase strand extension and single-strand nicking to produce many short oligonucleotides[32–35]. Any target amplification is allowed by inserting nicking enzyme recognition sites inside of the targeted sequence to introduce a strand break on only one strand of a double-stranded DNA cleavage site. On another side, the length of the final ONs is limited because the concept of this method is based on incapability of target ONs to form a stable duplex at reaction temperature while primers are long enough to hybridize for the next cycle of reaction (Scheme 4).



Scheme 4. Nicking enzyme amplification reaction.

1.3 Synthesis of functionalized nucleosides and nucleotides

In the past years, different modification strategies have been developed to synthesize functional nucleosides and nucleotides, that can be utilized in broad range of applications, as pharmaceutical candidates[36,37] or chemical probes for analyzing the structure and function of nucleic acids[38–41]. For enzymatic incorporation of modified nucleoside triphosphates into DNA, the position of functional groups in the base is essential. DNA polymerase tolerates modifications in the positions 5 of pyrimidines[42] and 7 of 7-deazapurine analogues[43], because functional groups in these positions are exposed to the major groove of DNA duplex and as result do not sterically interfere with the DNA helix (Figure 3). Kinetics experiments of competing incorporation of modified dNTPs bearing substituents of varying bulkiness in the presence of their natural counterparts (unmodified dNTPs) showed higher efficiency of 7-deazapurines bearing π-electron-containing substituens (ethynyl and phenyl, as well as 7-vinyl-7-deazaadenine) to the active site of the polymerase than their natural counterparts[44,45]. Later studies showed, that 2-substituted adenosine derivatives with small functional group (methyl, vinyl or ethynyl) can be incorporated into DNA with a location of substituents in the minor groove[46].

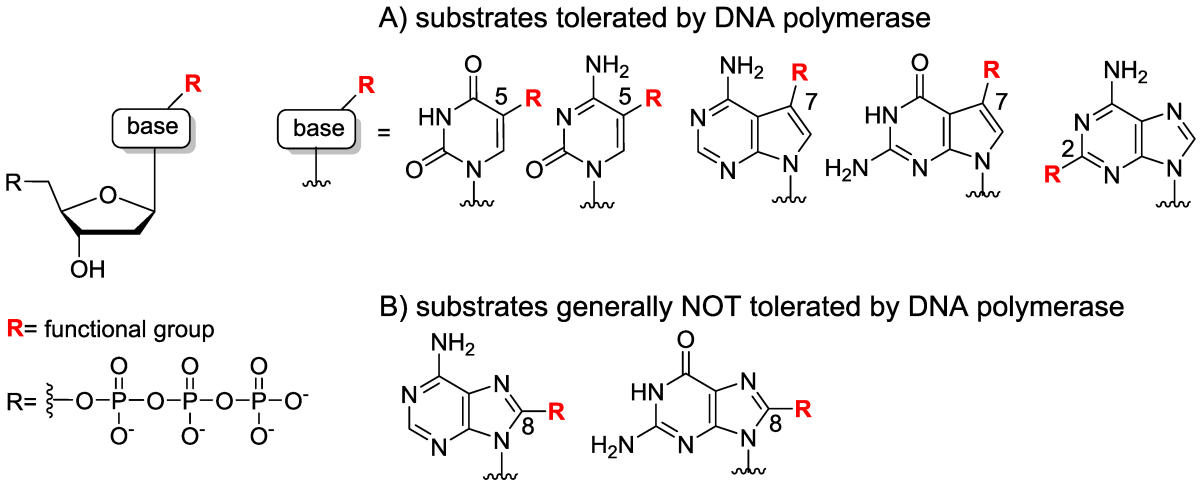
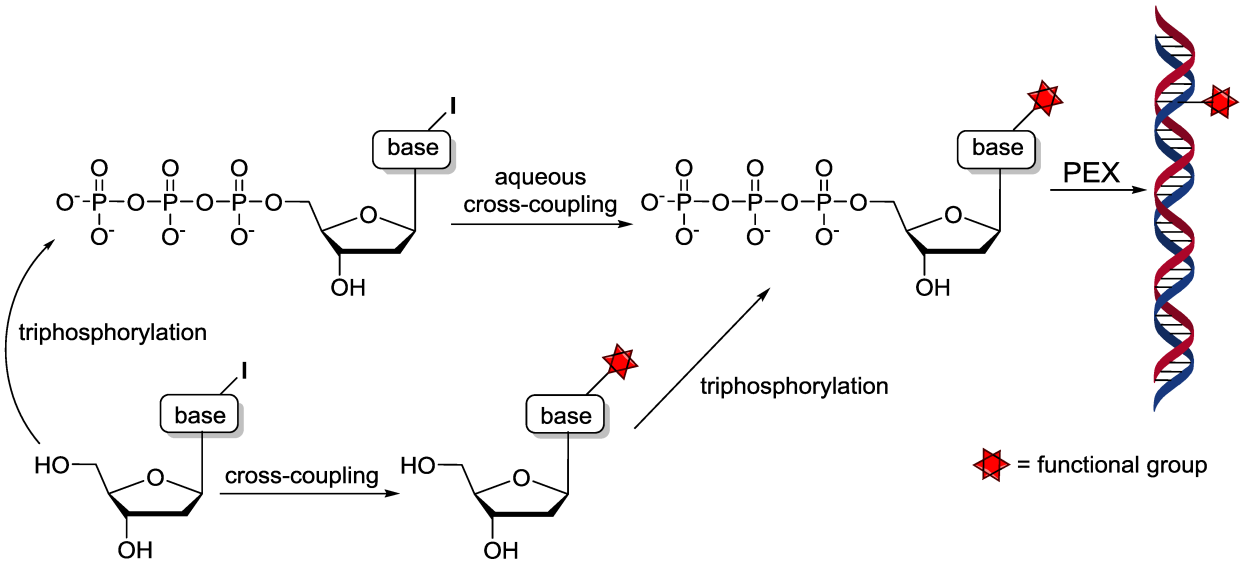


Figure 3. Positions of functional groups in dNTP favourable (A) or not (B) for enzymatic incorporation into DNA.

The functionalized nucleoside triphosphates can be synthesized by two possible pathways. (Scheme 5). Functional group can be attached to the nucleobase by palladium-catalyzed reaction with halogenated nucleoside followed by triphosphorylation of obtained modified nucleoside. This method can be problematic for substrates sensitive to acidic conditions applied during phosphorylation step. In alternative approach halogenated nucleosides undergo the

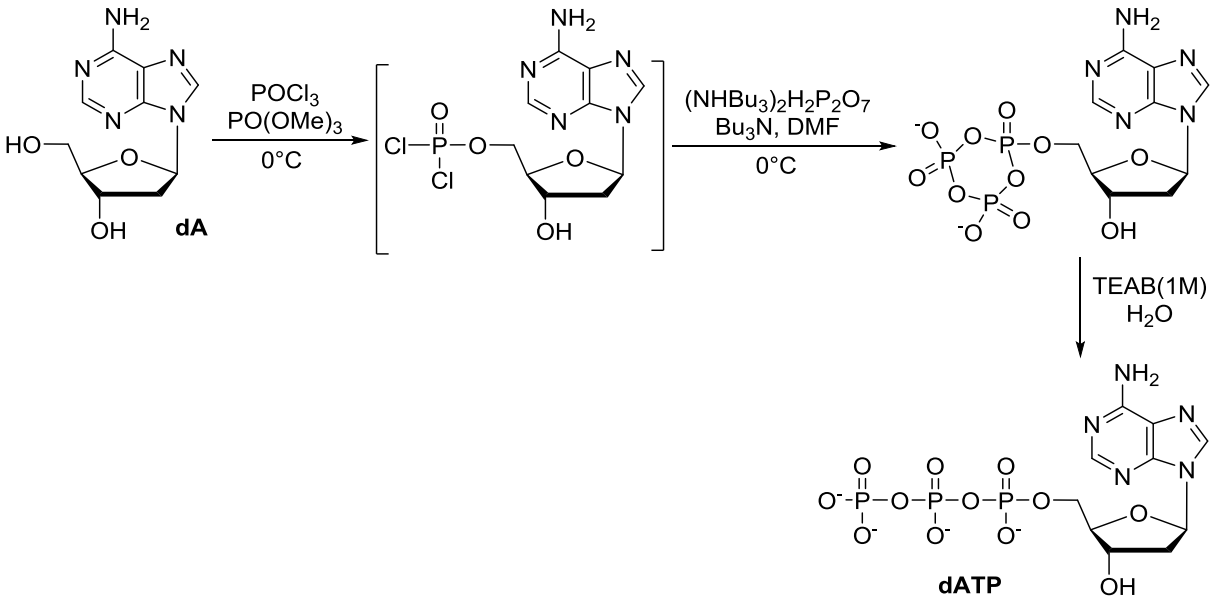
phosphorylation first, followed by cross-coupling step to get a final product. This methodology can be difficult for compounds poorly soluble in water.



Scheme 5. Two approaches for the preparation of functionalized nucleoside triphosphates.

1.3.1 Triphosphorylation of nucleosides

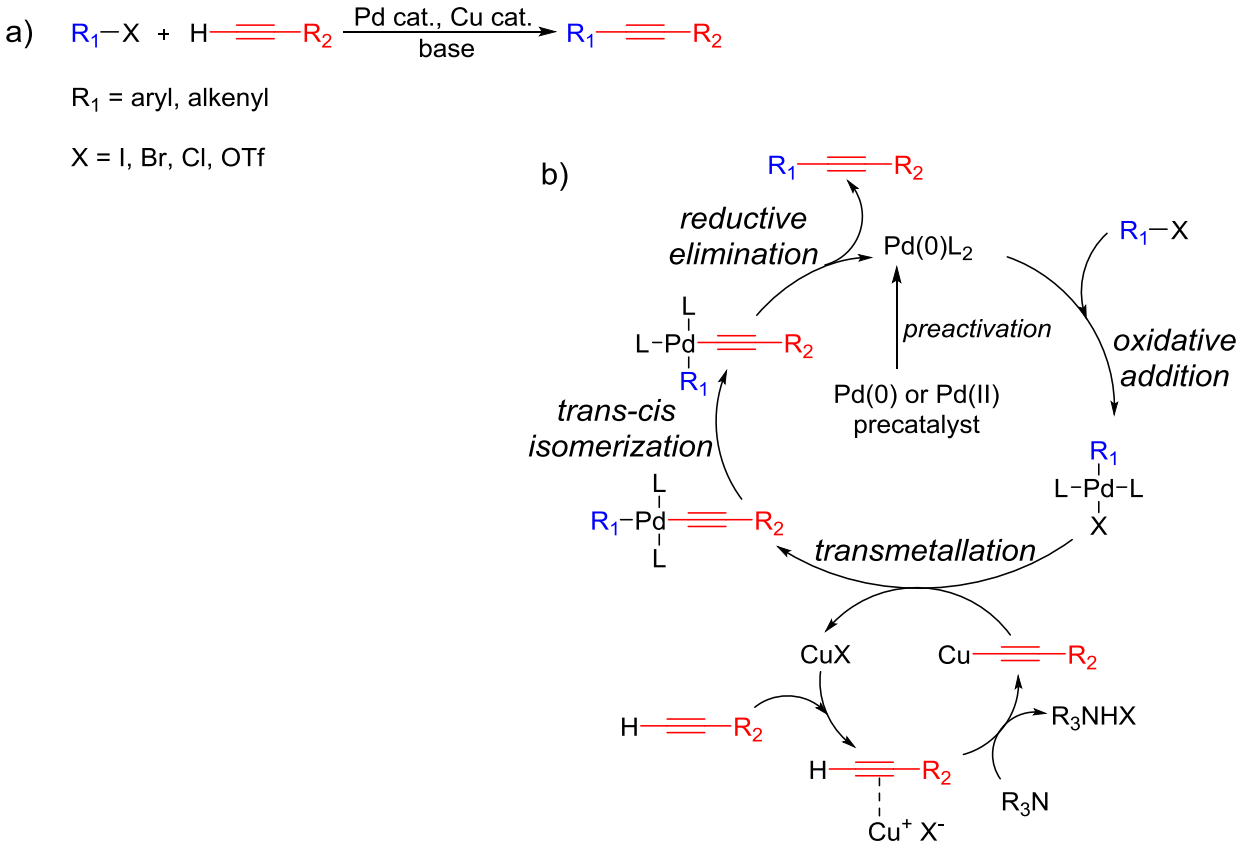
The first step on the way to the synthesis of modified triphosphates was done by Yoshikawa[47,48], who developed a phosphorylation procedure to give nucleoside monophosphates. It was identified the importance of using trialkyl phosphates in the reaction as accelerators of phosphorylation with phosphoryl chloride, as well as good solvents for the most of starting nucleosides. Yoshikawa's monophosphorylation method attracted further study due to the possibility of using chlorophosphate intermediate in reactions with the pyrophosphate ion to synthesize desired nucleoside triphosphates. In this way, the most widely used "one-pot, three-step" formation of triphosphates was designed by Ludwig's procedure[49] consisting of the reaction of nucleoside dichlorophoshates generated via Yoshikawa's methodology with bis(tri-*n*-butylammonium) pyrophosphate in dry DMF followed by quenching the reaction with triethylammonium bicarbonate (TEAB) buffer (Scheme 6). The importance of use tributhylamine as a base for increasing yield of the reaction is noted. A vast number of different protocols were developed later for optimization of reacting conditions[50–52], including a monophosphate activated to various phosphoramidites[53–55], phosphoroimidazolidates or phosphites[56], direct nucleophilic substitution of 5′-*O*-leaving groups of nucleobase with triphosphate ions[57] or solid-phase syntheses[58], but none of the protocols for making nucleoside triphosphates is universal and needs to be optimized for every triphosphate.



Scheme 6. "One-pot, three-step" triphosphorylation procedure for the synthesis of dATP.

1.3.2 Sonogashira cross-coupling reactions

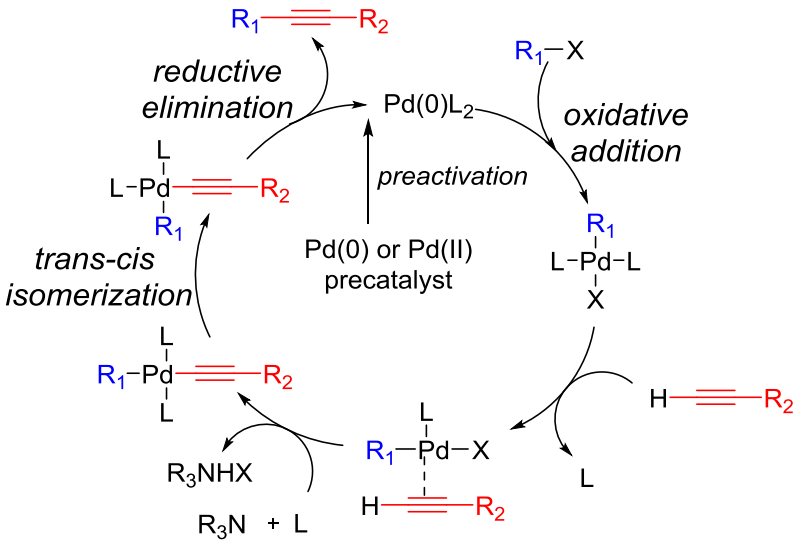
The Sonogashira reaction was first announced by Kenkichi Sonogashira in 1975 as an extension of Heck reaction[59,60]. Instead of using harsh conditions such as high temperature for carbon-carbon bond formation, Sonogashira suggested to use palladium and copper metal catalysts simultaneously that led to the increase of selectivity and as result ability to carry reaction at room temperature. Therefore, Sonogashira cross-coupling reaction became the most versatile and powerful way for alkynylation to generate arylalkynes which are widely used in the synthesis of pharmaceuticals and natural products where alkynyl-group is a required moiety[60,61]. The mechanism of reaction[62] takes place through two independent catalytic cycles as shown in Scheme 7. The palladium catalytic cycle starts with formation the initial palladium complex, 14-electron $Pd(0)L_2$, by reduction of different palladium-(II) complexes under influence of n-electron donors, such as phosphanes, amines, used as ligands and solvents. The resulting undercoordinated palladium(0) complex reacts with R_1-X substrate in the oxidative addition step, where the nature of the halide substrate is crutial. Iodide and triflate derivatives facilitate the reaction, as well as the presence of electron-withdrawing groups, which reduce electron density on the C-X bond, making oxidation addition step easier. The next step is transmetallation with the copper acetylide, which was produced in the copper cycle, to form a $R_1Pd(-C{\equiv}CR_2)L_2$ species, which give the final coupled alkyne after *trans-cis* isomerization of organic ligands and reductive elimination step with regeneration of the catalyst.



Scheme 7. Sonogashira reaction a) general equation; b) mechanism.

The second Cu-cycle is still poorly understood. The π-alkyne-Cu complex is formed to increase the acidity of alkyne proton for its easier abstraction by the base (usually tertiary amine) for construction a copper acetylide.

While a copper co-catalyst is added to the reaction to increase reactivity, the presence of copper can result in Glaser coupling as a side reaction leading to formation of alkyne dimers, what led to the development of alternative Cu-free protocol[63]. The mechanism of reaction starts with an oxidative addition of aryl- or alkenyl halides to the palladium(0) complex (Scheme 8). Since tertiary amines are not basic enough to deprotonate the reacting alkyne, Pd(0) catalyst requires a displacement of one ligand to create an intermediate complex. Following deprotonation of the terminal alkyne proton and subsequent ligand exchange with the leaving group X leads to the formation of a $R_1Pd(-C{\equiv}CR_2)L_2$ complex, which releases the desired coupling product by the reductive elimination step.

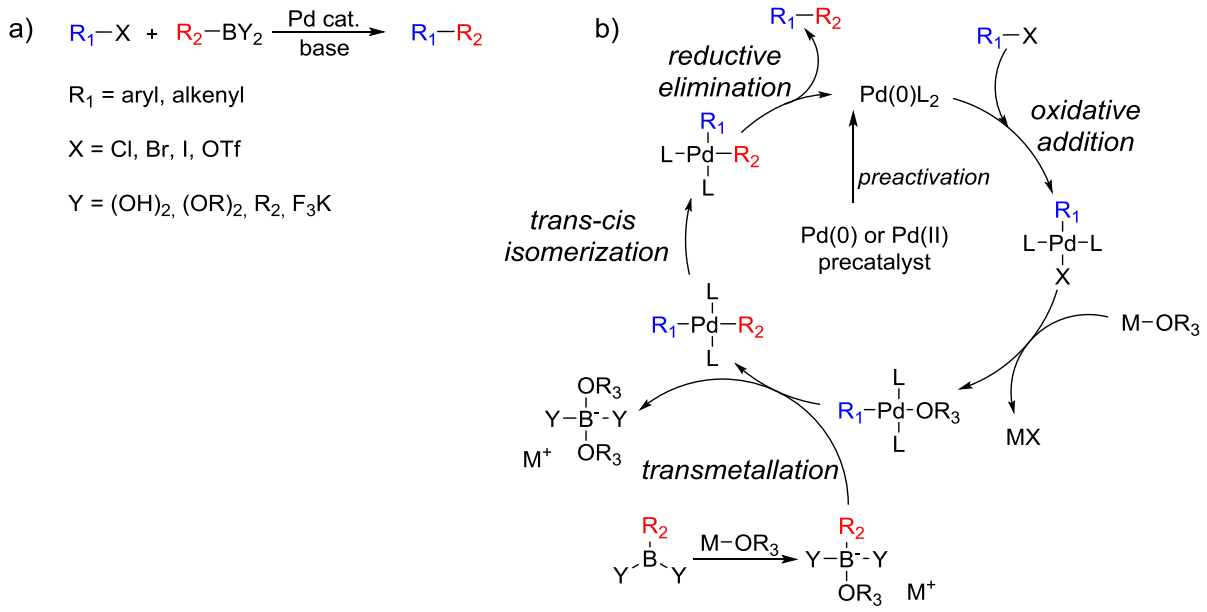


Scheme 8. Cu-free reaction mechanism.

1.3.3 Suzuki-Miyaura cross-coupling reactions

The Suzuki–Miyaura cross-coupling reaction was first discribed in 1979, where boronic compounds and organohalides were coupling partners for C-C bond formation[64]. In 2010 Akira Suzuki received a Nobel price together with Richard F. Heck and Eiichi Negishi for their effort for discovery and development of palladium-catalyzed cross couplings in organic synthesis.

The mechanism of reaction includes three fundamental steps: oxidative addition, transmetallation and reductive elimination (Scheme 9)[65]. In the first step Pd(0)-complex undergoes an oxidative addition with aryl- or alkenyl halide to form the organopalladium complex, which under attack of a base displaces a halide group. The oxidative addition is often the rate-determining step, where relative reactivity increases in the order of Cl < Br < OTf < I. The presence of electron withdrawing groups in aryl- or alkenyl halides reduces an electron density on the C-X bond, making the oxidation addition step faster. In transmetallation step, the boronic acid must be activated by reaction with a base forming a boron-ate complex, which reacts with a R_1-PdL_2-OR_3 complex. Reductive elimination is a final step, where the desired coupled product is released from Pd complex, which should be in cis-configuration.



Scheme 9. Suzuki-Miyaura reaction a) general equation; b) mechanism.

Extensive application of Suzuki reaction is favored by milder reaction conditions, commercial availability of the diverse boronic acids derivatives, ease of handling and removal of boron-containing byproducts compared to other organometallic reagents, especially in case of large-scale synthesis of a product[66].

1.3.4 Aqueous cross-coupling reactions for preparation of modified nucleotides

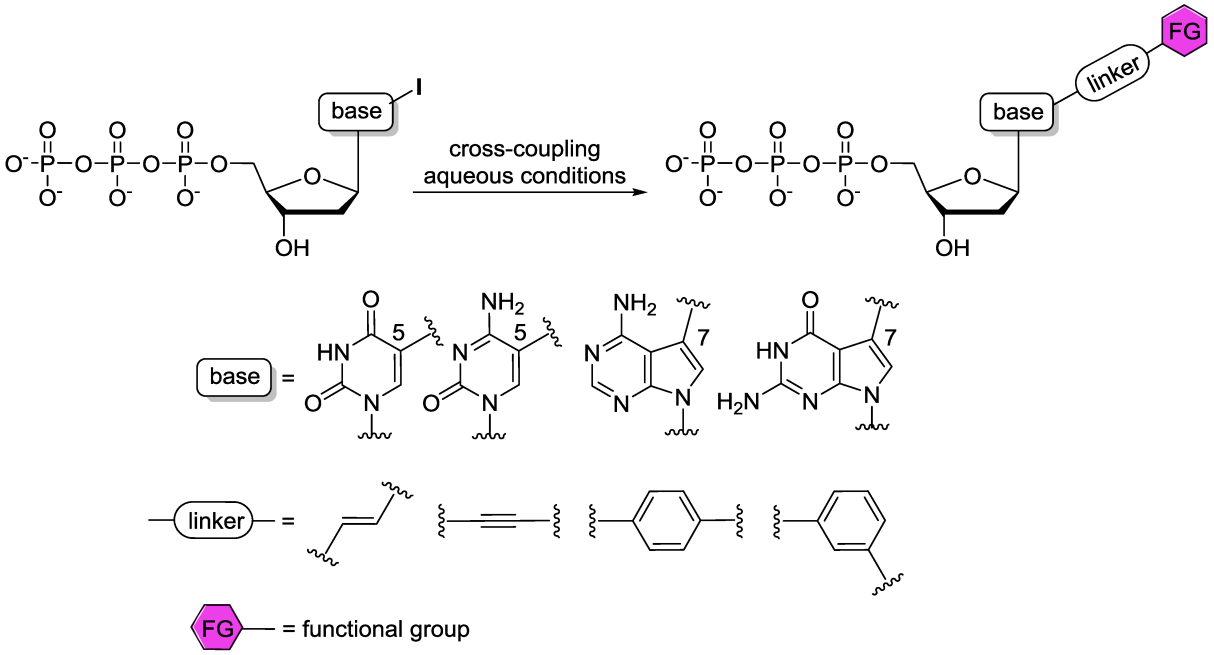
A vast number of modified nucleosides and nucleoside triphosphates were synthesized using aqueous conditions for palladium-catalyzed cross-coupling reactions to label dNTPs with a variety of functional groups.

The Sonogashira cross-coupling reaction was first applied in the field of nucleoside chemistry by Casalnuovo and Calabrese in 1990 for the synthesis of modified nucleosides and nucleoside monophosphates with propargylamide in aqueous acetonitrile solution to get desired product in good yield[67]. Later on, Burgess group presented the synthesis of modified thymidine triphosphates by reaction of 2'-deoxy-5-iodouridine triphosphates with fluorescein derivatives bearing terminal alkynes with use of water-soluble catalyst system for this transformation ($Pd(OAc)_2$ with TPPTS)[68]. After Garg et al. suggested a mild heterogeneous, ligand-free protocol to synthesize biologically important deoxynucleoside derivatives with use of resin-bound tertiary amine for easier purification process[69].

Wagenknecht group reported the first example of Suzuki coupling of 2'-deoxy-5-iodouridine with pyren-1-ylboronic acid[70]. Later the Shaughnessy group presented a synthesis of less reactive 2'-deoxy-8-bromoguanosine with boronic acids[71]. Suzuki reaction was also applied in

post-oligomerization strategy based on the reaction of short oligonucleotides, bearing 2'-deoxy-5-iodouridine moiety, with boronic ester reagents[72] and cross-coupling on a protein surface between peptide containing the amino acid *p*-iodobenzyl cysteine, and boronic acids[73]. Sonogashira coupling for the reaction of oligonucleotides containing 2'-deoxy-5-iodouridine with different ethynyl moieties was applied for tuning the melting point of DNA duplexes[74]. Very recently, peptide labeling approach was presented by using of Sonogashira cross-coupling with ethynyl modified fluorescent dye[75].

Many of the synthetic procedures involve combinations of charged ionic reagents with more lipophilic substrates, therefore appropriate reaction media is important for the synthesis and isolation of a charged water-soluble product from a mixture of hydrophilic and hydrophobic impurities. Suzuki or Sonogashira cross-coupling reactions for the straightforward synthesis of modified dNTPs are typically performed in the mixture of water/acetonitrile in the presence of base and palladium catalyst with a water-soluble ligand, usually tris(3-sulfonylphenyl)phosphine (Scheme 10).



Scheme 10. General approach for aqueous cross-coupling synthesis of modified nucleotides.

This method requires a short reaction time, because nucleoside triphosphates are not particularly robust and are prone to hydrolysis under basic conditions and at high temperatures. In Hocek group numerous nucleoside triphosphates bearing various functional moieties were synthesized using this methodology (Figure 4). These nucleotides were successfully incorporated into DNA for further use as fluorescent labels[76,77], protection groups against cleavage by restriction endonucleases[78,79], selective cross-linking of protein agents[80,81].

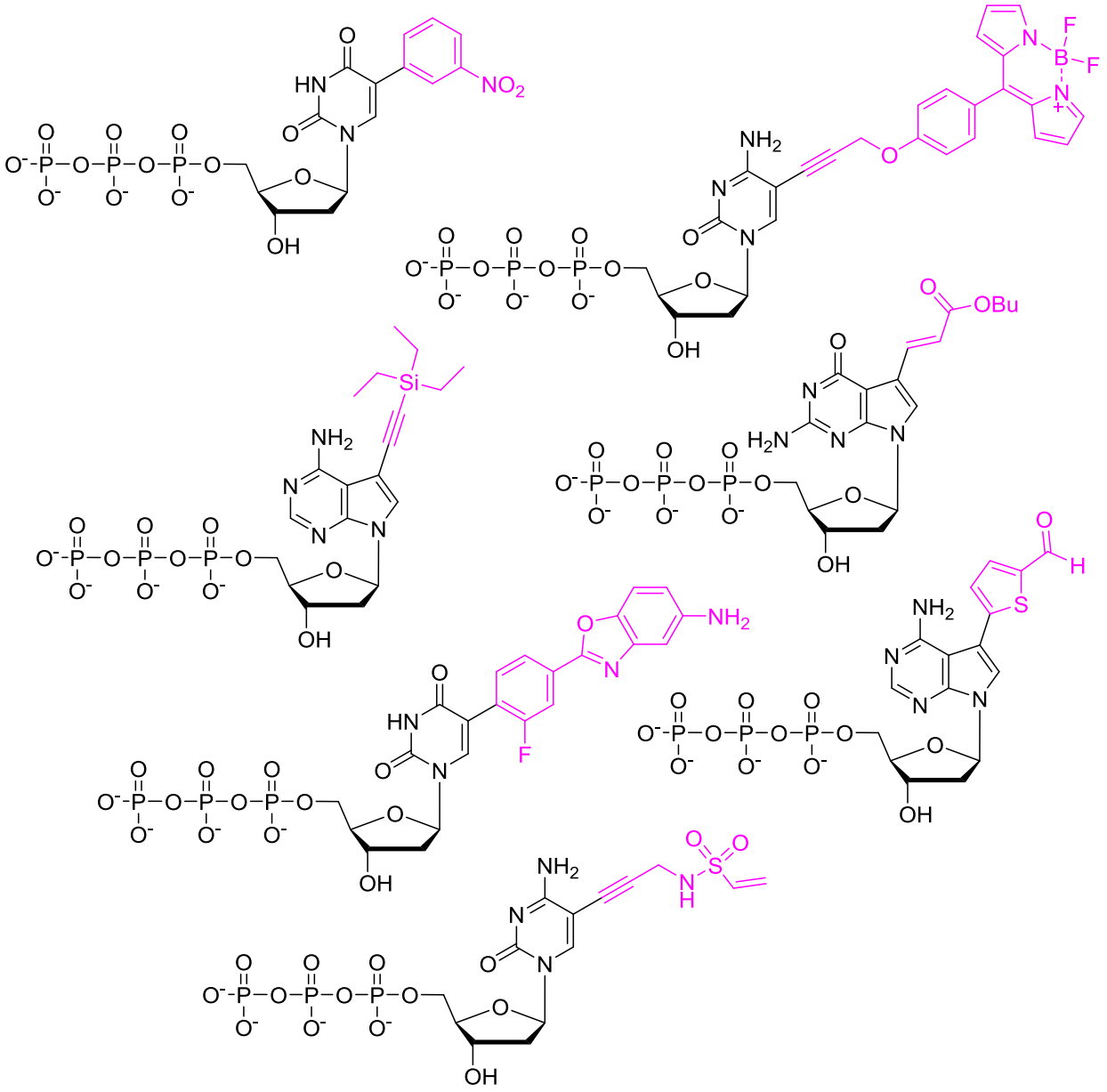


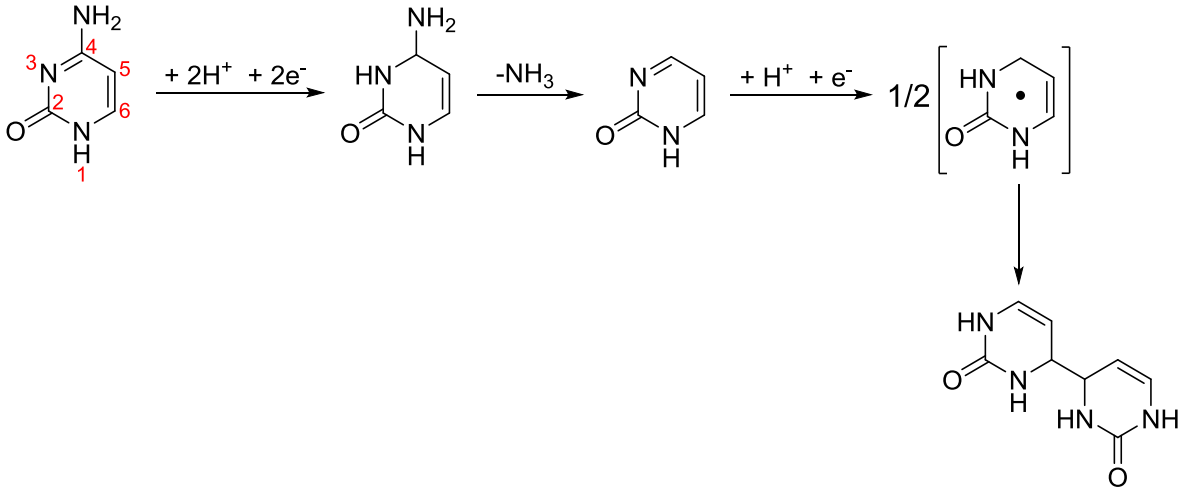
Figure 4. Selected examples of functionalized dNTPs.

1.4 Electrochemistry of nucleic acids

1.4.1 Electrochemical reduction of nucleobases

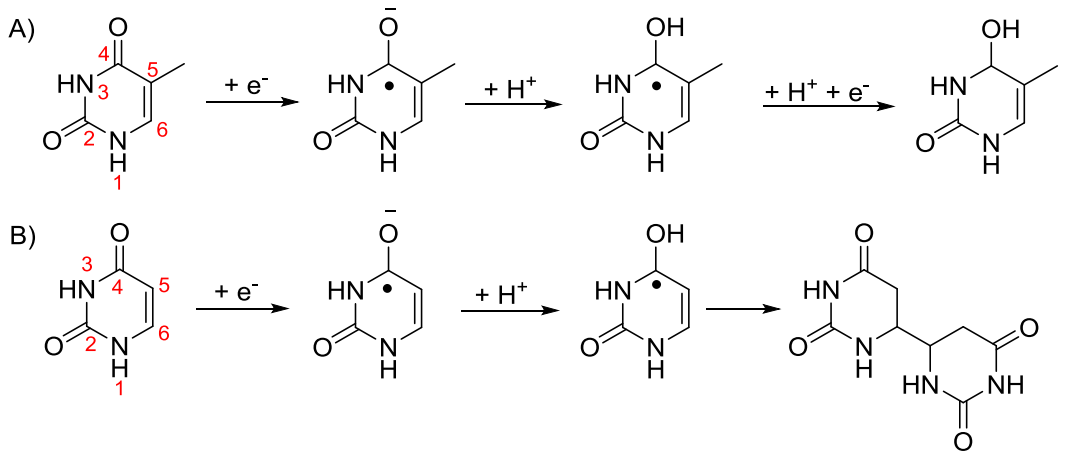
Electroactivity of nucleic acids was discovered more than 50 years ago by E. Paleček, who applied an oscillographic polarography for detecting of DNA[82]. Reduction of bases at mercury drop electrode is particularly sensitive to changes in DNA structure[83,84], while solid electrodes, especially carbon, are used for oxidation processes[85,86].

Electrochemical reduction of cytosine shows a $3e^-$ process (Scheme 11). The reduction mechanism is initiated by hydrogenation of the double bonds N(3)=C(4) with a formation of dihydropyrimidine followed by deamination to form a 2-oxypyrimidine, which immediately undergoes a le^- reduction resulting in the formation of a free radical which dimerizes to a 6,6'-bis(3,6-dihydropyrimid-2-one).



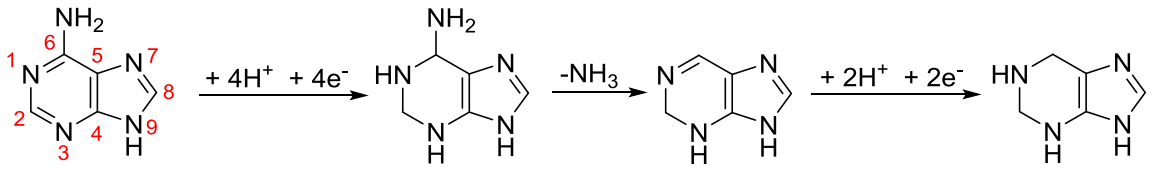
Scheme 11. Mechanism of $3e^-$ process of the cytosine reduction.

The reduction of uracil and thymine was not observed at mercury electrode in aqueous solutions because of complication by the reduction of protons (from the water molecules), however, the electrochemical reduction of uracil and thymine was studied in dimethyl sulfoxide[87,88]. Uracil and thymine undergo complicated reduction processes at highly negative potentials involving several radicals. The initial reduction in DMSO is suggested to be at the position C(4) of pyrimidine ring both for uracil and thymine. The resulting radical anion of uracil abstracts a proton from the nucleobase to form the conjugate base of uracil and the free radical which rapidly dimerizes making the protonation step effectively irreversible. The protonation reaction is considerably more rapid than the radical anion dimerization and consistent with the strong proton affinity of radical anions from the fact that a very weak acid (uracil or thymine) is the protonating agent in such a rapid reaction. The thymine free radical undergoes further reduction rather than dimerization because of steric hindrance of 5-methyl group (Scheme 12).



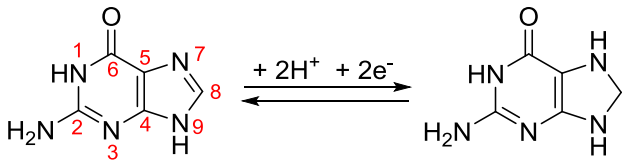
Scheme 12. Mechanism of the thymine (A) and uracil (B) electrochemical reduction.

Electrochemical reduction of adenine is a $6e^-$ process, which includes following steps: hydrogenation of double bonds N(1)=C(6) and C(2)=N(3), deamination and further reduction of the regenerated double bond (Scheme 13)[86].



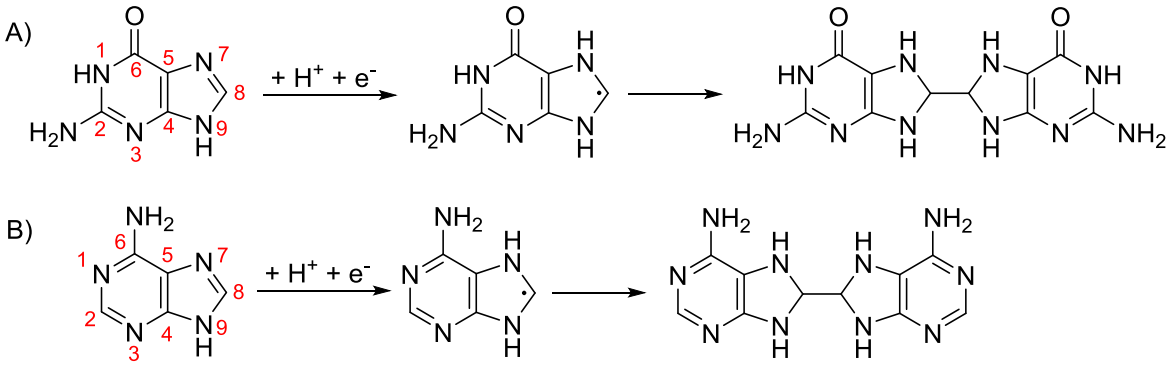
Scheme 13. Mechanism of the adenine electrochemical reduction.

Guanine, in common with adenine, is reducible at the position N(1)=C(6), but at the potentials close to the electrolyte discharge with a formation of an unstable, oxidizable product (Scheme 14)[86].



Scheme 14. Mechanism of the guanine electrochemical reduction.

Recent studies of the electrochemical behavior of the guanine and adenine at platinum electrodes in ionic liquids show the formation of the unstable radical anions, which undergo the dimerization. The reductive site is suggested to be at the position C(8) of the imidazole group in both purines and not at N(1)=C(6) bond of the pyrimidine ring as verified by its reduction in acid medium (Scheme 15)[89].

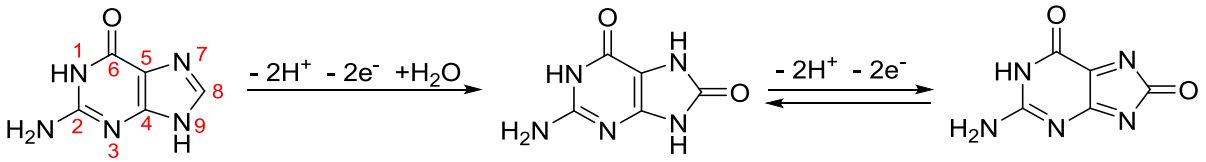


Scheme 15. Reduction mechanism for guanine (A) and adenine (B) on the platinum electrode in ionic liquid.

1.4.2 Electrochemical oxidation of nucleobases

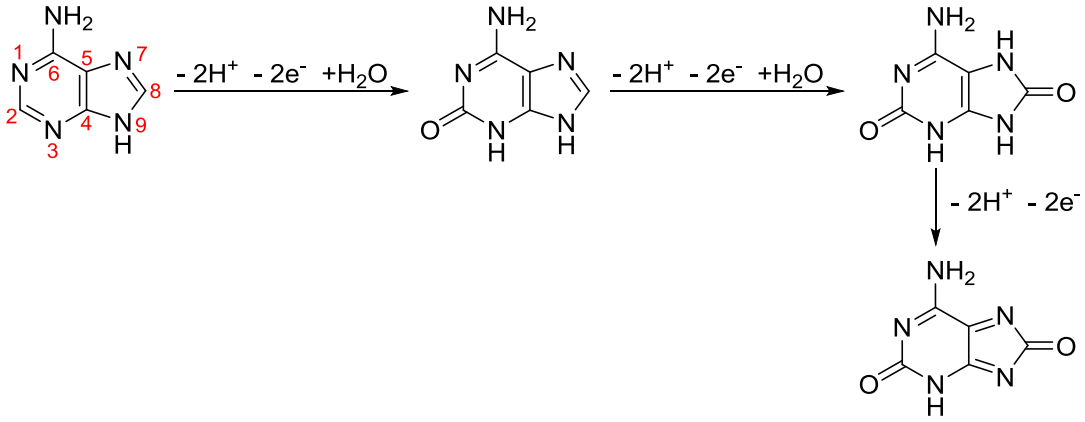
Although studies of the electrochemical oxidation of DNA have been performed using different electrodes such as silver[90], copper[91], gold[92] and mercury[93], carbon-based electrodes stay the most widely used electrodes for studying the direct oxidation of nucleobases, nucleosides and nucleotides[94].

Guanine is the nucleobase which is the easiest to oxidize. The oxidation process of guanine is irreversible and proceeds in two steps: oxidation of the doble bond N(7)=C(8) to 8-oxo-7,8-dihydroguanine, which requires two protons and two electrons and following oxidation of the double bond C(4)=C(5) with formation of the 8-oxo-7,8-dihydroguanine by using of another two protons and two electrons in a reversible reaction (Scheme 16).



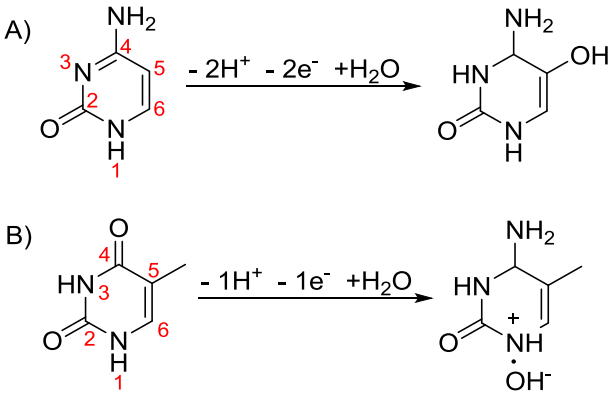
Scheme 16. Mechanism of electrochemical oxidation of guanine.

The electrochemical oxidation of adenine[95] is $6e^-$ process and consists on three steps. It is known that the products of the first two oxidations (8-oxoadenine and 2,8-dioxoadenine respectively) are more easily oxidized than the parent molecule, that makes all process irreversible (Scheme 17).



Scheme 17. Mechanism of electrochemical oxidation of adenine.

The oxidation peaks of cytosine and thymine at carbon electrodes were firstly observed by Oliveira-Brett group[96]. Cytosine undergoes one step 2e$^-$ oxidation process[97] and shows an oxidation peak at highly positive potential on the carbon electrode, while thymine electrochemical oxidation[98,99] is a 1e$^-$ process (Scheme 18).



Scheme 18. Mechanism of electrochemical oxidation of cytosine (A) and thymine (B).

1.5 Redox-active labels and their applications

Electrochemical detection is extensively used approach with a variety of applications in DNA sequencing and molecular diagnostics. Redox labeling of DNA is alternative to fluorescence detection or radiolabeling due to its simplicity, low cost and ease of miniaturization[100]. This research is based on the idea of developing a set of four orthogonal, independently detectable tags for electrochemical labeling of short DNA fragments, which could be used in detection of point mutations, when a single nucleotide base is changed, inserted or delited from a sequence of DNA[101,102]. Point mutations cause different genetic diseases as cystic fibrosis[103], different types of cancer[104], or neurofibromatosis[105], therefore simple method for diagnosis of mutant DNA is attractive alternative to existing protocols[106].

In Hocek group a lot of different modified triphosphates bearing different redox moieties like amino- and nitrobenzene[107], Ru- and Os(bpy)$_3$ complexes[108], tetrathiafulvalene[109], hydrazones[110], butyl acrylate[111], anthraquinone[112] or azidophenyl[113] were synthesized for redox labeling of DNA by enzymatic incorporation (Figure 5).

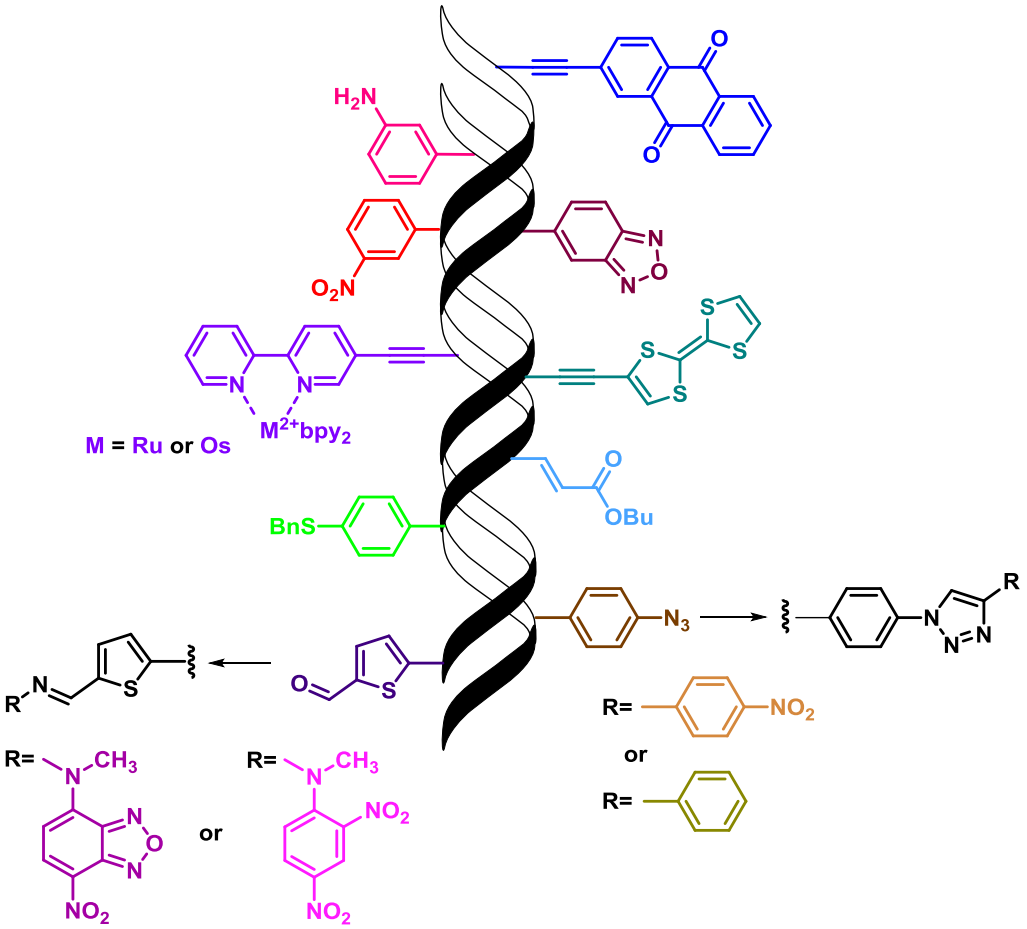


Figure 5. Selected examples of redox labels.

The electrochemical properties of labeled DNA have been studied by cyclic or square-wave voltammetry at hanging mercury drop and pyrolytic graphite electrodes[114]. Natural nucleic acids in aqueous media produce electrochemical signals of irreversible reduction of cytosine and adenine at mercury electrodes around -1.5 V. The peak of guanine reduction can not be observed directly because of the overlapping currents of background discharge, however the reduction product, 7,8-dihydroguanine, can be detected at +0.3 V due to the electrochemical oxidation back to guanine[115]. The advantages of using mercury electrodes for detection of electrochemical reduction are broad potential window in cathodic region and easily renewable surface, which prevents the electrode passivation and increases the sensitivity of the electrochemical detection[116]. Electrochemical oxidation of natural DNA nucleobases can be performed on pyrolytic graphite electrode. Purines produce oxidation signals at +1V for guanine and +1.2 V for adenine, while the oxidation of pyrimidines can be detected at more positive potentials, making difficult their distinction. The signal of background discharge at carbon electrod is at +0.2 V making challenging to find a combination of two oxidizable labels without overlapping with signals of electrochemical oxidation of natural nucleobases.

The oxidation of amino group gives the peak at +0.63-(+0.685) V due to irreversible $2e^-$ process to form the polyamine[117,118]. The irreversible nitro group reduction was observed at redox potential around -0.615-(-0.665) V with formation of hydroxylamine by $4e^-$ mechanism[119,120]. Both types of modifications can be independently detected either by oxidation of amino group or by reduction of nitro group.

Electrochemical measurements of Os^{2+} and Ru^{2+} complexes showed the redox potentials at +0.75 V and +1.1-(+1.25) V respectively. The disadvantage of these labels is that the reversible oxidation of $Ru^{2+/3+}$ complex is close to redox potential of guanine oxidation at +1.1 V, while enzymatic incorporation is also difficult for bulky metal complexes.

The redox potentials of tetrathiafulvalene-modified triphosphates were obtained by square-wave voltammetric technique. Two reversible peaks at +0.2-(+0.3) V and at +0.65 V correspond to two $1e^-$ redox oxidation processes with formation of the cation radical and dication[121]. Polymerase incorporation of tetrathiafulvalene-modified dNTPs was problematic probably due to DNA polymerase inhibition.

The reduction of formylthienyl and hydrazone groups was observed with use of cyclic voltammetry at the hanging mercury drop electrode. Nevertheless the aldehyde and hydrazone groups are rather reactive and/or unstable for biosensing applications.

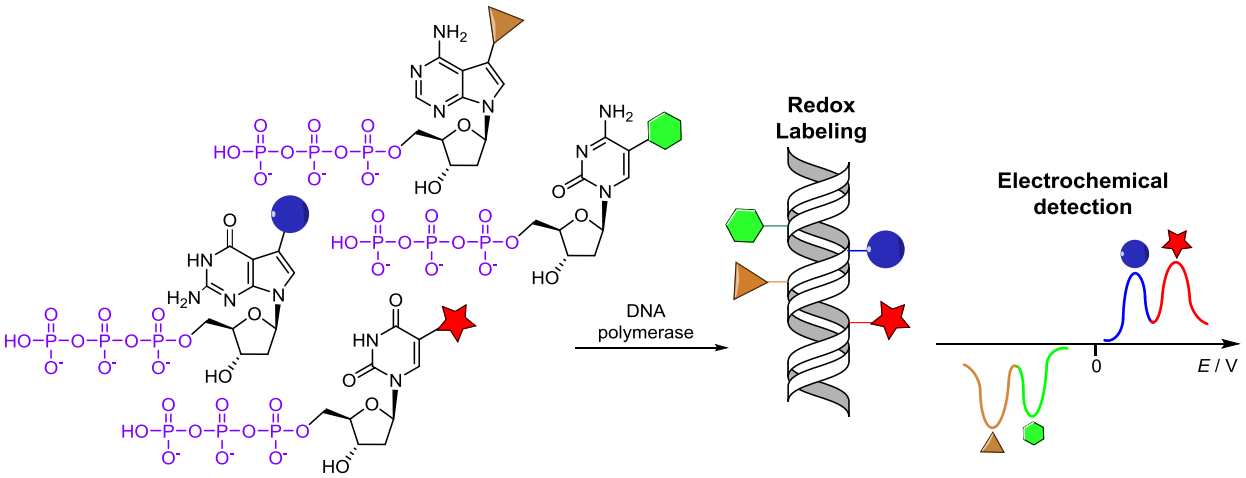
The redox potentials of butylacrylate-modified triphosphates were detected at potentials -1.4 V, that is close to redox potential of irreversible reduction of adenine and cytosine at -1.49 V.

The mechanism of electrochemical reduction of anthraquinone is $2e^-$ process with reversible formation of anthrahydroquinone moiety. The redox potential was observed at -0.41 V, however reduction of anthraquinone in combination with nitrophenyl group gave one broad reduction peak.

Azidophenyl modifications produce a strong reduction peak around -0.9 V under cyclic voltammetry and were used for electrochemical detection of protein-DNA interactions.

Recently a combination of two reducible labels, nitrobenzene and benzofurazane[122], was reported, where multi-electron electrochemical reduction signal of the furazane ring was detected at mercury electrode at -0.90 V, independently from the peak arising by reduction of nitrobenzene group at -0.60 V.

In order to develop the electrochemical detection of multiple nucleobase mutations in short DNA sequences, the library of redox labels should be extended to form an orthogonal set of two reducible and two oxidizable labels for coding of nucleobases, which can be promptly incorporated into DNA and be independently detected in the presence of all the other labels (Scheme 19).



Scheme 19. Set of four orthogonal labels for multicolor redox coding of DNA.

2 Specific aims of the thesis

1. Synthesis of 2,3-dihydrobenzofuran- and 4-hydroxy-3-methoxyphenyl-modified 2′-deoxyribonucleosides and dNTPs substituted in 5-position of cytidine and 7-position of 7-deazaadenine, study on their incorporation into DNA by enzymatic methods and investigation of electrochemical properties of labeled DNA and further examination of methoxyphenol group in combination of benzofurazane and aminophenyl labels for multicolor coding of DNA.
2. Synthesis of directly linked or through an acetylene tether phenothiazine modified derivatives of nucleosides and nucleoside triphosphates, their enzymatic incorporation into DNA and electrochemical study with combination of nitrophenyl and benzofurazane labels.
3. Synthesis of nuleosides, nucleotides and DNA labeled with substituted ferrocenes to tune of their electrochemical behavior depending on the nature of substituents on the ferrocene ring.

2.1 Rationale of the specific aims

Several redox labels were previously reported in our group. Recent discovery of the combination of two reducible labels (nitrophenyl and benzofurazane) made possible the electrochemical minisequensing of short DNA. However, for orthogonal coding of DNA bases, all four building blocks of DNA should be modified by four different labels with strong, easily differentiated electrochemical peaks. Therefore my task was to prepare another two oxidizable labels with independent electrochemical detection without significant mutual interference with signals from natural nucleobases and previously discovered redox labels. For that reasons methoxyphenol-, dihydrobenzofuran- and phenothiazine-labeled nucleosides, nucleotides and DNA were synthesized and their electrochemical properties were studied by cyclic and square-wave voltammetry. Next, methoxyphenol- and phenothiazine tags were tested in combination with previously discovered labels for electrochemical DNA labeling.

Ferrocene is a well known electrochemical label with analytically useful signals in redox potentials[123]. Therefore I have focused on the synthesis of ferrocene labels with different substituents for tunning the electrochemical signals of modified nucleosides, nucleotides and DNA.

3 Results and discussion

3.1 MOP and DHB-labeled nucleosides and dNTPs. Synthesis, enzymatic incorporation and electrochemical detection

3.1.1 Introduction

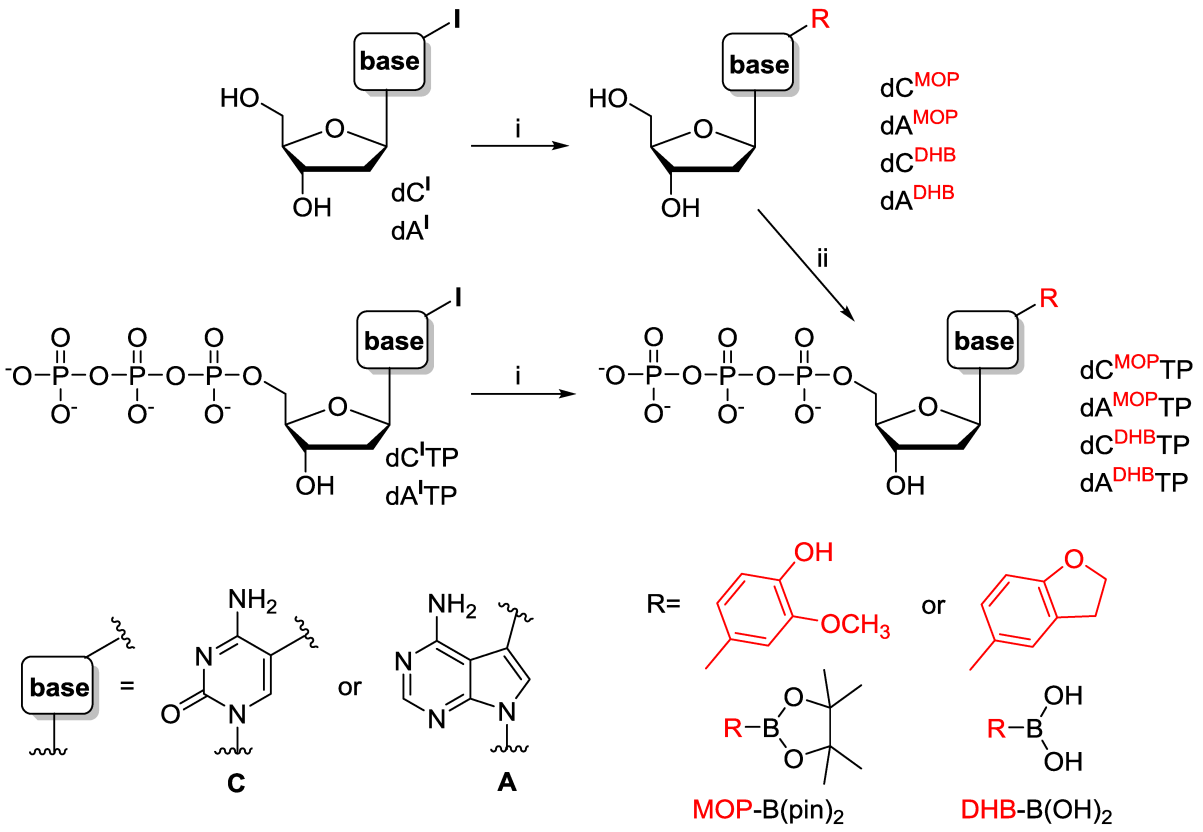
Electrochemical detection is an alternative method of DNA analysis. Number of different oxidizable and reducible labels were mentioned in the chapter 1.5, but most of them suffer from problems with weak sensitivity, limited stability, or difficult incorporation of some labels into DNA. Recently, first orthogonal set of two reducible labels was reported, where nitrophenyl and benzofurazane groups were applied for electrochemical minisequencing of short DNA stretches. However, full orthogonal set of four labels that can be readily incorporated into DNA by polymerase requires another two labels, therefore two oxidizable labels should be developed for a multicolor redox coding of DNA.

2,3-Dihydrobenzofuran (DHB) derivatives are widely used compounds due to their capability to exhibit useful biological activities[124]. The electrochemistry of this class of compounds was not deeply investigated, but they are known to be easily oxidized to aromatic benzofurans[125]. 2-Methoxyphenol (MOP) is known to undergo $2e^-$ electrooxidation to o-benzoquinone[126]. The application of DHB and MOP groups in nucleoside chemistry was not reported before.

3.1.2 Synthesis

The synthesis of base-modified nucleosides and nucleoside triphosphates was performed by aqueous-phase Suzuki-Miyaura cross-coupling reactions of the corresponding halogenated nucleosides or dNTPs with commercially available 2,3-dihydrobenzofuran-5-boronic acid **DHB-**$B(OH)_2$ or 4-hydroxy-3-methoxyphenylboronic acid pinacol ester **MOP-**B(pin) (Scheme 20).

At first, the reactions were performed with nucleosides as models for further use of optimized conditions in the synthesis of modified dNTPs. Thus the reactions of either $\mathbf{dA^I}$ or $\mathbf{dC^I}$ with **MOP-**B(pin) and **DHB-**$B(OH)_2$ were carried out in the mixture of water/acetonitrile (1:2) in the presence of $Pd(OAc)_2$, 3,3′,3″-phosphinidynetris(benzenesulfonic acid) trisodium salt (TPPTS) as a water-soluble phosphine ligand, and Cs_2CO_3 as a base, at 75°C for 1 hour. The desired aryl-substituted nucleosides $\mathbf{dA^{MOP}}$, $\mathbf{dC^{MOP}}$, $\mathbf{dA^{DHB}}$, and $\mathbf{dC^{DHB}}$ were obtained in acceptable yields of 42–78% after chromatographic separation (Table 1).



Scheme 20. Synthesis of modified nucleosides and nucleotides by Suzuki reaction. Reagents and conditions: i) **MOP**-B(pin) or **DHB**-$B(OH)_2$, $Pd(OAc)_2$, TPPTS, Cs_2CO_3, $MeCN/H_2O$ (2:1), 1 h, 75°C for nucleosides; 40 min, 50°C for nucleoside triphosphates; ii) 1) $PO(OMe)_3$, $POCl_3$, 3 h, 0°C; 2) $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 1.5 h, 0°C; 3) 2M TEAB (phosphorylation of modified nucleosides to triphosphates was applied only in the case of **dC^{MOP}TP**).

The same reactions were then performed with iodinated dNTPs (**dA^{I}TP** and **dC^{I}TP**) for 40 minutes at 50 °C (to avoid hydrolysis of the triphosphates). The convertion to desired labeled dNTPs (**dA^{MOP}TP**, **dC^{MOP}TP**, **dA^{DHB}TP**, and **dC^{DHB}TP**) after isolation by semipreparative HPLC proceeded in approximately 40% yields (Table 1). Only in the case of **dC^{MOP}TP** the isolation of modified nucleoside triphosphate was complicated by a similar retention time to TPPTS. Therefore, this compound was synthesized by an alternative approach. Corresponding modified nucleoside **dC^{MOP}** underwert a chemical triphosphorylation by reaction with $POCl_3$ in trimethyl phosphate at 0 °C followed by the addition of $(NHBu_3)_2H_2P_2O_7$ in DMF in the presence of Bu_3N. The reaction was quenched with triethylammonium bicarbonate (TEAB) and the desired product **dC^{MOP}TP** was isolated in pure form in 28% yield.

Table 1. Synthesis of nucleosides and nucleoside triphosphates bearing 2,3-dihydrobenzofuran (**DHB**) or 4-hydroxy-3-methoxyphenyl (**MOP**) derivatives.

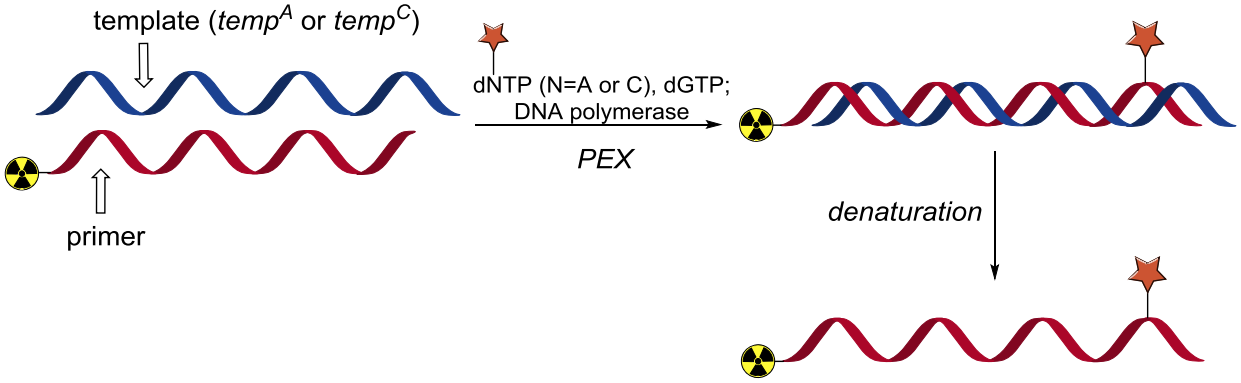
Entry	Starting compound	Reagent	Product	yield(%)[a]
1	dA^{I}	DHB-$B(OH)_2$	$\mathbf{dA^{DHB}}$	78
2	dC^{I}	DHB-$B(OH)_2$	$\mathbf{dC^{DHB}}$	69
3	dA^{I}	MOP-B(pin)	$\mathbf{dA^{MOP}}$	77
4	dC^{I}	MOP-B(pin)	$\mathbf{dC^{MOP}}$	83
5	$dA^{I}TP$	DHB-$B(OH)_2$	$\mathbf{dA^{DHB}TP}$	42
6	$dC^{I}TP$	DHB-$B(OH)_2$	$\mathbf{dC^{DHB}TP}$	41
7	$dA^{I}TP$	MOP-B(pin)	$\mathbf{dA^{MOP}TP}$	42
8	$dC^{I}TP$	MOP-B(pin)	$\mathbf{dC^{MOP}TP}$	35[b]
9	$\mathbf{dC^{MOP}}$	1) $PO(OMe)_3$, $POCl_3$, 0°C; 2) $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0°C; 3) TEAB (2M)	$\mathbf{dC^{MOP}TP}$	28

[a] Yield of isolated product. [b] Product was not separable from TPPTS.

3.1.3 Enzymatic incorporation of modified dNTPs into DNA

The enzymatic synthesis of modified dNTPs ($\mathbf{dA^{MOP}TP}$, $\mathbf{dC^{MOP}TP}$, $\mathbf{dA^{DHB}TP}$, and $\mathbf{dC^{DHB}TP}$) was studied by primer extension experiments (PEX) using KOD XL and Pwo polymerases. The templates and primer were designed in order to introduce one ($\mathbf{ON^{1X}}$) or four modifications ($\mathbf{ON^{4X}}$) to the extended primer strand (for sequences of primer and templates, see Table 2).

At first, single nucleotide incorporation of each of the $\mathbf{dN^{X}TP}$s into a 15-nucleotide (nt) primer was followed by three natural dG using templates $temp^{A}$ or $temp^{C}$ (Scheme 21).



Scheme 21. Single nucleotide incorporation by primer extension experiments.

All four functionalized triphosphates were successfully incorporated into DNA and fully extended products were observed by polyacrylamide gel electrophoresis (Figure 6).

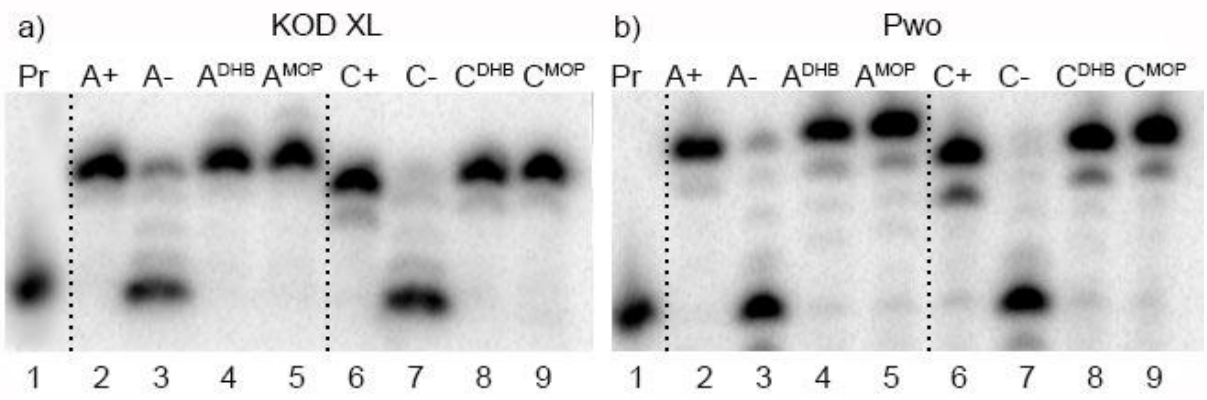


Figure 6. Primer extension with a) KOD XL and b) Pwo polymerases using prim^{rnd}, temp^{A}, and temp^{C}: (Pr) primer (5'-^{32}P-end labeled); (A+) dATP, dGTP; (A-) dGTP; (A^{DHB}) **dA^{DHB}TP**, dGTP; (A^{MOP}) **dA^{MOP}TP**, dGTP; (C+) dCTP, dGTP; (C-) dGTP; (C^{DHB}) **dC^{DHB}TP**, dGTP; (C^{MOP}) **dC^{MOP}PTP**, dGTP.

Table 2. List of sequences of templates, primer, and products*

Oligonucleotide	Sequence
primer^{rnd}	5'-CATGGGCGGCATGGG-3'
temp^{rnd16}	5'-**CTAGCATGAGCTCAGT**CCCATGCCGCCCATG-3'
temp^{4A}	5'-**TTTT**CCCATGCCGCCCATG-3'
temp^{4A1C}	5'-**GTTTT**CCCATGCCGCCCATG-3'
temp^{4C}	5'-**GGGG**CCCATGCCGCCCATG-3'
temp^{A}	5'-**CCCT**CCCATGCCGCCCATG-3'
temp^{C}	5'-**CCCG**CCCATGCCGCCCATG-3'
temp^{termA}	5'-**T**CCCATGCCGCCCATG-3'
temp^{termC}	5'-**G**CCCATGCCGCCCATG-3'
ON^{rnd16} **A^{DHB}**	5'-CATGGGCGGCATGGGA^{DHB}CTGA^{DHB}GCTCA^{DHB}TGCTA^{DHB}G-3'
ON^{rnd16} **A^{MOP}**	5'-CATGGGCGGCATGGGA^{MOP}CTGA^{MOP}GCTCA^{MOP}TGCTA^{MOP}G-3'
ON^{rnd16} **C^{DHB}**	5'-CATGGGCGGCATGGGA**C**^{DHB}TGAG**C**^{DHB}**C**^{DHB}ATG**C**^{DHB}TAG-3'
ON^{rnd16} **C^{MOP}**	5'-CATGGGCGGCATGGGA**C**^{MOP}TGAG**C**^{MOP}T**C**^{MOP}ATG**C**^{MOP}TAG-3'
ON^{rnd16} **A^{DHB} C^{MOP}**	5'-CATGGGCGGCATGGGA^{DHB}**C**^{MOP}TGA^{DHB}G**C**^{MOP}T**C**^{MOP}A^{DHB}TG**C**^{MOP}TA^{DHB}G-3'
ON^{rnd16} **A^{MOP} C^{DHB}**	5'-CATGGGCGGCATGGGA^{MOP}**C**^{DHB}TGA^{MOP}G**C**^{DHB}T**C**^{DHB}A^{MOP}TG**C**^{DHB}TA^{MOP}G-3'
ON^{rnd16} **A^{NH2} C^{MOP}**	5'-CATGGGCGGCATGGGA^{NH2}**C**^{MOP}TGA^{NH2}G**C**^{MOP}T**C**^{MOP}A^{NH2}TG**C**^{MOP}TA^{NH2}G-3'
ON^{rnd16} **A^{BF} C^{MOP}**	5'-CATGGGCGGCATGGGA^{BF}**C**^{MOP}TGA^{BF}G**C**^{MOP}T**C**^{MOP}A^{BF}TG**C**^{MOP}TA^{BF}G-3'
ON^{4A} **A^{DHB}**	5'-CATGGGCGGCATGGGA^{DHB}A^{DHB}A^{DHB}A^{DHB} -3'
ON^{4A} **A^{MOP}**	5'-CATGGGCGGCATGGGA^{MOP}A^{MOP}A^{MOP}A^{MOP}-3'
ON^{4A1C} **A^{DHB}**	5'-CATGGGCGGCATGGGA^{DHB}A^{DHB}A^{DHB}A^{DHB}-3'
ON^{4A1C} **A^{MOP}**	5'-CATGGGCGGCATGGGA^{MOP}A^{MOP}A^{MOP}A^{MOP}-3'
ON^{4C} **C^{DHB}**	5'-CATGGGCGGCATGGG**C**^{DHB}**C**^{DHB}**C**^{DHB}**C**^{DHB} -3'
ON^{4C} **C^{MOP}**	5'-CATGGGCGGCATGGG**C**^{MOP}**C**^{MOP}**C**^{MOP}**C**^{MOP} -3'
ON^{A} **A^{DHB}**	5'-CATGGGCGGCATGGGA^{DHB}GGG-3'
ON^{A} **A^{MOP}**	5'-CATGGGCGGCATGGGA^{MOP}GGG-3'
ON^{C} **C^{DHB}**	5'-CATGGGCGGCATGGG**C**^{DHB}GGG-3'
ON^{C} **C^{MOP}**	5'-CATGGGCGGCATGGG**C**^{MOP}GGG-3'
ON^{termA} **A^{DHB}**	5'-CATGGGCGGCATGGGA^{DHB} -3'
ON^{termA} **A^{MOP}**	5'-CATGGGCGGCATGGGA^{MOP} -3'
ON^{termC} **C^{DHB}**	5'-CATGGGCGGCATGGG**C**^{DHB} -3'
ON^{termC} **C^{MOP}**	5'-CATGGGCGGCATGGG**C**^{MOP} -3'

*In the template ONs the segments forming duplex with the primer are underlined, the replicated segments are in bold. For magnetic separation of the extended primer strands, the templates were 5´-end biotinylated. Acronyms used in the text for primer extension products are analogous to those introduced for the templates.

Next, kinetic experiments were performed to compare the efficiency of incorporation of modified and natural dNTPs in primer extension reactions using KOD XL polymerase and different templates. At first, simple kinetics study involved single-nucleotide PEX reactions in order to compare the rate of incorporation of the MOP- and DHB-modified dNTPs in comparison with the natural ones (Figures 7-10). Kinetic experiments showed that the modified dCTPs (**dC^{DHB}TP** and **dC^{MOP}TP**) were incorporated with only slightly slower rate than dCTP, while modified dATPs (**dA^{DHB}TP** and **dA^{MOP}TP**) tended to form N+1 products (Figures 7, 9).

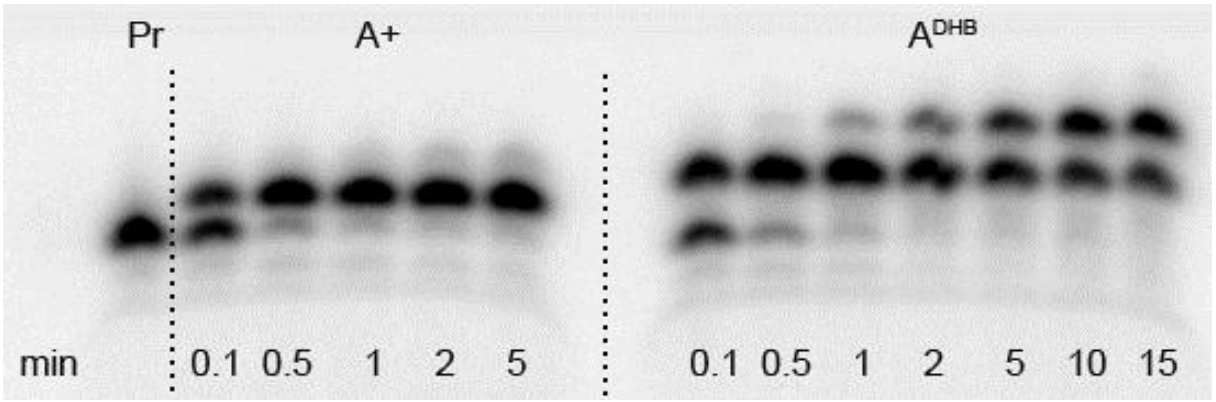


Figure 7. Comparison of incorporation of natural and modified dATPs to DNA with *temp^{termA}* using KOD XL polymerase: (**Pr**) primer; (**A+**) dATP; (**A^{DHB}**) **dA^{DHB}TP**.

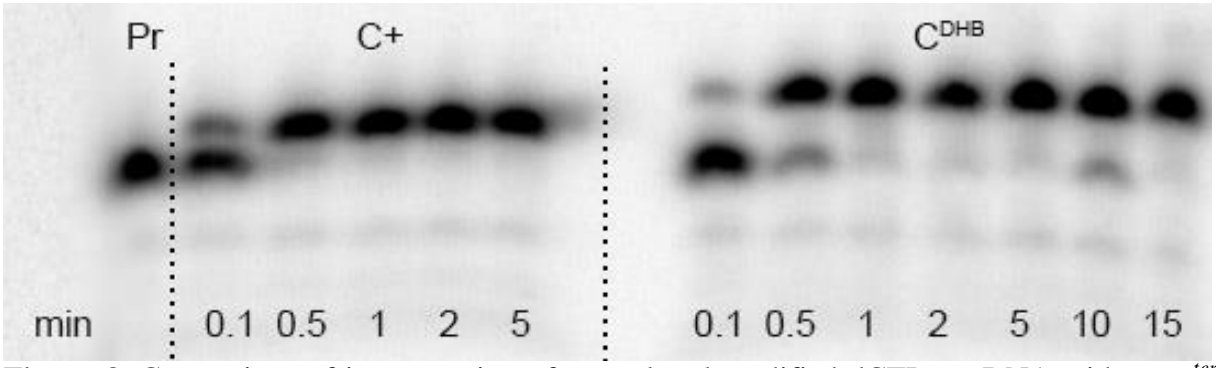


Figure 8. Comparison of incorporation of natural and modified dCTPs to DNA with *temp^{termC}* using KOD XL polymerase: (**Pr**) primer; (**C+**) dCTP; (**C^{DHB}**) **dC^{DHB}TP**.

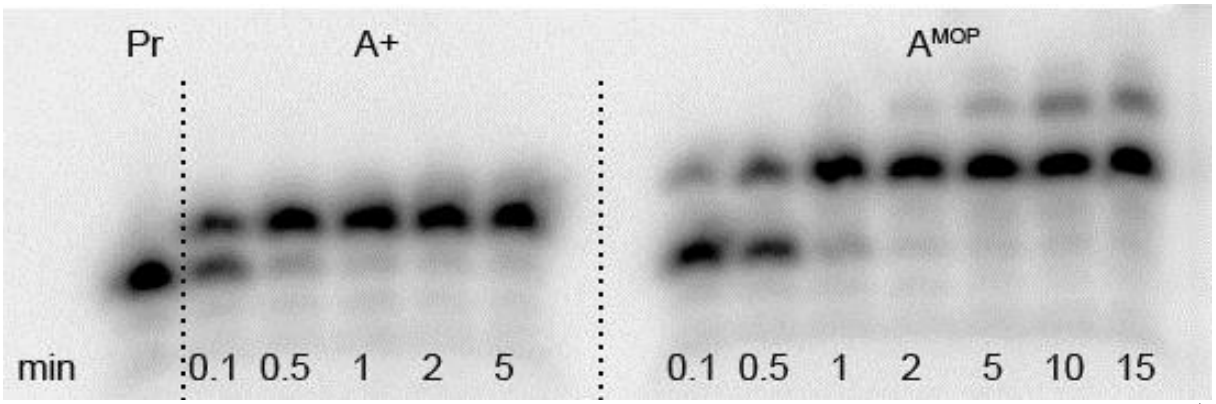


Figure 9. Comparison of incorporation of natural and modified dATPs to DNA with *temp^{termA}* using KOD XL polymerase: (**Pr**) primer; (**A+**) dATP; (**A^{MOP}**) **dA^{MOP}TP**.

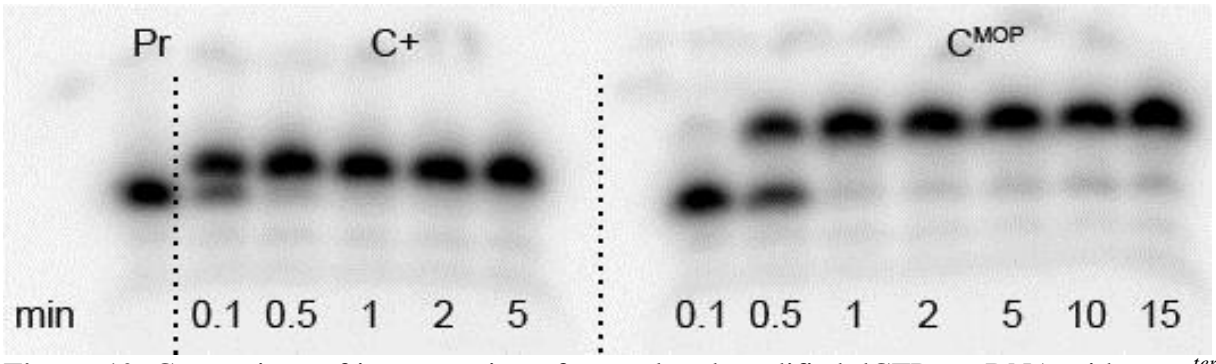


Figure 10. Comparison of incorporation of natural and modified dCTPs to DNA with *temp*^{termC} using KOD XL polymerase: (**Pr**) primer; (**C+**) dCTP; (**C**$^{\mathbf{MOP}}$) **dC**$^{\mathbf{MOP}}$**TP**.

Next, KOD XL and Pwo polymerases were used for incorporation of four modified dNTPs at adjacent positions. The incorporation of the natural dATPs as well as modified **dA**$^{\mathbf{DHB}}$**TPs** and **dA**$^{\mathbf{MOP}}$**TPs** using temp^{4A1C} and KOD XL polymerase was finished within 30 seconds. Using Pwo polymerase incorporation of the natural dATPs was finished in 2 minutes and modified **dA**$^{\mathbf{X}}$**TP**s were incorporated in 5 minutes. Despite the fact that template temp^{4A1C} was designed to avoid the extra incorporation of modified **dA**$^{\mathbf{X}}$**TP**s by adding G nucleotide in the 5´-end of the template the formation of n+1 products was still observed both for KOD XL and Pwo polymerases (Figure 11-14).

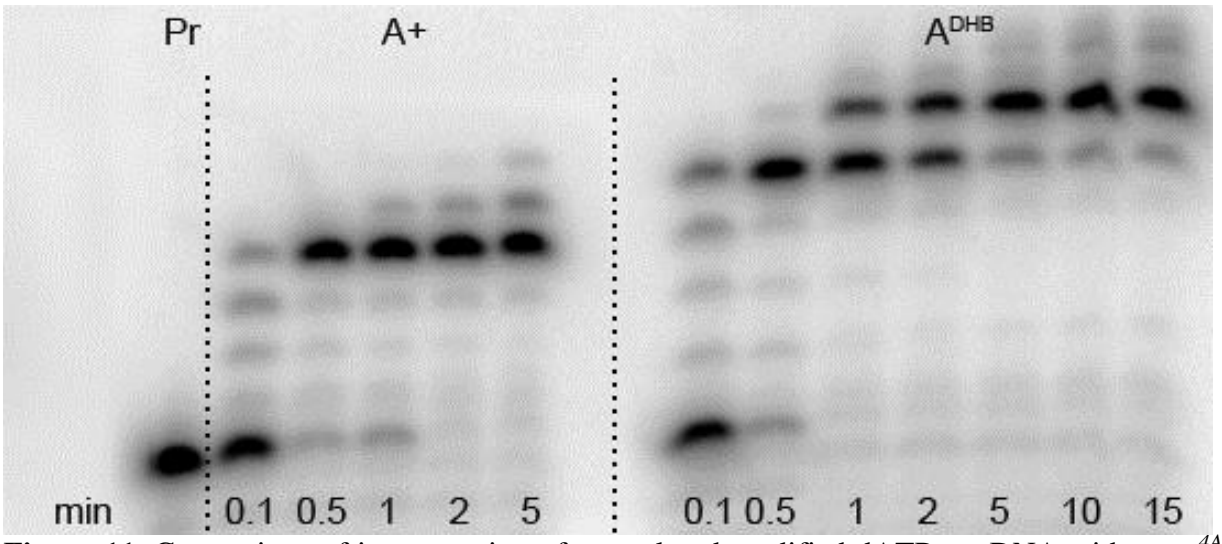


Figure 11. Comparison of incorporation of natural and modified dATPs to DNA with *temp*^{4A1C} using KOD XL polymerase: (**Pr**) primer; (**A+**) dATP; (**A**$^{\mathbf{DHB}}$) **dA**$^{\mathbf{DHB}}$**TP**.

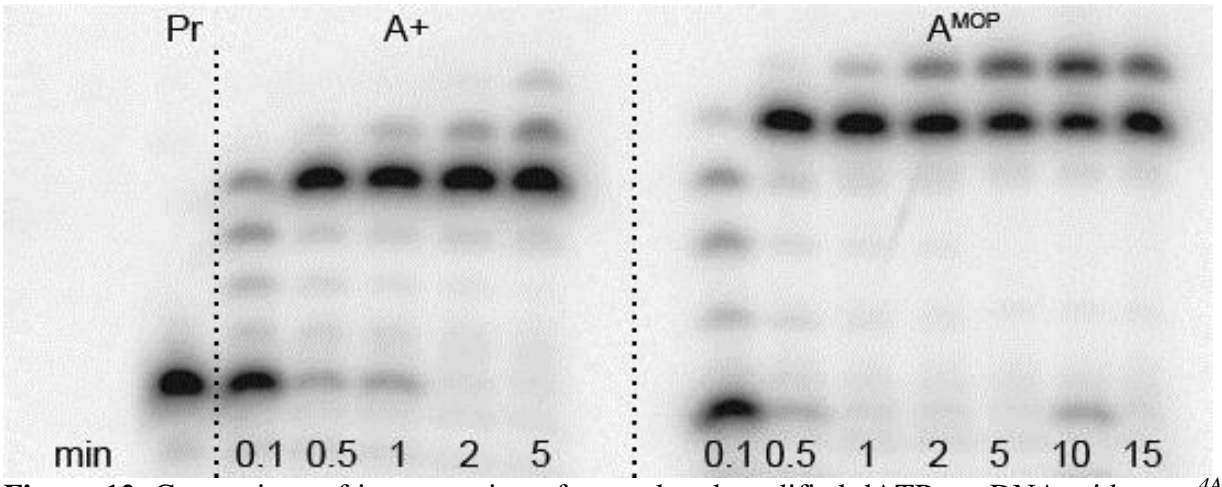


Figure 12. Comparison of incorporation of natural and modified dATPs to DNA with *temp*^{4A1C} using KOD XL polymerase: (**Pr**) primer; (**A+**) dATP; (**A**$^{\mathbf{MOP}}$) **dA**$^{\mathbf{MOP}}$**TP**.

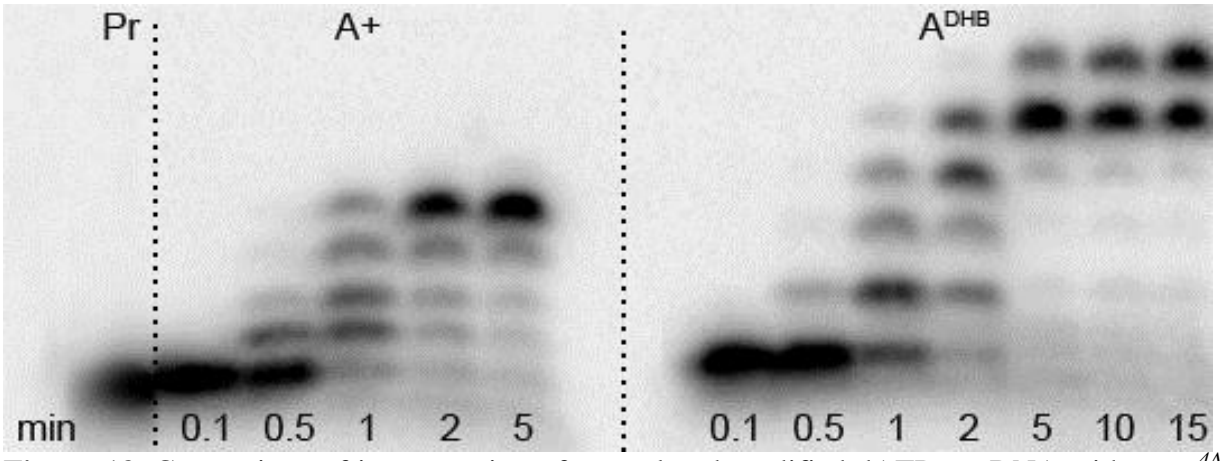


Figure 13. Comparison of incorporation of natural and modified dATPs to DNA with *temp*^{4A1C} using Pwo polymerase: (**Pr**) primer; (**A+**) dATP; (**A**$^{\mathbf{DHB}}$) **dA**$^{\mathbf{DHB}}$**TP**.

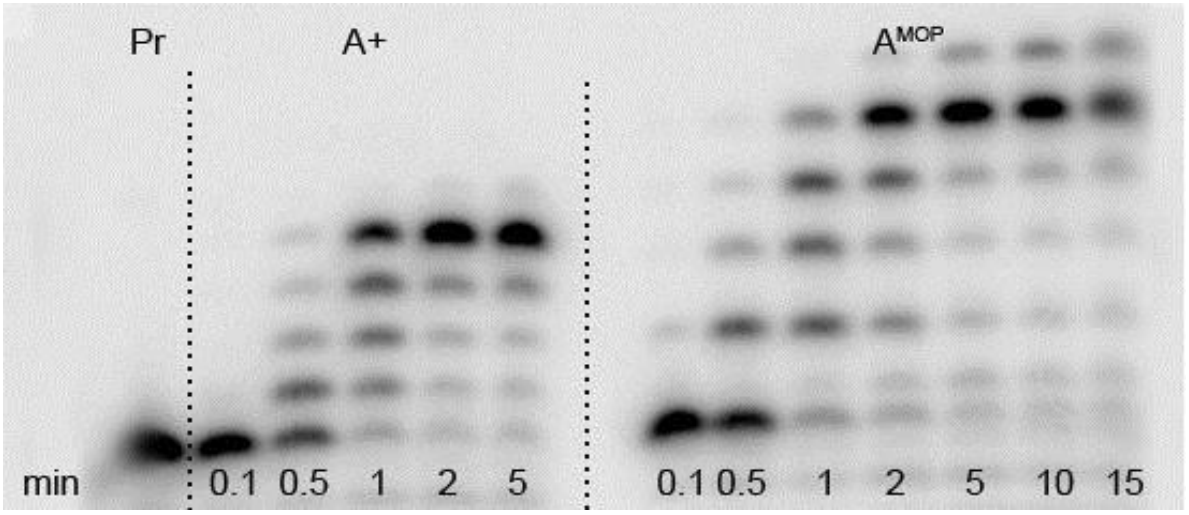


Figure 14. Comparison of incorporation of natural and modified dATPs to DNA with *temp*^{4A1C} using Pwo polymerase: (**Pr**) primer; (**A+**) dATP; (**A**$^{\mathbf{MOP}}$) **dA**$^{\mathbf{MOP}}$**TP**.

The incorporation of the natural dCTPs using temp^{4C}, KOD XL or Pwo polymerases was performed within 5 minutes whereas reactions with modified **dC**$^{\mathbf{DHB}}$**TPs** and **dC**$^{\mathbf{MOP}}$**TPs**

required much longer time to complete the incorporation. The PEX with **dC^{DHB}TP** took 15 minutes to complete using KOD XL polymerase and 1hour using PWO polymerase (Figure15-16). In case of using the **dC^{MOP}TP** in PEX experiment the mixture of full products and n-1 products was observed even after 90 minutes of incubation (Figure17-18).

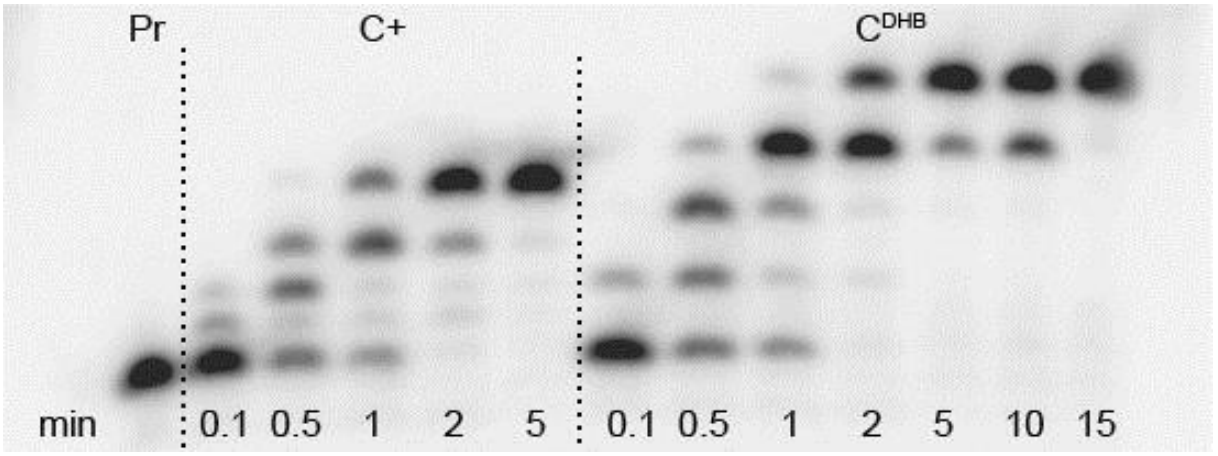


Figure 15. Comparison of incorporation of natural and modified dCTPs to DNA with *temp*^{4C} using KOD XL polymerase: (**Pr**) primer; (**C+**) dCTP; (**C^{DHB}**) **dC^{DHB}TP**.

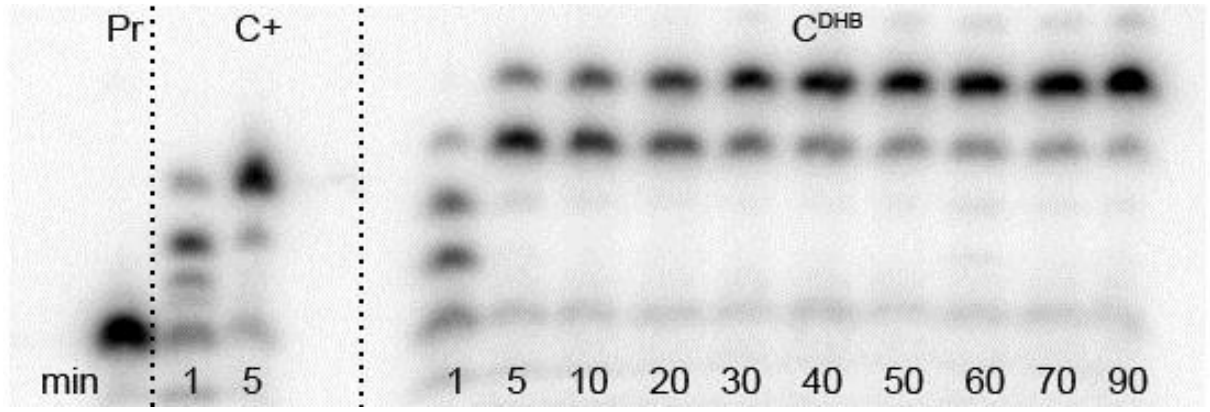


Figure 16. Comparison of incorporation of natural and modified dCTPs to DNA with *temp*^{4C} using Pwo polymerase: (**Pr**) primer; (**C+**) dCTP; (**C^{DHB}**) **dC^{DHB}TP**.

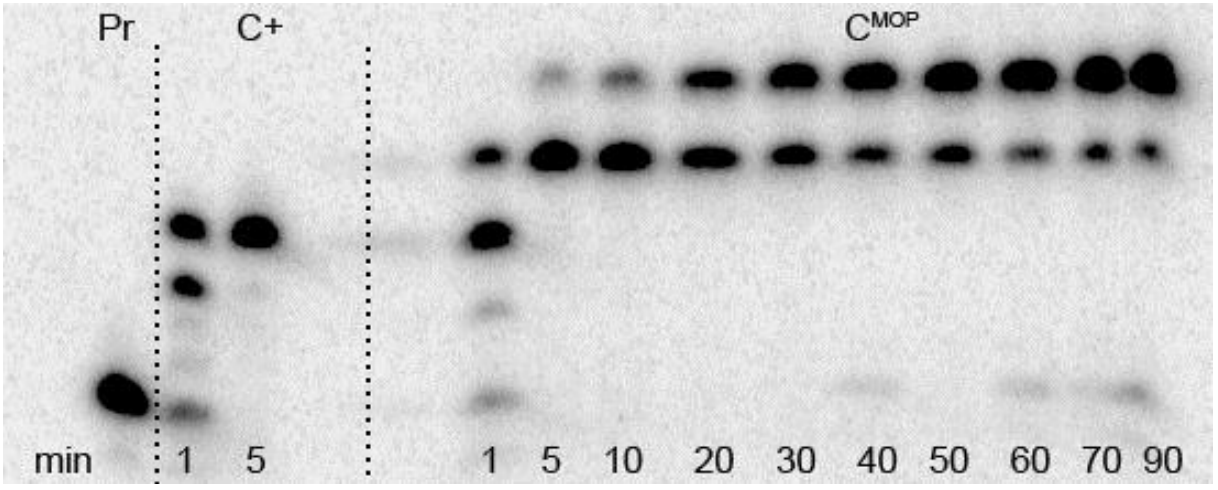


Figure 17. Comparison of incorporation of natural and modified dCTPs to DNA with *temp*^{4C} using KOD XL polymerase: (**Pr**) primer; (**C+**) dCTP; (**C^{MOP}**) **dC^{MOP}TP**.

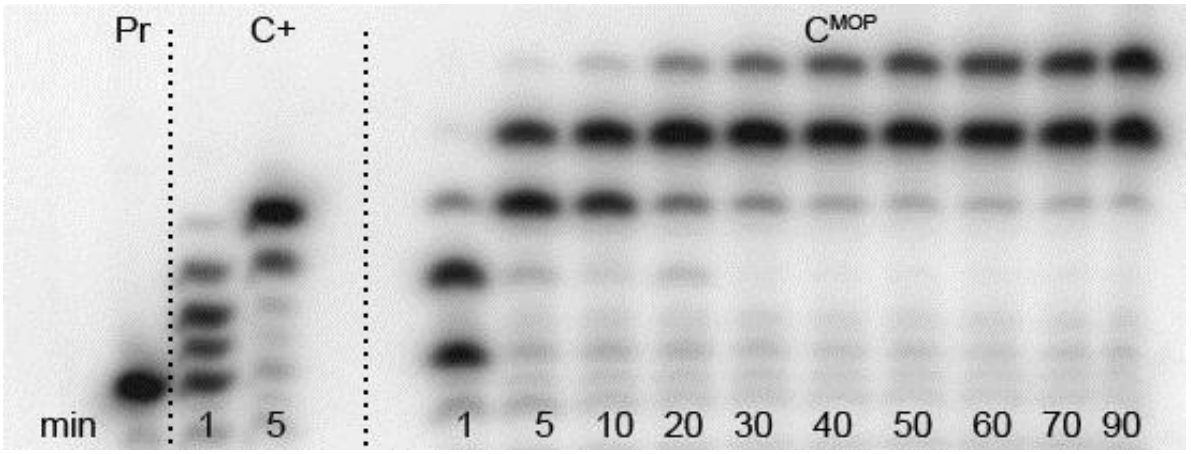
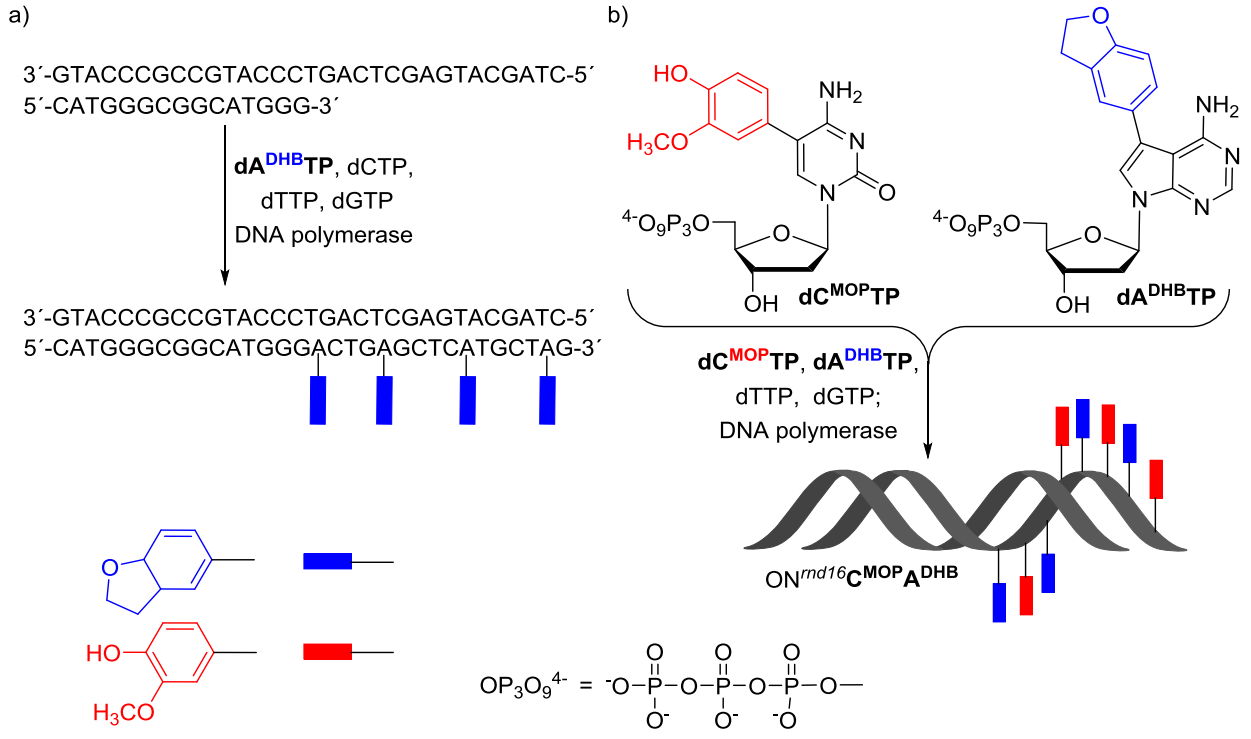


Figure 18. Comparison of incorporation of natural and modified dCTPs to DNA with *temp*^{4C} using Pwo polymerase: (**Pr**) primer; (**C+**) dCTP; (**C^{MOP}**) **dC^{MOP}TP**.

Then, the **dN^{X}TP**s were tested for multiple incorporations into a longer oligonucleotides by using temp^{rnd16} designed for possible encoding of four modified triphosphates of each nucleobase (Scheme 22a). The experiments were performed using KOD XL and Pwo polymerases giving the full-length products in all cases (Figure 19). Also, a combination of a modified A and modified C (**dA^{DHB}TP+dC^{MOP}TP** or **dA^{MOP}TP+dC^{DHB}TP**) was successfully applied to give full-length ONs bearing combinations of two different modifications (Scheme 22b; Figure 19, lines 9). These results confirm that all four modified **dN^{X}TP**s are very good substrates both for KOD XL and Pwo polymerases.



Scheme 22. a) Example of multiple nucleotide incorporation of modified triphosphate **dA^{DHB}TP** by primer extension experiments; b) Multiple nucleotide incorporation of combination of modified triphosphates **dA^{DHB}TP+dC^{MOP}TP** by primer extension experiments.

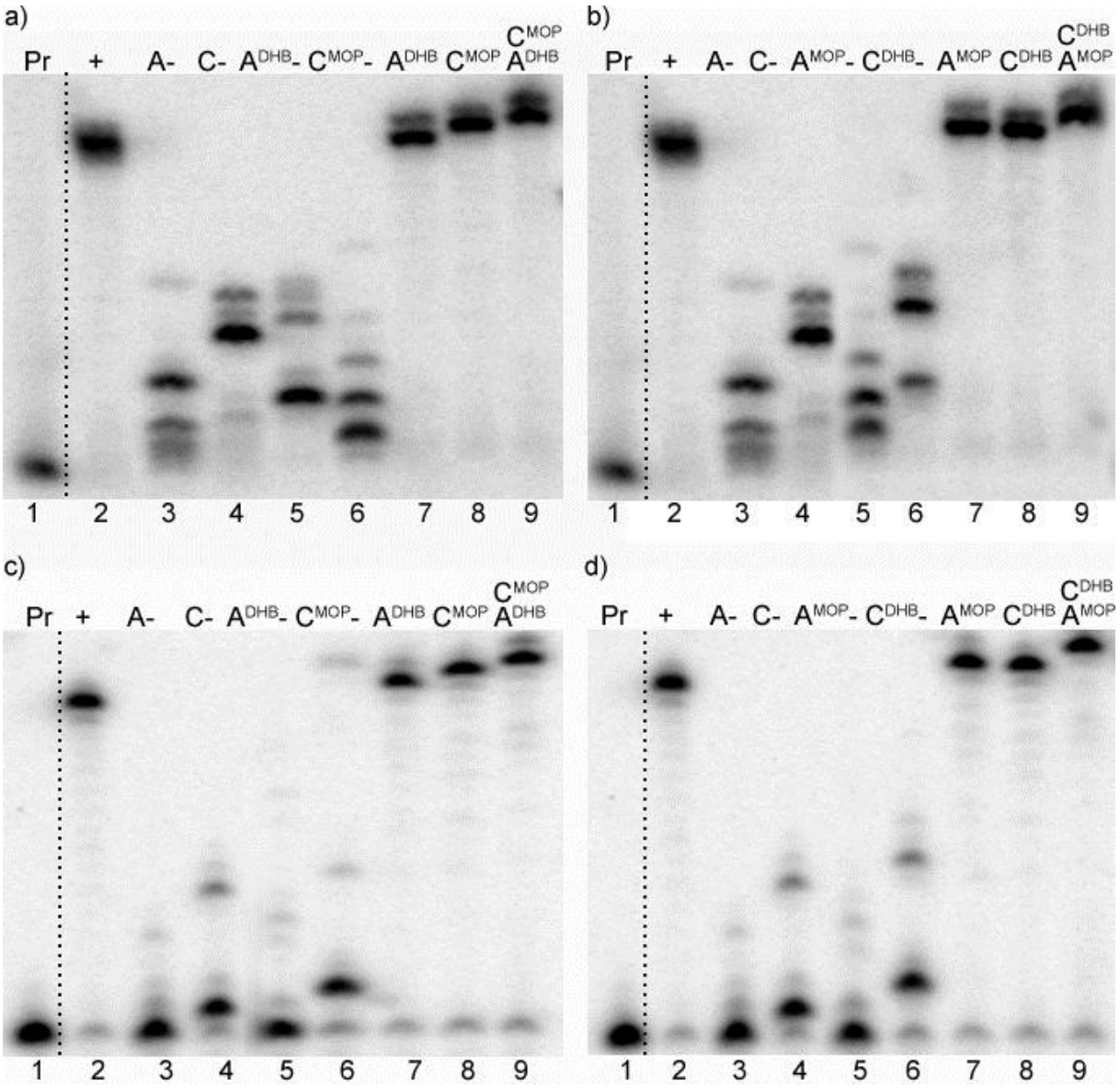


Figure 19. Primer extension with KOD XL (a and b) and Pwo (c and d) polymerases using prim^{rnd}, temp^{rnd16}: (Pr) primer (5'-^{32}P-end labeled); (+) natural dNTPs; (A-) dCTP, dTTP, dGTP; (C-) dATP, dTTP, dGTP; (A^{DHB}-) **dC^{MOP}TP**, dTTP, dGTP; (A^{MOP}-) **dC^{DHB}TP**, dTTP, dGTP; (C^{MOP}-) **dA^{DHB}TP**, dTTP, dGTP; (C^{DHB}-) **dA^{MOP}TP**, dTTP, dGTP; (A^{DHB}) **dA^{DHB}TP**, dCTP, dTTP, dGTP; (A^{MOP}) **dA^{MOP}TP**, dCTP, dTTP, dGTP; (C^{MOP}) **dC^{MOP}TP**, dATP, dTTP, dGTP; (C^{DHB}) **dC^{DHB}TP**, dATP, dTTP, dGTP; (A^{DHB}C^{MOP}) **dA^{DHB}TP**, **dC^{MOP}TP**, dTTP, dGTP; (A^{MOP}C^{DHB}) **dA^{MOP}TP**, **dC^{DHB}TP**, dTTP, dGTP.

All modified ONs (bearing either one, four or eight modifications) prepared by PEX with biotinylated template using KOD XL DNA polymerase were isolated by magnetoseparation for characterization by MALDI-TOF mass spectrometry and for futher use in the electrochemical studies (data are summarized in Table 3).

Table 3. List of MALDI data of midified ONs.

Oligonucleotide	M calcd. (Da)	M found (Da)
ON^{rnd16} **A**$^{\mathbf{DHB}}$	10085.9	10085.9
ON^{rnd16} **A**$^{\mathbf{MOP}}$	10101.86	10101.3
ON^{rnd16} **C**$^{\mathbf{DHB}}$	10089.9	10090.6
ON^{rnd16} **C**$^{\mathbf{MOP}}$	10105.86	10107.0
ON^{rnd16} **A**$^{\mathbf{DHB}}$ **C**$^{\mathbf{MOP}}$	10574,46	10575.2
ON^{rnd16} **A**$^{\mathbf{MOP}}$ **C**$^{\mathbf{DHB}}$	10574.46	10575.3
ON^{rnd16} **A**$^{\mathbf{NH2}}$ **C**$^{\mathbf{MOP}}$	10466.34	10 467.8
ON$^{\mathrm{rnd16}}$ **A**$^{\mathbf{BF}}$ **C**$^{\mathbf{MOP}}$	10574.26	10 575.8
ON^{4A} **A**$^{\mathbf{DHB}}$	6395.5	6396.7
ON^{4A} **A**$^{\mathbf{MOP}}$	6411,46	6433.3*
ON^{4C} **C**$^{\mathbf{DHB}}$	6303.4	6304.9
ON^{4C} **C**$^{\mathbf{MOP}}$	6319.36	6320.7
ON^{A} **A**$^{\mathbf{DHB}}$	6092.05	6093.4
ON^{A} **A**$^{\mathbf{MOP}}$	6096.04	6097.2
ON^{C} **C**$^{\mathbf{DHB}}$	6069.05	6070.1
ON^{C} **C**$^{\mathbf{MOP}}$	6073.04	6074.0

* Found mass corresponds to the presence of one atom of sodium in the sample $[M+Na]^+$

Also the enzymatic synthesis of modified oligonucleotides with four modifications in a row was developed by using temp^{4C} or temp^{4A}. The use of KOD XL polymerase has shown full-length products in all cases, whereas Pwo gave mixtures of full-length and n+1 products in the case of incorporation of modified A bases (**dA**$^{\mathbf{DHB}}$**TP** and **dA**$^{\mathbf{MOP}}$**TP**: Figure 20b, line 4 and 5) and mixtures of full-length and n-1 products with **dC**$^{\mathbf{MOP}}$**TP** (Figure 20b, line10).

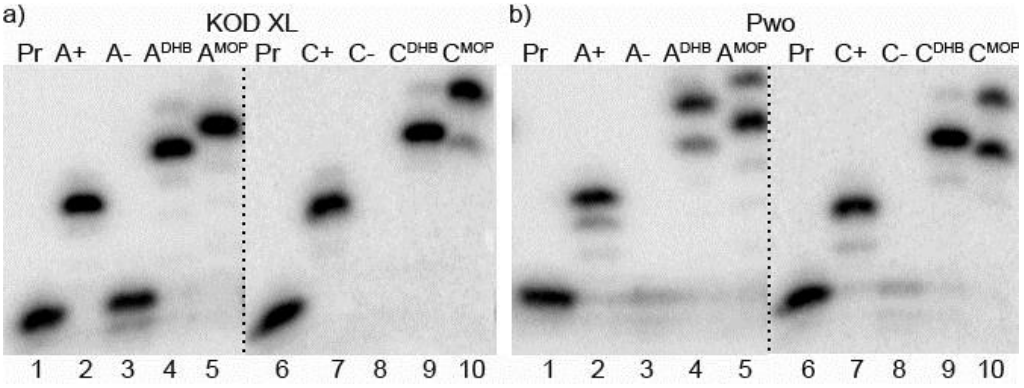
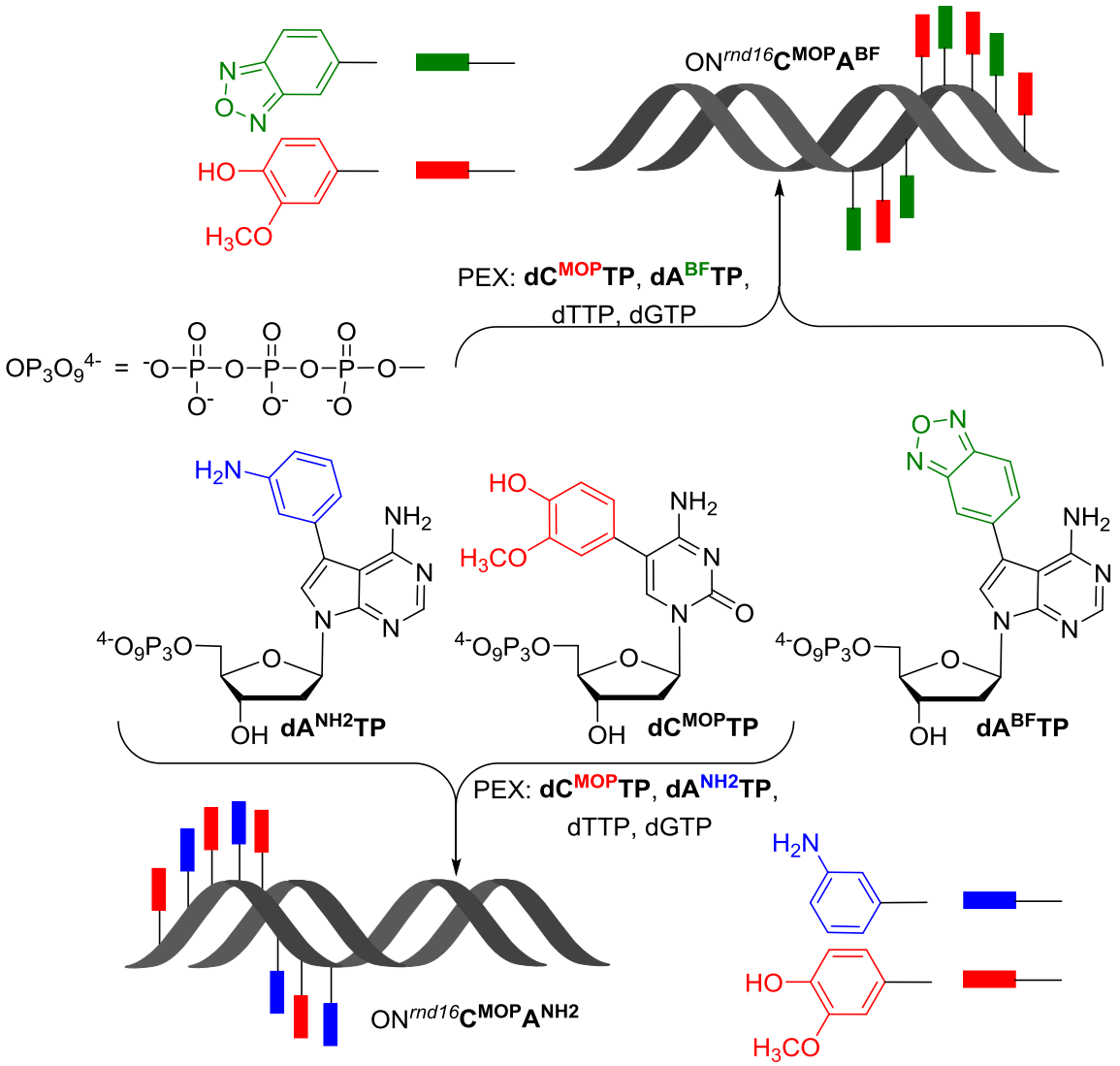


Figure 20. Primer extension with a) KOD XL and b) Pwo polymerases using prim^{rnd}, temp^{4A} and temp^{4C}: (Pr) primer (5'-^{32}P-end labeled); (A+) dATP; (A-) no dNTPs; (A^{DHB}) **dA**$^{\mathbf{DHB}}$**TP**; (A^{MOP}) **dA**$^{\mathbf{MOP}}$**TP**; (C+) dCTP; (C-) no dNTPs; (C^{DHB}) **dC**$^{\mathbf{DHB}}$**TP**; (C^{MOP}) **dC**$^{\mathbf{MOP}}$**TP**.

To investigate the compatibility of the **MOP** label with previously reported aminophenyl and benzofurazane moieties, PEX products were prepared containing combinations of **C**$^{\mathbf{MOP}}$ with 7-(3-aminophenyl)-7-deazaadenine (**A**$^{\mathbf{NH2}}$) or with 7-(benzofurazan-5-yl)-7-deazaadenine (**A**$^{\mathbf{BF}}$) (Scheme 23).



Scheme 23. Redox coding of DNA bases: structures of redox labeled **dN^{X}TPs** and DNA products of the mixed PEX.

Modified oligonucleotides bearing **dC^{MOP}TP** and **dA^{NH2}TP** were enzymatically synthesized using KOD XL or Pwo polymerases and template temp^{rnd16} (Figure 21). Full-length products bearing two different labels were obtained both by KOD XL and Pwo polymerases. Similarly, the incorporations of **dC^{MOP}TP** in combination with **dA^{BF}TP** were performed (Figure 22). KOD XL polymerase gave clean full-length products for each modified DNA, whereas use of Pwo gave a mixture of full-length product with some shorter ON side-products (Figure 22b, line 9). Therefore, for further study of electrochemical properties of labeled ONs KOD XL polymerase was used in enzymatic synthesis.

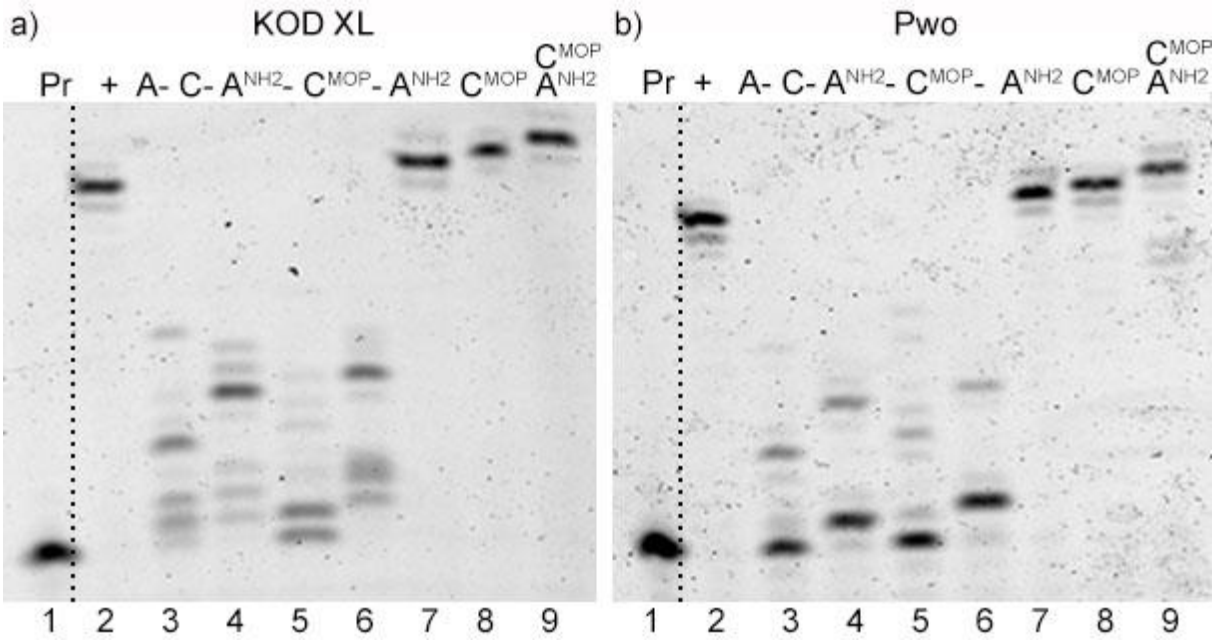


Figure 21. Primer extension with a) KOD XL and b) Pwo polymerases, prim^{rnd}, and temp^{rnd16}: (Pr) primer (5'-FAM-labeled); (+) natural dNTPs; (A-) dCTP, dTTP, dGTP; (C-) dATP, dTTP, dGTP; (A^{NH2}-) **$dC^{MOP}TP$**, dTTP, dGTP; (C^{MOP}-) **$dA^{NH2}TP$**, dTTP, dGTP; (A^{NH2}) **$dA^{NH2}TP$**, dCTP, dTTP, dGTP; (C^{MOP}) **$dC^{MOP}TP$**, dATP, dTTP, dGTP; ($A^{NH2}C^{MOP}$) **$dA^{NH2}TP$**, **$dC^{MOP}TP$**, dTTP, dGTP.

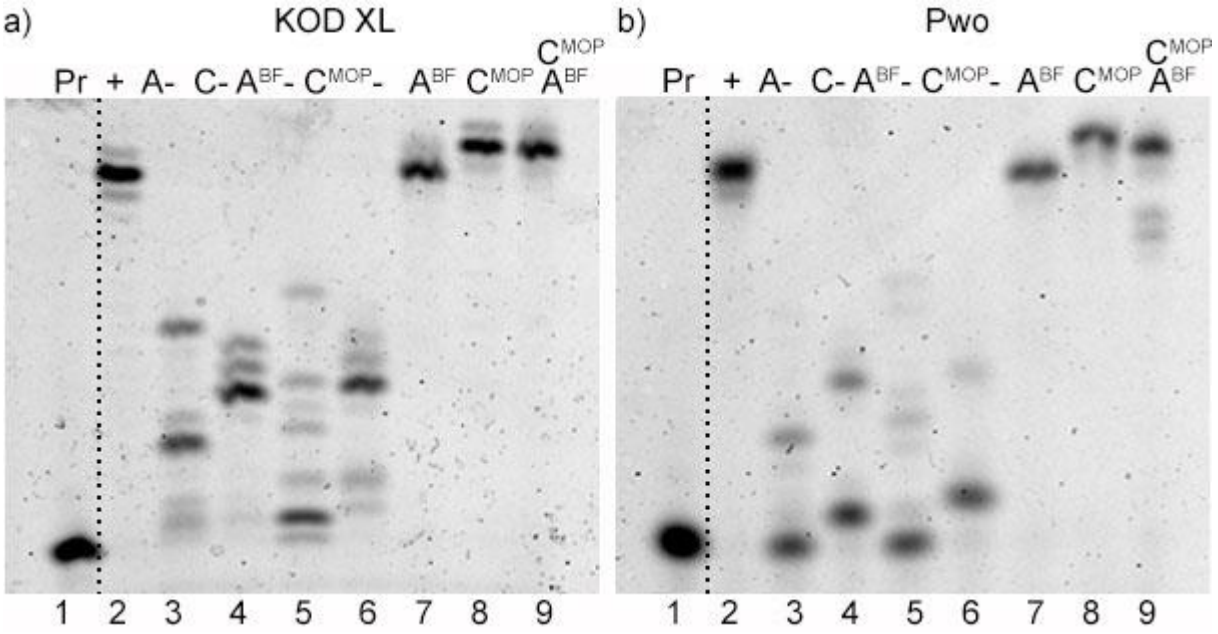


Figure 22. Primer extension with a) KOD XL and b) Pwo polymerases, prim^{rnd}, and temp^{rnd16}: (Pr) primer (5'-FAM-labeled); (+) natural dNTPs; (A-) dCTP, dTTP, dGTP; (C-) dATP, dTTP, dGTP; (A^{BF}-) **$dC^{MOP}TP$**, dTTP, dGTP; (C^{MOP}-) **$dA^{BF}TP$**, dTTP, dGTP; (A^{BF}) **$dA^{BF}TP$**, dCTP, dTTP, dGTP; (C^{MOP}) **$dC^{MOP}TP$**, dATP, dTTP, dGTP; (A^{BF} C^{MOP}) **$dA^{BF}TP$**, **$dC^{MOP}TP$**, dTTP, dGTP.

The incorporation of a large number of modifications into double-stranded DNA was performed by the polymerase chain reaction (PCR) using 98-mer template (sequences of primers and

template used in PCR were summarized in Table 4) and KOD XL polymerase. The amplification of the modified DNAs proceeded well for each of the modified **dN^{X}TPs** (Figure 23). Next, combinations of the modified A and C (**dA^{DHB}TP** + **dC^{MOP}TP** or **dA^{MOP}TP** + **dC^{DHB}TP**) were also tested in the amplification of DNA with 98-mer template (Figure 23, lines 9 and 10). According to obtained results all four modified **dN^{X}TPs** were very good substrates when KOD XL polymerase was used.

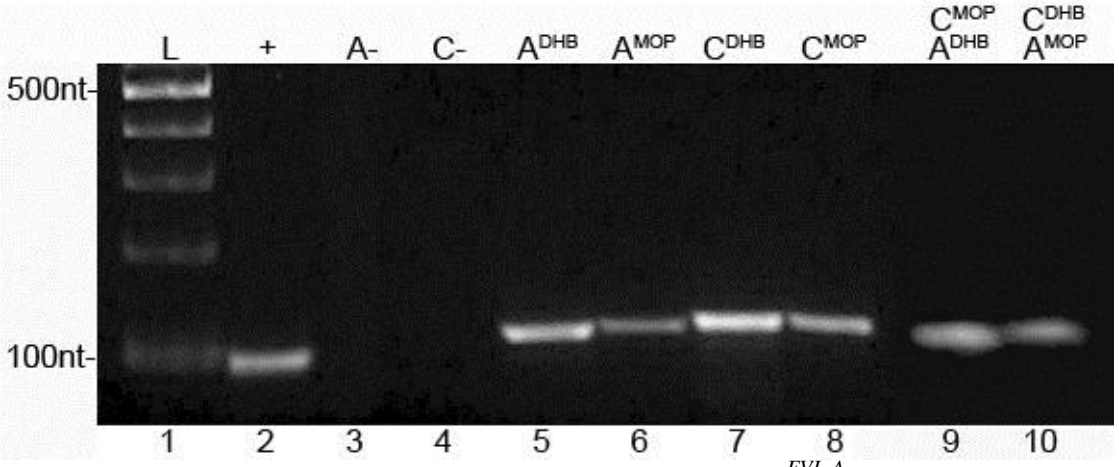


Figure 23. PCR synthesis of 98-mer with template Temp^{FVL-A} by KOD XL polymerase. L: ladder (mix of dsDNA with specific number of base pairs); (+) all natural dNTPs; (A-) dTTP, dGTP, dCTP; (C-) dTTP, dGTP, dATP; (A^{DHB}) **A^{DHB}TP**, dTTP, dGTP, dCTP; (A^{MOP}) **dA^{MOP}TP**, dTTP, dGTP, dCTP; (C^{DHB}) **dC^{DHB}TP**, dTTP, dGTP, dATP; (C^{MOP}) **dC^{MOP}TP**, dTTP, dGTP, dATP; (A^{DHB}+ C^{MOP}) **A^{DHB}TP**, **dC^{MOP}TP**, dTTP, dGTP; (A^{MOP}+C^{DHB}) **dA^{MOP}TP**, **dC^{DHB}TP**, dTTP, dGTP; 2 % agarose gel, stained by GelRed.

Table 4. List of sequences of template and primers used in PCR study.

Oligonucleotide	Sequence
Prim^{LT25TH}	5´-CAAGGACAAAATACCTGTATTCCTT -3´
Prim^{L20-}	5´-GACATCATGAGAGACATCGC -3´
Temp^{FVL-A}	5´-GACATCATGAGAGACATCGCCTCTGGGCTAATAGGACTAC TTCTAATCTGTAAGAGCAGATCCCTGGACAGGCAAGGAATA CAGGTATTTTGTCCTTG-3´

In the template the segments forming duplex with the primers are underlined.

3.1.4 Electrochemical studies of modified nucleosides, dNTPs and DNA (in collaboration with Doc. Fojta research group)

At first, electrochemical measurements were performed on modified nucleosides **dN^{X}** (**dA^{MOP}**, **dC^{MOP}**, **dA^{DHB}**, and **dC^{DHB}**) and **dN^{X}TPs** (**dA^{MOP}TP**, **dC^{MOP}TP**, **dA^{DHB}TP**, and **dC^{DHB}TP**) and compared with electrochemical behavior of the starting boronic acids/ pinacol borates **DHB**-B(OH)$_2$ and **MOP**-B(pin) by using of a square-wave voltammetry (SWV) at the basal-plane pyrolytic graphite electrode (Figure 24).

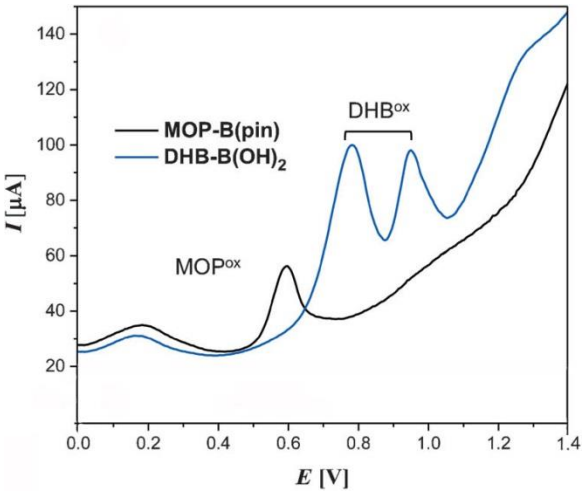


Figure 24. Square-wave voltammograms of boronic acids.

The experiments were done in 0.2M acetate buffer (pH 5.0) with 40mM concentration of samples. The **MOP**-B(pin) produced under these conditions a single irreversible oxidation peak around 600 mV (peak **MOP**^{ox}, Figure 24), whereas **DHB**-B(OH)$_2$ produced two irreversible anodic signals at 785 and 950 mV (peaks **DHB**^{ox}, Figure 24). The electrochemical oxidation of **MOP** group in both nucleosides were observed at less positive potentials than for the corresponding pinacol boronic acid, around 450 mV for **dA**^{MOP} and around 510 mV for **dC**^{MOP} (Figure 25). Additional poorly developed oxidizable double peaks were detected around 750 mV for **dC**^{MOP} and 880 mV for **dA**^{MOP} and the oxidation of the 7-deazaadenine moiety[127] was detected at 1045 mV (peak A*^{ox}, Figure 25). Irreversable oxidation of **DHB**-labeled nucleosides gave signals around 1150 mV for **dC**^{DHB} and 845 mV for **dA**^{DHB} (peaks **DHB**^{ox}(C) and **DHB**^{ox}(A), Figure 25). While the separate signal corresponding to the 7-deazaadenine oxidation was not detected in the electrochemical measurement of **dA**^{DHB}.

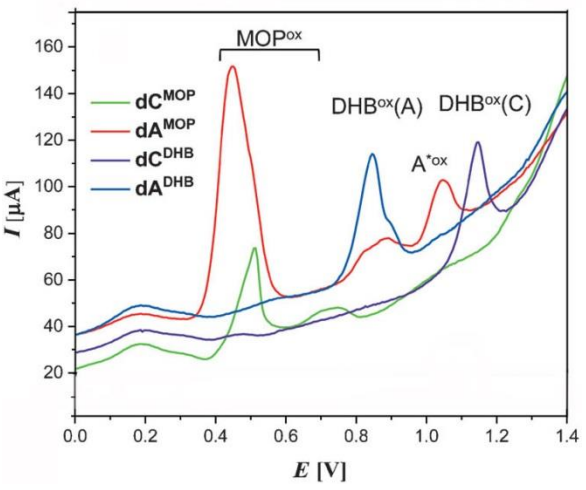


Figure 25. Square-wave voltammograms of modified nucleosides.

Electrochemical characteristics of modified nucleoside triphosphates showed the similar behavior with the oxidation of corresponding nucleosides. Only **dC^{MOP}TP** was oxidized with formation of two well-developed anodic signals in the potential region around 300 mV and 500 mV (Figure 26).

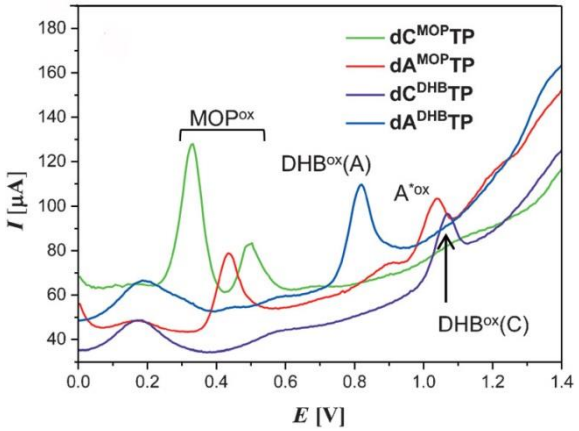


Figure 26. Square-wave voltammograms of nucleoside triphosphates.

Variations in the intensity and potential of the oxidative peaks produced by oxidation of the corresponding boronic acids/pinacol borates, nucleosides and nucleoside triphosphates can be attributed to the nature of corresponding species. Different nucleobase types or negatively charged triphosphate groups influence on the adsorbability of the given substances at the positively charged electrode surface and accessibility of the oxidizable moieties for the electrode reaction.

Next, the single-stranded DNAs obtained by PEX reaction with following magnetoseparation procedure and each containing four modifications were applied for electrochemical measurement by adsorptive transfer stripping (AdTS) SWV (Figure 27). Electrochemical studies of each oligonucleotide showed the oxidation peaks of natural guanine and adenine nucleobases at potentials of 1100 mV and 1370 mV respectively (peaks G^{ox} and A^{ox}).

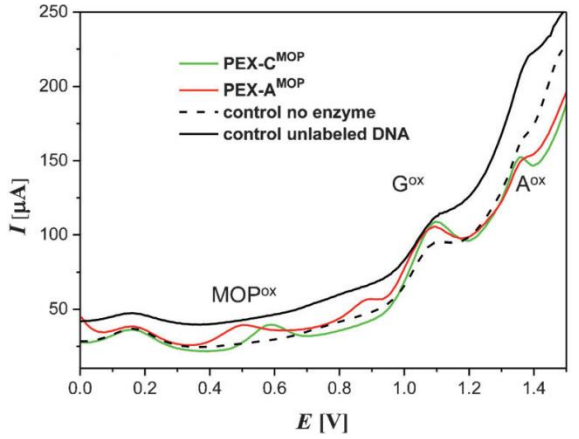


Figure 27. AdTS square-wave voltammograms of single-stranded PEX products bearing **MOP** labels.

PEX products containing **MOP** labels attached to cytidine produced anodic signal around 590 mV, while ONs labeled by **MOP** moiety through adenine nucleobases showed two signals at potentials of 510 mV and 900 mV. Electrochemical measurements of $\mathbf{A}^{\mathbf{DHB}}$-labeled ON detected the oxidation peak at 870 mV, whereas for ON containing $\mathbf{C}^{\mathbf{DHB}}$ conjugates did not show any separate peak besides the oxidation of natural purine nucleobases, but great increase of peak intensity at 1100 mV, what can be explained by overlapping of **DHB** oxidation peak with the peak of guanine oxidation (Figure 28). Redox potentials of **DHB** and **MOP** moieties in boronic acids, nucleosides, dNTPs and ONs are summarized in Table 5.

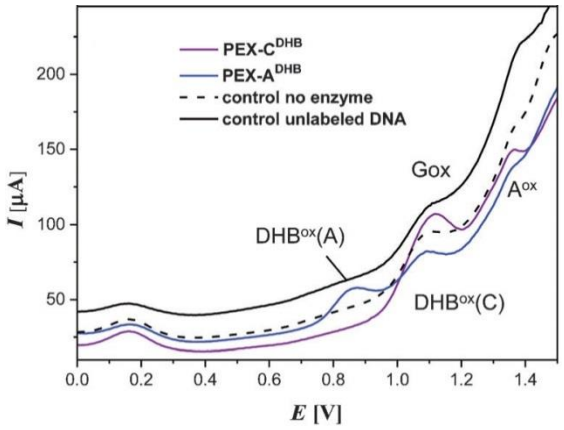


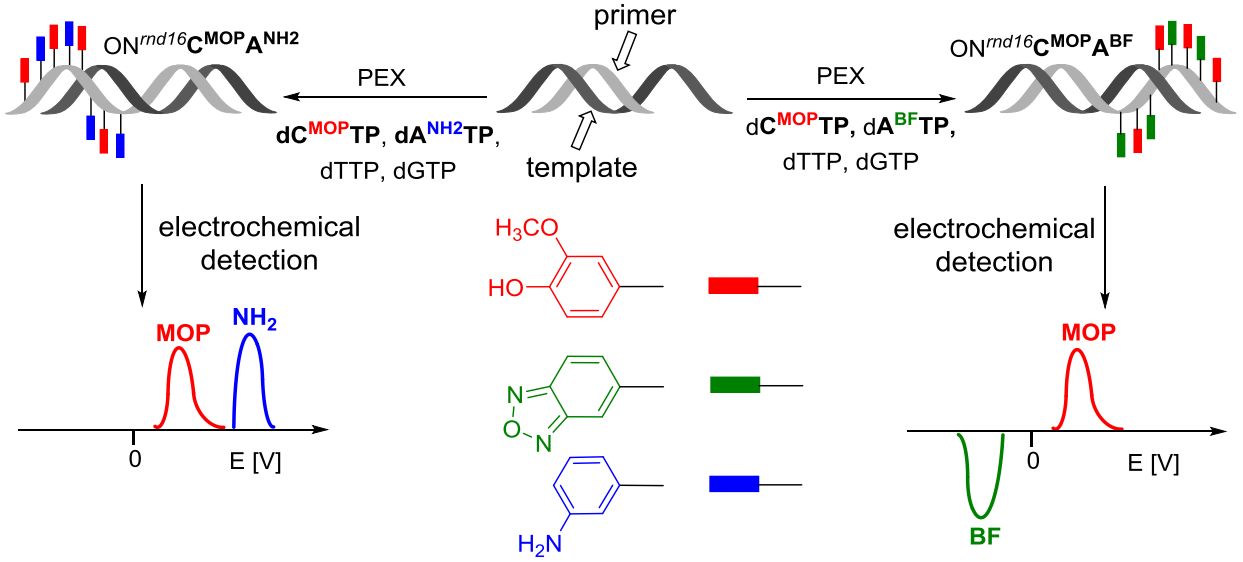
Figure 28. AdTS square-wave voltammograms of single-stranded PEX products bearing **DHB** labels.

Table 5. Redox potentials of **DHB** and **MOP** moieties in boronic acids, nucleosides, dNTPs and ONs (in mV).

Sample	Peaks				
	MOP^{ox}	DHB^{ox}	A^{*ox}	G^{ox}	A^{ox}
MOP-B(pin)	600	-	-	-	-
DHB-$B(OH)_2$	-	785, 950	-	-	-
$\mathbf{dC}^{\mathbf{MOP}}$	510	-	-	-	-
$\mathbf{dA}^{\mathbf{MOP}}$	450	-	1045	-	-
$\mathbf{dC}^{\mathbf{DHB}}$	-	1150	-	-	-
$\mathbf{dA}^{\mathbf{DHB}}$	-	845	-	-	-
$\mathbf{dC}^{\mathbf{MOP}}\mathbf{TP}$	330, 500	-	-	-	-
$\mathbf{dA}^{\mathbf{MOP}}\mathbf{TP}$	435	-	1040	-	-
$\mathbf{dC}^{\mathbf{DHB}}\mathbf{TP}$	-	1070	-	-	-
$\mathbf{dA}^{\mathbf{DHB}}\mathbf{TP}$	-	820	-	-	-
ON^{rnd16} $\mathbf{C}^{\mathbf{MOP}}$	590	-	-	1100	1360
ON^{rnd16} $\mathbf{A}^{\mathbf{MOP}}$	510, 900	-	-	1095	1370
ON^{rnd16} $\mathbf{C}^{\mathbf{DHB}}$	-	1100[a]	-	1100[a]	1360
ON^{rnd16} $\mathbf{A}^{\mathbf{DHB}}$	-	870	-	1090	1360
unlabeled PEX		-	-	1100	1370

[a] Overlapping signals.

3.1.5 The combination of MOP with BF or $PhNH_2$ labels in DNA and their electrochemical studies (in collaboration with Doc. Fojta research group)



Scheme 24. Electrochemical studies of ON-products of mixed PEX experiments.

Electrochemical studies of labeled PEX products showed that the MOP group is potentially useful label both for cytosine and adenine nucleobases since the corresponding conjugates show oxidation signals in a potential region different from anodic signals of natural DNA components (natural guanine, adenine or 7-deazaadenine). In addition, modified DNAs can be distinguished by using $\mathbf{C}^{\mathbf{MOP}}$ or $\mathbf{A}^{\mathbf{MOP}}$ conjugates because of their difference in peak potentials. Therefore **MOP**-group was chosen for the testing of multipotential DNA coding in combination with previously reported oxidizable or reducible labels (Scheme 24). At first, a PEX product containing two oxidizable labels $\mathbf{C}^{\mathbf{MOP}}$ and $\mathbf{A}^{\mathbf{NH2}}$ underwent electrochemical study. Two independently readable peaks were observed at potentials of 550 mV and 830 mV that corresponded to oxidation of **MOP** group and aminophenyl moiety respectively (Figure 29).

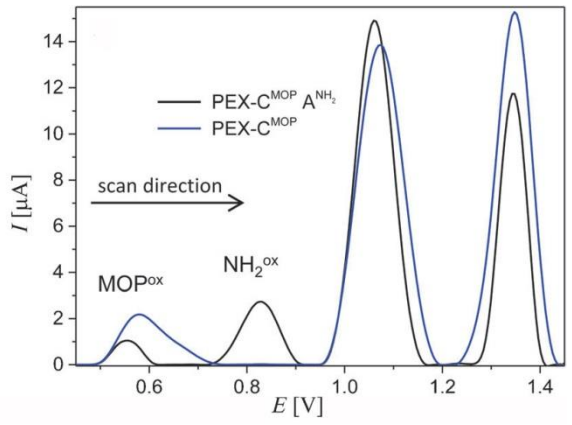


Figure 29. AdTS square-wave voltammograms of PEX products labeled with $\mathbf{C}^{\mathbf{MOP}}$ in combination with $\mathbf{A}^{\mathbf{NH2}}$.

Then, electrochemical measurement of modified DNAs bearing oxidizable **MOP** moiety in combination with reducible benzofurazane label (**BF**) was performed (Figure 30). Voltammetric responses were obtained by sequent measurement of modified DNA at first on mercury electrode for detection of benzofurazane group in the cathodic potential and then on graphite electrode for detection of **MOP**-derivative in the anodic potential, making the differentiation even more obvious.

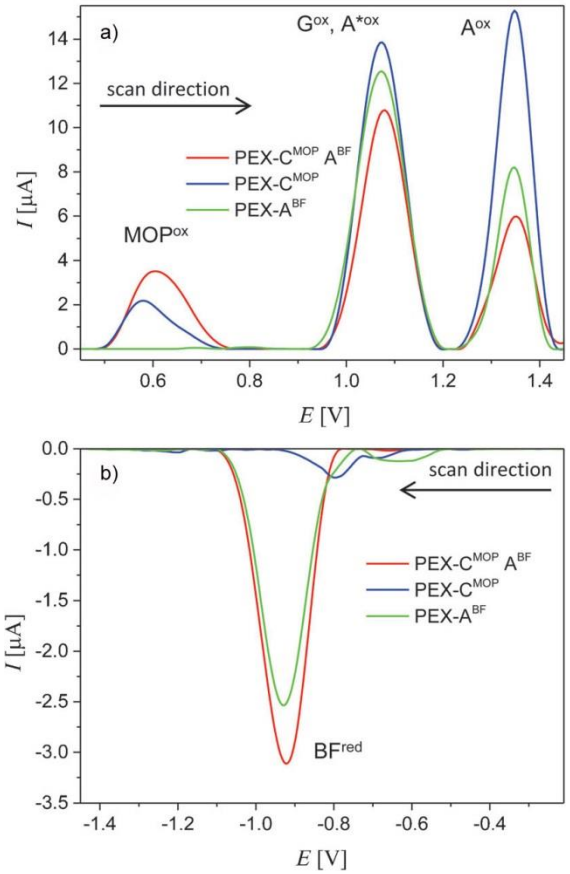


Figure 30. AdTS square-wave voltammograms of PEX products labeled with $\mathbf{C^{MOP}}$ in combination with $\mathbf{A^{BF}}$.

Electrochemical measurements of modified DNA with **DHB** labels showed a limited applicability of **DHB** group, because $\mathbf{C^{DHB}}$ oxidation peak is overlapping with guanine oxidation. Whereas multipotential DNA labeling with $\mathbf{A^{DHB}}$ in combination with earlier introduced oxidizable labels is also difficult owing to the coincidence of $\mathbf{A^{DHB}}$ oxidation with oxidation signals of aminophenyl group and purine nucleobases.

3.1.6 Conclusion

4-Hydroxy-3-methoxyphenyl and 2,3-dihydrobenzofuran groups were attached to nucleosides and dNTPs by the aqueous-phase Suzuki cross-coupling reactions with efficient yields. The corresponding modified $\mathbf{dN^XTPs}$ were good substrates for DNA polymerases and served as

building blocks in primer extension experiments or polymerase chain reactions for producing modified oligonucleotides. Electrochemical measurements of the labeled nucleosides and dNTPs showed distinct signals resulting from oxidation of **MOP** and **DHB** moieties. However, the electrochemical studies of modified DNA showed that only the **MOP** tag behaves as useful label, since its oxidation signals are different from oxidation peaks of natural nucleobases. The **MOP** redox label appeared to be a candidate for multipotential DNA labeling in combination with other oxidizable or reducible labels. While use of **DHB** label was limited because of overlapping of the corresponding cytosine conjugate oxidation peak ($\mathbf{C}^{\mathbf{DHB}}$) with oxidation of guanine.

3.2 PT-labeled nucleosides and dNTPs. Synthesis, enzymatic incorporation and electrochemical detection

3.2.1 Introduction

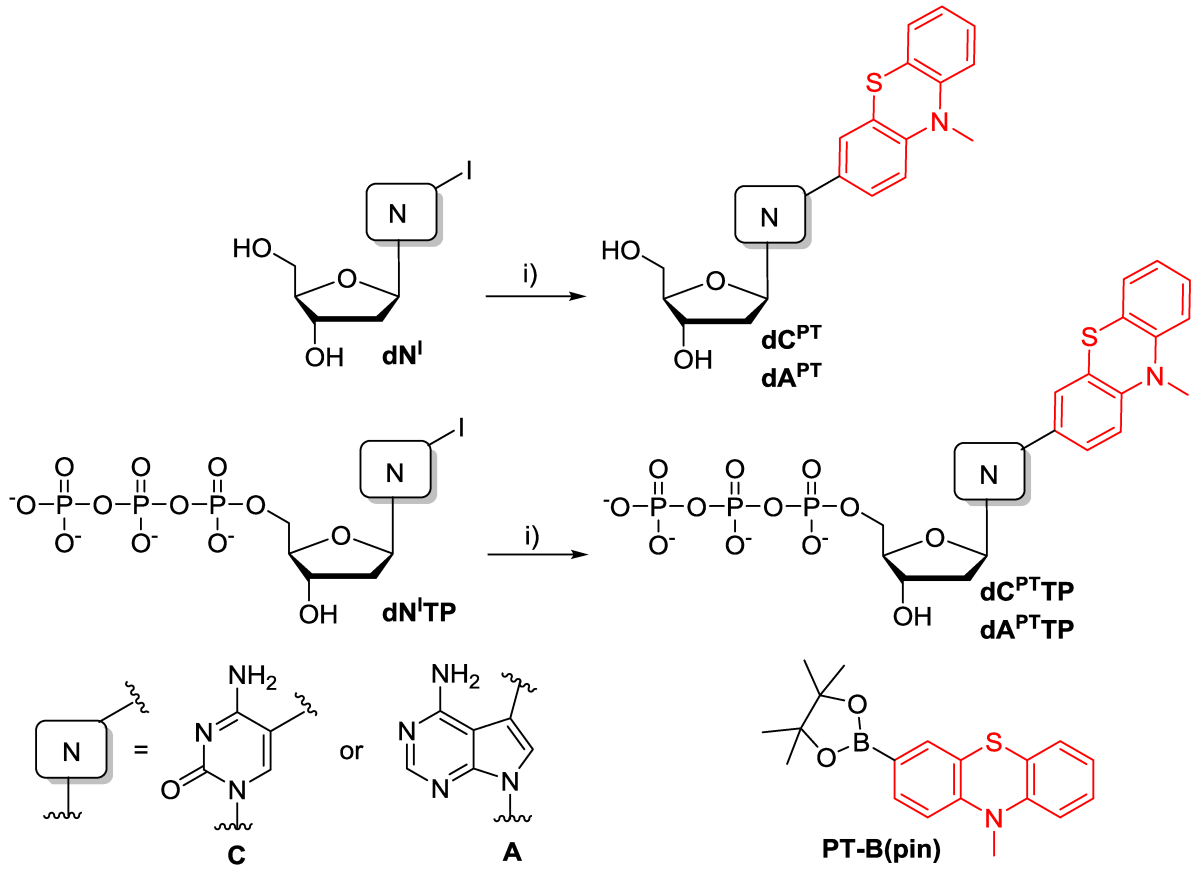
Phenothiazine (PT) derivatives were used in a broad range of applications, as potential antitumor[128] or antipsychotic[129] drugs, insecticidal, antifungal or anthelmintic agents[130]. Due to its electrochemical properties, phenothiazine is widely used as redox label for surface functionalization[131–134]. Some studies involved use of PT derivatives in biochemistry as markers for DNA, mostly for photosensitising[135] or charge-transfer study[136–141]. Recently one example of modified dNTP connected to phenothiazine through a flexible non-conjugate amide linker was presented for use in redox coding for the detection of single nucleotide polymorphisms[142,143]. However, the PT-modified dNTP was not fully characterized and the enzymatic studies were not reported either. Therefore, we focused on the synthesis of two types of PT-modified nucleosides and dNTPs either directly linked or tethered through acetylene linker for enzymatic incorporation into DNA and the study of their electrochemical properties as potentially useful redox labels for DNA.

3.2.2 Synthesis of PT-modified nucleosides and dNTPs

The synthesis of directly linked phenothiazine-modified derivatives of nucleosides was performed in one step by Suzuki-Miyaura cross-coupling of unprotected halogenated nucleosides 5-iodocytidine and 7-deaza-7-iodoadenosine with 10-methyl-10H-phenothiazine-3-boronic acid pinacol ester (**PT**-B(pin)) under aqueous conditions in the presence of $Pd(OAc)_2$, TPPTS, and Cs_2CO_3 at 50 °C for 40 minutes to give labeled nucleosides **dC^{PT}** and **dA^{PT}** in good yields of 75% and 96 % respectively (Table 6, Scheme 25). Under the same aqueous conditions of the Suzuki-Miyaura cross-coupling of halogenated dNTPs (dC^{I}TP and dA^{I}TP) with **PT**-B(pin) desired arylated **dN^{X}TPs** were isolated in moderate yields (**dC^{PT}TP** in 53% and **dA^{PT}TP** in 68%).

Table 6. Synthesis of nucleosides and nucleoside triphosphates bearing directly linked phenothiazine labels.

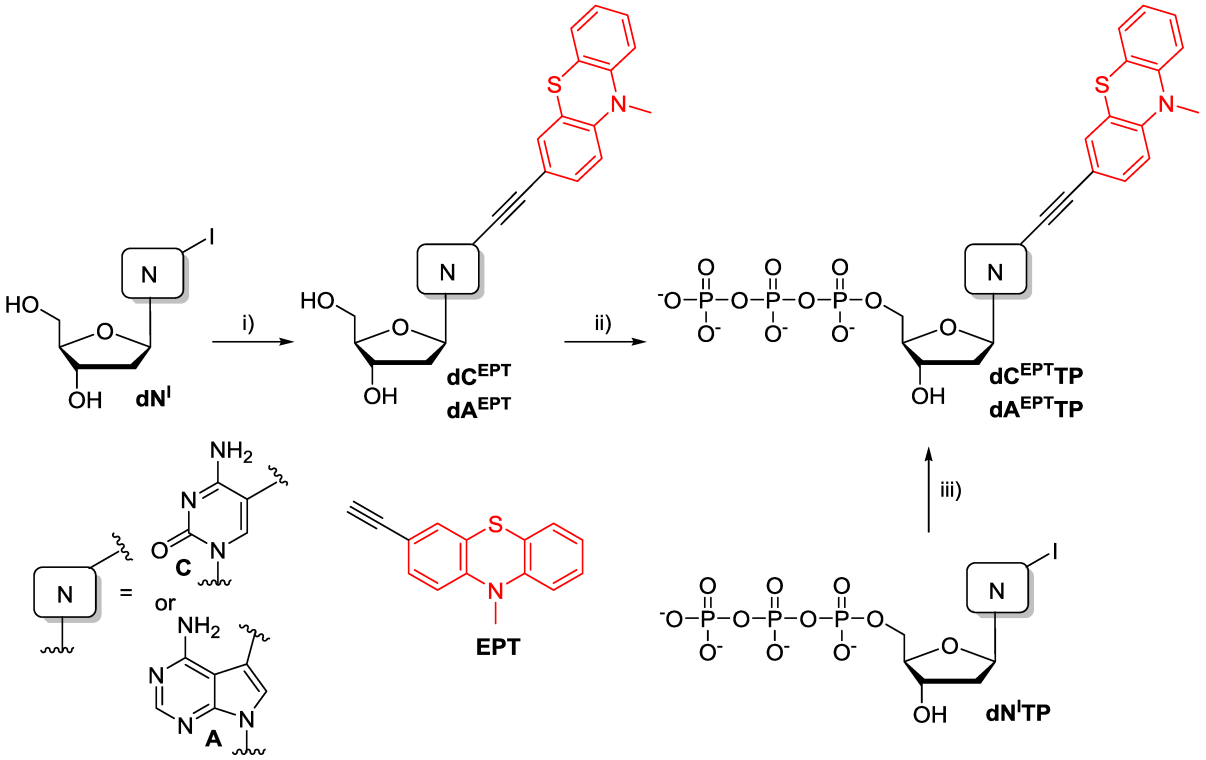
Starting compound	Reagent	Catalyst	Solvent	Base	Product	Yield (%)
dA^{I}	**PT**-B(pin)	$Pd(OAc)_2$, TPPTS	MeCN/H_2O (1/1)	Cs_2CO_3	**dA^{PT}**	96
dC^{I}					**dC^{PT}**	75
dA^{I}TP					**dA^{PT}TP**	68
dC^{I}TP					**dC^{PT}TP**	53



Scheme 25. Reagents and conditions: i) **PT**-B(pin), $Pd(OAc)_2$, TPPTS, Cs_2CO_3, MeCN/H_2O (1:1), 50°C, 40 min.

For the attachment of phenothiazine moiety linked through an acetylene tether the Sonogashira cross-coupling reaction of 3-ethylnyl-10-methyl-10H-phenothiazine (**EPT**) with unprotected halogenated nucleosides in the presence of $Pd(PPh_3)_2Cl_2$ and CuI in DMF was applied to give labeled nucleosides **dC**$^{\mathbf{EPT}}$ and **dA**$^{\mathbf{EPT}}$ in good yields (Sheme 26, Table 7). The aqueous Sonogashira cross-coupling reactions of dN$^{\mathrm{I}}$TPs with **EPT** resulted in moderate yields of desired **PT**-acetylene-linked **dN**$^{\mathbf{EPT}}$**TP**s (**dC**$^{\mathbf{EPT}}$**TP** in 48% and **dA**$^{\mathbf{EPT}}$**TP** in 49%).

In order to synthesize a larger quantities of **dN**$^{\mathbf{EPT}}$**TP**s (**dC**$^{\mathbf{EPT}}$**TP** and **dA**$^{\mathbf{EPT}}$**TP),** the alternative approach was applied by triphosphorylation of the corresponding nucleosides **dN**$^{\mathbf{EPT}}$ in moderate yields (**dA**$^{\mathbf{EPT}}$**TP** in 43% and **dC**$^{\mathbf{EPT}}$**TP** in 45%). In all cases, the **PT**-labeled **dN**$^{\mathbf{PT}}$**TP**s or **dN**$^{\mathbf{EPT}}$**TP**s were isolated by HPLC and fully characterized.



Scheme 26. Reagents and conditions: i) **EPT**, $Pd(PPh_3)_2Cl_2$, CuI, Et_3N, DMF, 75°C, 1 h; ii) 1. $POCl_3$, $PO(OMe)_3$, 0°C, 3 h; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0°C,1.5 h; 3. TEAB; iii) **EPT**, $Pd(OAc)_2$,CuI, TPPTS, Et_3N, MeCN/H_2O (1:1), 75°C, 1 h .

Table 7. Synthesis of nucleosides and nucleoside triphosphates bearing acetylene-linked phenothiazine labels.

Starting compound	Reagent	Catalyst	Solvent	Base	Product	Yield (%)
dA^I	**EPT**	$Pd(PPh_3)_2Cl_2$, CuI	DMF	Et_3N	**dA^{EPT}**	93
dC^I					**dC^{EPT}**	76
dA^ITP		$Pd(OAc)_2$, CuI, TPPTS	MeCN/H_2O (1/1)		**$dA^{EPT}TP$**	49
dC^ITP					**$dC^{EPT}TP$**	48
dA^{EPT}	1) $PO(OMe)_3$, $POCl_3$, 0°C; 2) $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0°C; 3) TEAB (2M)				**$dA^{EPT}TP$**	43
dC^{EPT}					**$dC^{EPT}TP$**	45

3.2.3 Enzymatic incorporation of PT-modified dNTPs into DNA

The four new dNTPs (**$dA^{PT}TP$**, **$dC^{PT}TP$**, **$dA^{EPT}TP$** and **$dC^{EPT}TP$**) then were applied as substrates for DNA polymerases in primer extension experiments (PEX) using thermostable DNA polymerases: KOD XL, Vent (exo-) and Pwo (for sequences of primer, templates and products, see Table 8). The templates were chosen in order to analyze the PEX incorporation of compounds **dN^XTP**s forming products both with single modification and four modifications in

the sequence. Each PEX experiment was analyzed by polyacrylamide gel electrophoresis using 6-carboxyfluorescein-(6-FAM-) labeled primer.

Table 8. List of sequences of templates, primer, and products.

Name	Sequence
primer^{rnd}	5'-CATGGGCGGCATGGG-3'
temp^{rnd16}	5'-CTAGCATGAGCTCAGTCCCATGCCGCCCATG-3'
temp^{A}	5'-CCCTCCCATGCCGCCCATG-3'
temp^{C}	5'-CCCGCCCATGCCGCCCATG-3'
temp^{termA}	5'-TCCCATGCCGCCCATG-3'
temp^{termC}	5'-GCCCATGCCGCCCATG-3'
ON^{rnd16} **A^{PT}**	5'-CATGGGCGGCATGGG**A^{PT}**CTG**A^{PT}**GCTC**A^{PT}**TGCT**A^{PT}**G-3'
ON^{rnd16} **A^{EPT}**	5'-CATGGGCGGCATGGG**A^{EPT}**CTG**A^{EPT}**GCTC**A^{EPT}**TGCT**A^{EPT}**G-3'
ON^{rnd16} **C^{PT}**	5'-CATGGGCGGCATGGGA**C^{PT}**TGAG**C^{PT}**T**C^{PT}$**ATG**C^{PT}$**TAG-3'
ON^{rnd16} **C^{EPT}**	5'-CATGGGCGGCATGGGA**C^{EPT}**TGAG**C^{EPT}**T**C^{EPT}$**ATG**C^{EPT}$**TAG-3'
ON^{rnd16} **A^{PT} C^{EBF}**	5'-CATGGGCGGCATGGG**A^{PT}C^{EBF}**TGA**^{PT}**G**C^{EBF}$**T**C^{EBF}$**A^{PT}**TG**C^{EBF}$**TA^{PT}**G-3'
ON^{rnd16} **A^{PT} U^{NO2}**	5'-CATGGGCGGCATGGG**A^{PT}C**U^{NO2}**GA^{PT}**GC**U^{NO2}**CA^{PT}**U^{NO2}**GC**U^{NO2}**A^{PT}**G-3'
ON^{A} **A^{PT}**	5'-CATGGGCGGCATGGG**A^{PT}**GGG-3'
ON^{A} **A^{EPT}**	5'-CATGGGCGGCATGGG**A^{EPT}**GGG-3'
ON^{C} **C^{PT}**	5'-CATGGGCGGCATGGG**C^{PT}**GGG-3'
ON^{C} **C^{EPT}**	5'-CATGGGCGGCATGGG**C^{EPT}**GGG-3'
ON^{termA} **A^{PT}**	5'-CATGGGCGGCATGGG**A^{PT}**-3'
ON^{termA} **A^{EPT}**	5'-CATGGGCGGCATGGG**A^{EPT}**-3'
ON^{termC} **C^{PT}**	5'-CATGGGCGGCATGGG**C^{PT}**-3'
ON^{termC} **C^{EPT}**	5'-CATGGGCGGCATGGG**C^{EPT}**-3'

In the template ONs the segments forming duplex with the primer are underlined; for magnetic separation of the extended primer strands the templates were 5´-end biotinylated; acronyms used in the text for primer extension products are analogous to those introduced for the templates.

At first the single nucleotide incorporation was performed by extension of 15-nt primer with one of each **dN^{X}TP**s followed by three natural dG using templates temp^{A} or temp^{C} (Figure 31, Scheme 27a). In all cases, the fully extended products were obtained.

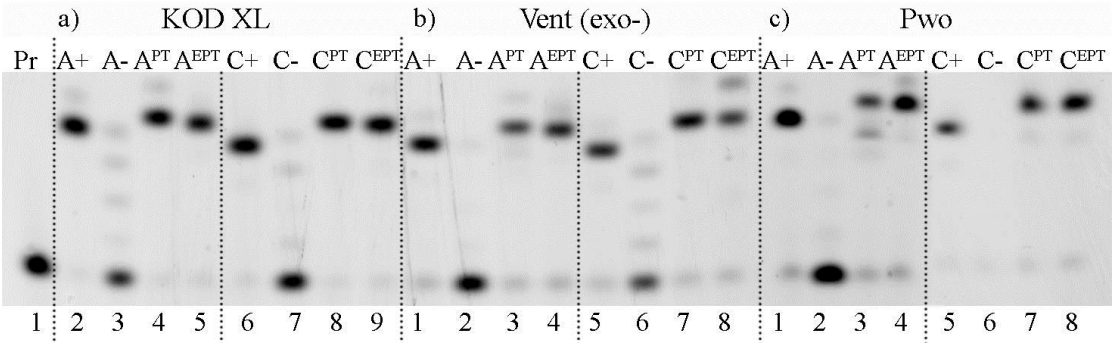
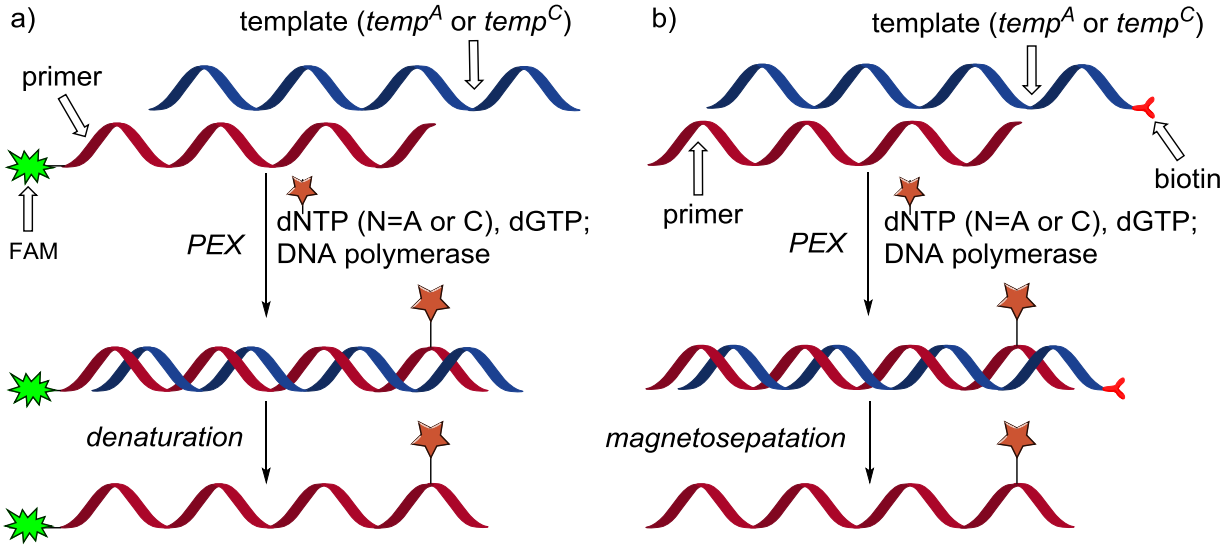


Figure 31. Primer extension with a) KOD XL, b)Vent (exo-) and c) Pwo polymerases using prim^{rnd}, temp^{A}, and temp^{C}: (Pr) primer (5'-FAM-labeled); (A+) dATP, dGTP; (A-) dGTP; (A^{PT}) **dA^{PT}TP**, dGTP; (A^{EPT}) **dA^{EPT}TP**, dGTP; (C+) dCTP, dGTP; (C-) dGTP; (C^{PT}) **dC^{PT}TP**, dGTP; (C^{EPT}) **dC^{EPT}PTP**, dGTP.

The single stranded DNAs were then obtained using 5′-end biotinylated templates and magnetoseparation procedure and were characterized by MALDI-TOF (Scheme 27b, Table 9).



Scheme 27. a) Single nucleotide incorporation by primer extension experiments; b) Primer extension with following magnetosepatation procedure for the synthesis of single stranded DNAs with a single incorporation of modified nucleoside triphosphates.

Table 9. List of MALDI data of ONs bearing phenothiazine labels.

oligonucleotide	M calcd. (Da)	M found (Da)
ON^{rnd16} $\mathbf{A^{PT}}$	10458.46	10459.00
ON^{rnd16} $\mathbf{A^{EPT}}$	10554.54	10555.3
ON^{rnd16} $\mathbf{C^{PT}}$	10462.46	10463.8
ON^{rnd16} $\mathbf{C^{EPT}}$	10558.54	10559.30
ON^{rnd16} $\mathbf{A^{PT}C^{EBF}}$	11031,10	11032,9
ON^{rnd16} $\mathbf{A^{PT}U^{NO2}}$	10886,94	10887,3
ON^{Nick_1A} $\mathbf{A^{PT}}$	3388.29	3389.4
ON^{Nick_1A} $\mathbf{A^{EPT}}$	3412.31	3413.4
ON^{Nick_1C} $\mathbf{C^{PT}}$	3373.69	3374.5
ON^{Nick_1C} $\mathbf{C^{EPT}}$	3397.41	3398.4
ON^{Nick_2A} $\mathbf{A^{PT}}$	3517.58	3519.3
ON^{Nick_2A} $\mathbf{A^{EPT}}$	3565.62	3566.8
ON^{Nick_2C} $\mathbf{C^{PT}}$	3519.58	3520.4
ON^{Nick_2C} $\mathbf{C^{EPT}}$	3567.62	3568.7
ON^{Nick_4A} $\mathbf{A^{PT}}$	5801.36	5802.8
ON^{Nick_4A} $\mathbf{A^{EPT}}$	5897.44	5898.7
ON^{Nick_4C} $\mathbf{C^{PT}}$	5805.36	5806.6
ON^{Nick_4C} $\mathbf{C^{EPT}}$	5901.44	5902.5
ON^{A} $\mathbf{A^{PT}}$	6185.2	6186.5
ON^{A} $\mathbf{A^{EPT}}$	6209.2	6210.6
ON^{C} $\mathbf{C^{PT}}$	6162.2	6163.5
ON^{C} $\mathbf{C^{EPT}}$	6186.2	6187.5

The kinetics studies in the presence of KOD XL polymerase were performed to examine the incorporation efficiency of modified **dN^{X}TP**s by PEX experiments in comparison with natural dNTPs (Figures 32-35). The rates of the PEX with natural dNTPs or modified nucleotides were revealed using templates temp^{termA} or temp^{termC} and primer^{rnd}. The incorporation of natural dATP or dCTP were completed in 1 minute and phenothiazine-labeled nucleotides were fully incorporated within max. 5-10 minutes. The slowest extension was observed in the experiment with **dA^{EPT}TP**.

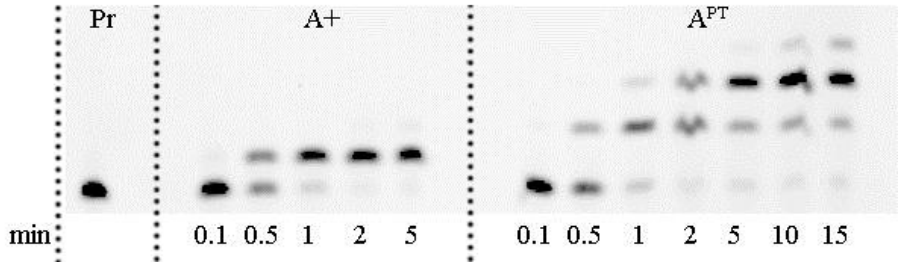


Figure 32. Comparison of incorporation of natural and modified dATPs to DNA with *temp^{termA}*: (**Pr**) primer; (**A+**) dATP; (**A^{PT}**) **dA^{PT}TP**.

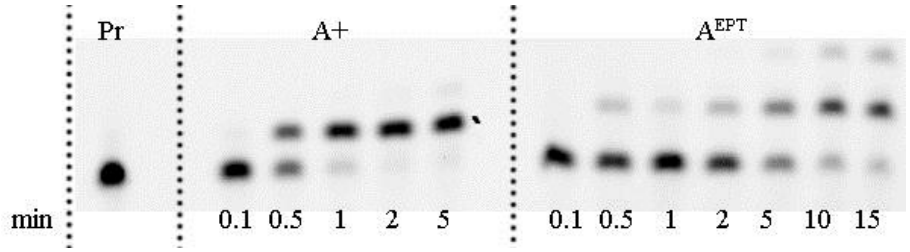


Figure 33. Comparison of incorporation of natural and modified dATPs to DNA with *temp^{termA}*: (**Pr**) primer; (**A+**) dATP; (**A^{EPT}**) **dA^{EPT}TP**.

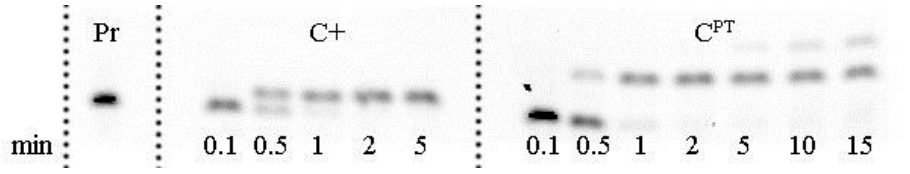


Figure 34. Comparison of incorporation of natural and modified dCTPs to DNA with *temp^{termC}*: (**Pr**) primer; (**C+**) dCTP; (**C^{PT}**) **dC^{PT}TP**.

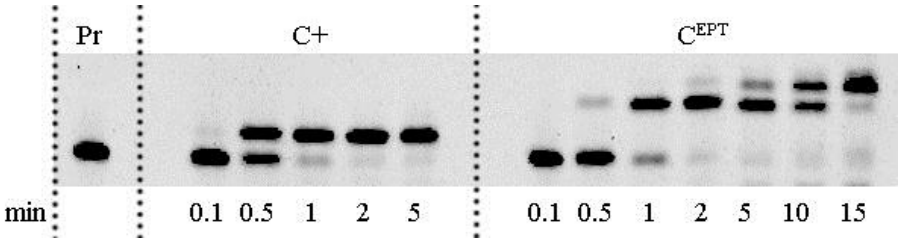


Figure 35. Comparison of incorporation of natural and modified dCTPs to DNA with *temp^{termC}*: (**Pr**) primer; (**C+**) dCTP; (**C^{EPT}**) **dC^{EPT}TP**.

Next, each of the modified **dN^{X}TPs** was used for a multiple incorporation into a longer 31-mer oligonucleotide (ON) by several DNA polymerases: KOD XL, Vent (*exo*-) and PWO using temp^{rnd16}. The sequence of the template was designed to encode for 4 incorporations at each of the four nucleotides and 4 modifications if one of dNTPs is modified. PAGE analysis showed full length products in the presence of all tested polymerases (Figure 36). The PEX products containing labels at adenosine nucleobases had slightly different electrophoretic mobility from cytidine modified products, however their correct length and sequence were verified by MALDI (Table 9).

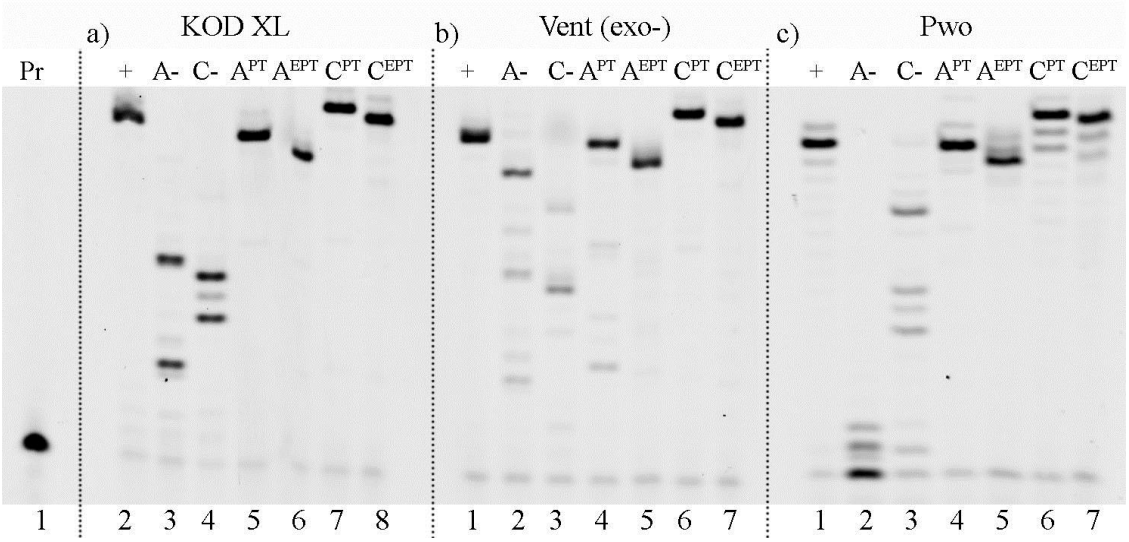
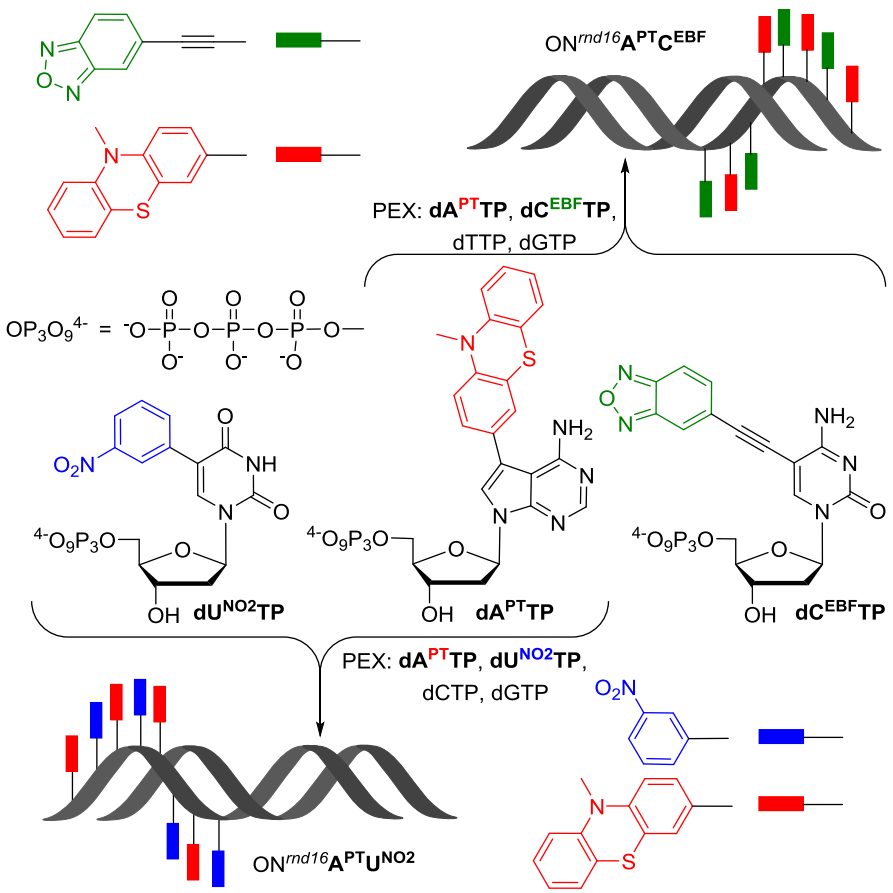


Figure 36. Primer extension with a) KOD XL, b)Vent (exo-) and c) Pwo polymerases using prim^{rnd} and temp^{rnd16}: (Pr) primer (5'-FAM-labeled); (+) natural dNTPs; (A-) dCTP, dGTP, dTTP; (C-) dATP, dGTP, dTTP; (A^{PT}) **dA^{PT}TP**, dCTP, dGTP, dTTP; (A^{EPT}) **dA^{EPT}TP**, dCTP, dGTP, dTTP; (C^{PT}) **dC^{PT}TP**, dGTP, dTTP, dATP; (C^{EPT}) **dC^{EPT}PTP**, dATP, dTTP, dGTP.

To examine the possibility of using PT-labeled nucleotides as potential candidates for redox coding of DNA bases, the incorporation of **dA^{PT}TP** was tested in combination with previously reported nitrophenyl and benzofurazane groups (**A^{PT}+C^{EBF}** or **A^{PT}+U^{NO2}**). Multipotential coding of DNA is challenging because the polymerase may have difficulties in recognition of modified **dN^{X}TP**s as subtrates and must be able to extend the primer by incorporation of modified nucleotides next to each other. Combination of **dA^{PT}TP** with **dC^{EBF}TP** or **dU^{NO2}TP** in the PEX experiments with template temp^{rnd16} resulted in formation of full-length ONs bearing four **A^{PT}** labels and either four **C^{EBF}** or four **U^{NO2}** modifications (Figure 37 and Sheme 28). These ON products were also characterized by MALDI (Table 9).



Scheme 28. Redox coding of DNA bases: structures of redox labeled dNTPs and DNA products of the mixed PEX.

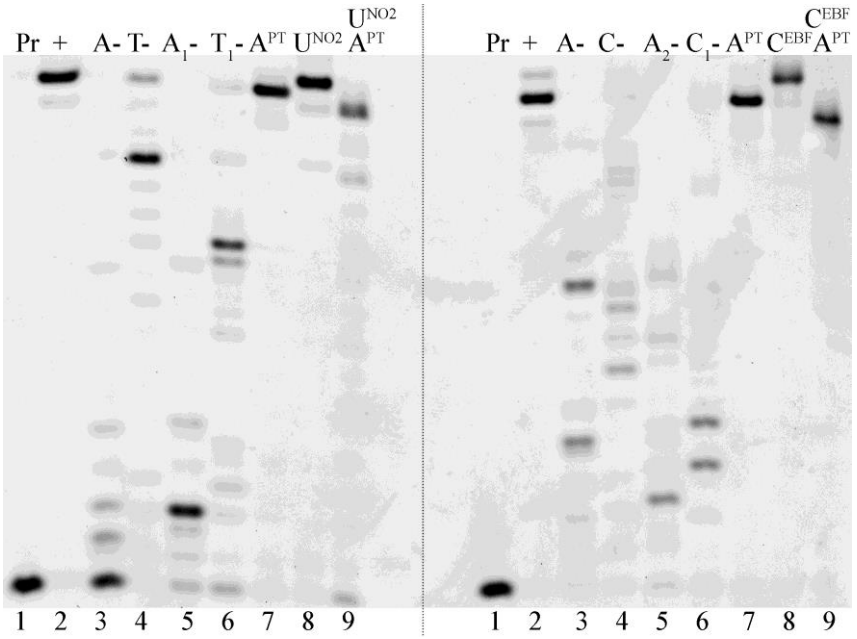


Figure 37. Primer extension with a KOD XL polymerase using prim^{rnd} and temp^{rnd16}: (Pr) primer (5'-FAM-labeled); (+) natural dNTPs; (A-) dCTP, dGTP, dTTP; (T-) dATP, dCTP, dGTP; (A_1-) **dU^{NO2}TP**, dCTP, dGTP; (T_1-) **dA^{PT}TP**, dCTP, dGTP; (A^{PT}) **dA^{PT}TP**, dCTP, dGTP, dTTP; (U^{NO2}) **dU^{NO2}TP**, dATP, dCTP, dGTP; ($A^{PT}U^{NO2}$) **dA^{PT}TP**, **dU^{NO2}TP**, dCTP,

dGTP; (C-) dATP, dGTP, dTTP; (A_2-) **dC^{EBF}TP**, dTTP, dGTP; (C_1-) **dA^{PT}TP**, dTTP, dGTP; (C^{EBF}) **dC^{EBF}TP**, dATP, dTTP, dGTP; ($A^{PT}C^{EBF}$) **dA^{PT}TP**, **dC^{EBF}TP**, dTTP, dGTP.

Phenothiazine-labeled nucleotides were also applied in PCR amplifications, but the PCR reactions using KOD XL did not give significant amount of product (Figure 38, Table 10). Since the **dN^{X}TP**s were showen as reasonably good substrates in the PEX experiments, the low yield of amplified products can be explain by difficulty for enzymes to read through the **PT**-modified templates. Therefore the experiment of PCR reactions with different percentage of natural dATP and modified **dA^{PT}TP** or **dA^{EPT}TP** was performed , where the only mixture of 50% of **dA^{PT}TP** / 50% of natural dATP gave significant product of amplification (Figures 39).

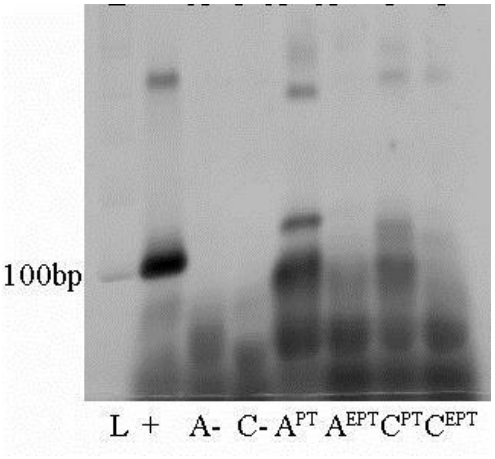


Figure 38. PCR synthesis of 98-mer by KOD XL polymerase (L) DNA ladder; (+) natural dNTPs; (A-): dCTP, dGTP, dTTP; (C-) dATP, dGTP, dTTP; (A^{PT}) **dA^{PT}TP**, dCTP, dGTP, dTTP; (A^{EPT}) **dA^{EPT}TP**, dCTP, dGTP, dTTP; (C^{PT}) **dC^{PT}TP**, dATP, dGTP, dTTP; (C^{EPT}) **dC^{EPT}TP**, dATP, dGTP, dTTP.

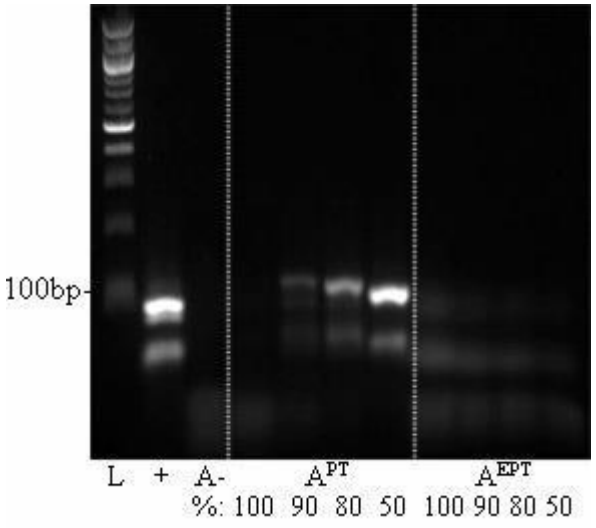


Figure 39. PCR synthesis of 98-mer by KOD XL polymerase. (L) DNA ladder; (+)natural dNTPs; (A-) dCTP, dGTP, dTTP; (A^{PT}) **dA^{PT}TP**, dCTP, dGTP, dTTP; (A^{EPT}) **dA^{EPT}TP**, dCTP, dGTP, dTTP. The percentage corresponds to the proportion of modified triphosphates (**dA^{PT}TP** or **dA^{EPT}TP**) in combination with natural dATP in the PCR reaction mixture.

Table 10. List of sequences of template and primers used in PCR study.

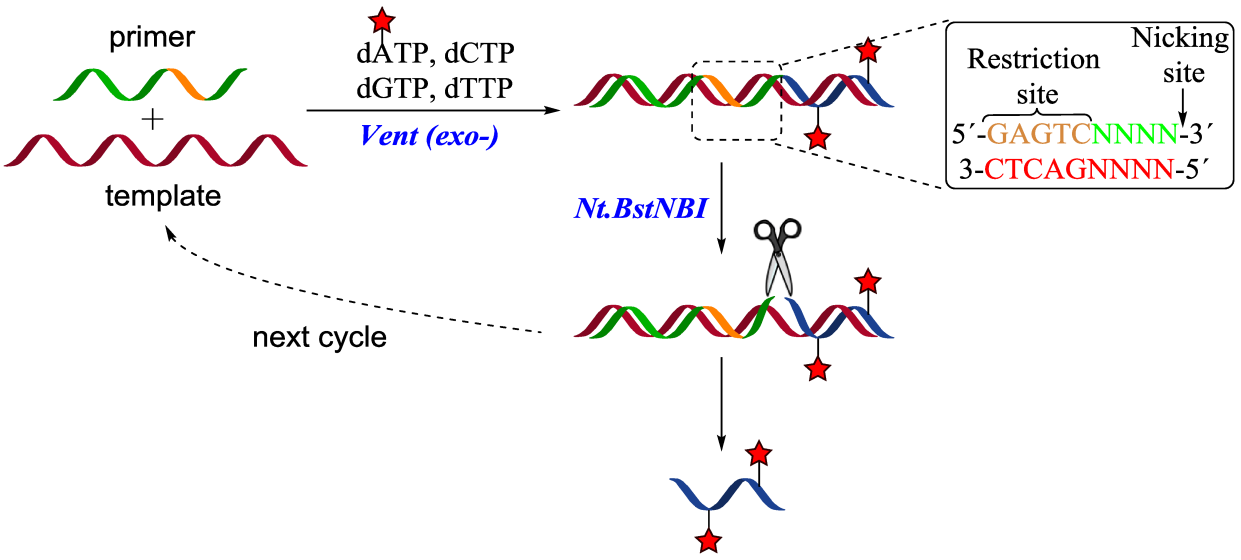
Oligonucleotide	Sequence
Prim^{LT25TH}	5′-CAAGGACAAAATACCTGTATTCCTT -3′
Prim^{L20-}	5′-GACATCATGAGAGACATCGC -3′
Temp^{FVL-A}	5′-GACATCATGAGAGACATCGCCTCTGGGCTAATAGGACTACTTCT AATCTGTAAGAGCAGATCCCTGGACAGGCAAGGAATACAGGTATT TTGTCCTTG-3′

In the template ON the segments forming duplex with the primers are underlined.

Based on the fact that new **dN^{X}TP**s are good substrates for PEX reaction but not the best candidates for PCR, nicking enzyme amplification reaction (NEAR) was used for enzymatic synthesis of short single-stranded ONs (Scheme 29). This isothermal amplification method for amplifying DNA combines polymerase strand extension and cleavage of the extended primer by nicking endonuclease, releasing the modified ssON. Previous studies have shown that Vent (exo-) is the best enzyme for primer extension step of NEAR. Different templates were designed for the synthesis of 10-mer ON containing either one or two modifications or 16-mer ON containing four modifications (Table 11). The enzymatic synthesis of ssONs containing one modification was efficient in case of each of the four **dN^{X}TP**s, whereas linear amplification reactions for the synthesis of ON containing two or four labels gave lower yields of products (Figure 40).

Table 11. List of sequences of templates, primer, and products.

Name	Sequence
primer NICK	5′-CCGATCTAGTGAGTCCTCG-3′
Nick_1A	5'-CACTCACGACcgagGACTCACTAGATCGG-3′
Nick_1C	5′-CACTCATGACcgagGACTCACTAGATCGG-3′
Nick_2A(2C)	5′-CAGTCATGAAcgagGACTCACTAGATCGG-3′
Nick_4A(4C)	5′-CATGATCAGTACGTACcgagGACTCACTAGATCGG-3′
ON^{Nick_1A} **A^{PT}**	5'-P-GTCGTG**A^{PT}**GTG-3'
ON^{Nick_1A} **A^{EPT}**	5'-P-GTCGTG**A^{EPT}**GTG-3'
ON^{Nick_1C} **C^{PT}**	5'-P-GT**C^{PT}**ATGAGTG -3'
ON^{Nick_1C} **C^{EPT}**	5'-P-GT**C^{EPT}**ATGAGTG-3'
ON^{Nick_2A} **A^{PT}**	5'-P-TTC**A^{PT}**TG**A^{PT}**CTG-3'
ON^{Nick_2A} **A^{EPT}**	5'-P-TTC**A^{EPT}**TG**A^{EPT}**CTG-3'
ON^{Nick_2C} **C^{PT}**	5'-P-TT**C^{PT}**ATGA **C^{PT}**TG -3'
ON^{Nick_2C} **C^{EPT}**	5'-P-TT**C^{EPT}**ATGA **C^{EPT}**TG-3'
ON^{Nick_4A} **A^{PT}**	5'-P-T **A^{PT}**GCA**^{PT}**TGCT**A^{PT}**CGTCA**^{PT}**G-3'
ON^{Nick_4A} **A^{EPT}**	5'-P-T **A^{EPT}**GCA**^{EPT}**TGCT**A^{EPT}**CGTCA**^{EPT}** G-3'
ON^{Nick_4C} **C^{PT}**	5'-P-TAG**C^{PT}**ATG**C^{PT}**TA**C^{PT}**GT**C^{PT}**AG-3'
ON^{Nick_4C} **C^{EPT}**	5'-P-TAG**C^{EPT}**ATG**C^{EPT}**TA**C^{EPT}**GT**C^{EPT}**AG-3'



Scheme 29. Nicking enzyme amplification reaction.

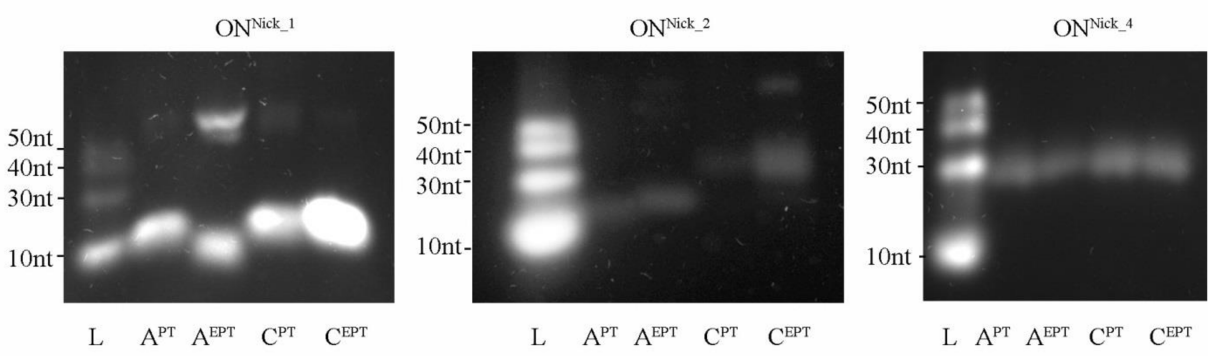
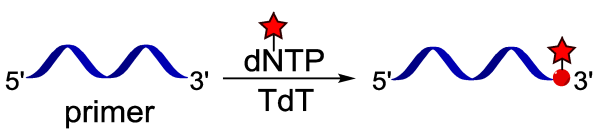


Figure 40. Incorporation of modified dNTPs by NEAR using Nick_1A, Nick_1C, Nick_2A(2C) and Nick_4A(4C) templates: (L) DNA ladder; (A^{PT}) **$dA^{PT}TP$**, dCTP, dGTP, dTTP; (A^{EPT}) **$dA^{EPT}TP$**, dCTP, dGTP, dTTP; (C^{PT}) **$dC^{PT}TP$**, dATP, dGTP, dTTP; (C^{EPT}) **$dC^{EPT}TP$**, dATP, dGTP, dTTP.

The last enzymatic synthesis was performed using terminal deoxynucleotidyl transferase (TdT). This method allows the extension of primers in the absence of template. After 4 hours of reaction catalyzed by TdT product of single nucleotide incorporation (SNI) was observed by PAGE analysis by using **$dA^{PT}TP$** and **$dC^{PT}TP$,** whereas elongation of primer using **$dN^{EPT}TP$**s was less efficient (Figure 41). SNI can be potentially used for specific elongation of 3'-end of ON with one PT-redox label for diagnostics (Scheme 30).



Scheme 30. Nontemplated 3′-tail labeling of ONs by terminal deoxynucleotidyl transferase (TdT).

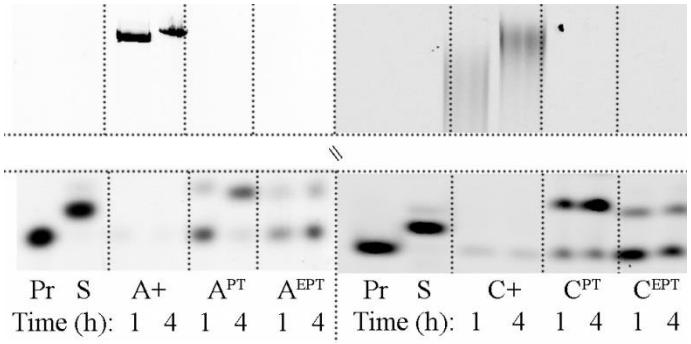


Figure 41. TdT-catalyzed DNA chain elongation. (Pr) primer^{rnd} (5'-FAM-labeled); (S) standard (PEX product of temp^{termA} with dATP or temp^{termC} with dCTP); A+, A^{PT} and A^{EPT}: products of primer^{rnd} elongation using terminal transferase and either dATP, **dA^{PT}TP** or **dA^{EPT}TP** respectively; C+, C^{PT} and C^{EPT}: products of primer^{rnd} elongation using terminal transferase and either dCTP, **dC^{PT}TP** or **dC^{EPT}TP** respectively. (for the full gel image, see Appendix 1).

3.2.4 Fluorescence measurements of PT-modified nucleosides, triphosphates and ON

In order to test the possibility of using the EPT-groups in fluorescence labeling of DNA, the absorption and emission spectra of corresponding nucleosides and triphosphates were measured. The notable fluorescence was observed by analysis of the nucleosides in ethanol with emission maxima at 462-476 nm (Figure 42), while fluorescence measurements of **dN^{EPT}TP**s in water had a negligible effect (Table 12).

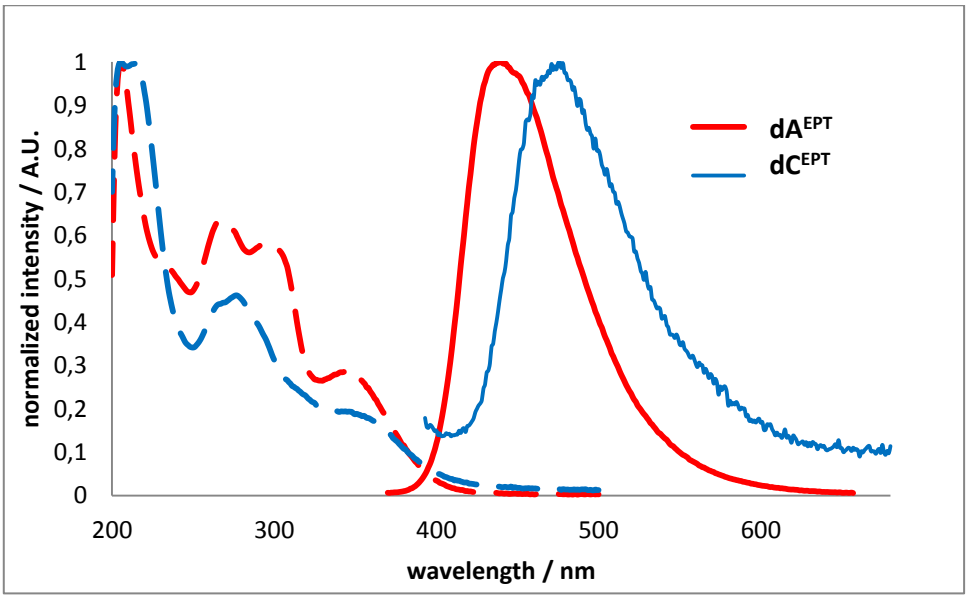


Figure 42. Normalized absorption (dashed lines) and fluorescence (solid lines) of compounds **dA^{EPT}** and **dC^{EPT}** in EtOH.

The fluorescence of the PEX product ON^{rnd16}**A^{EPT}** containing four **A^{EPT}** modifications was moderate (Figure 43). The difference between EPT-modified ON and control indicates that **dA^{EPT}TP** is accepted as a substrate by DNA polymerase and does not bind unspecifically to DNA.

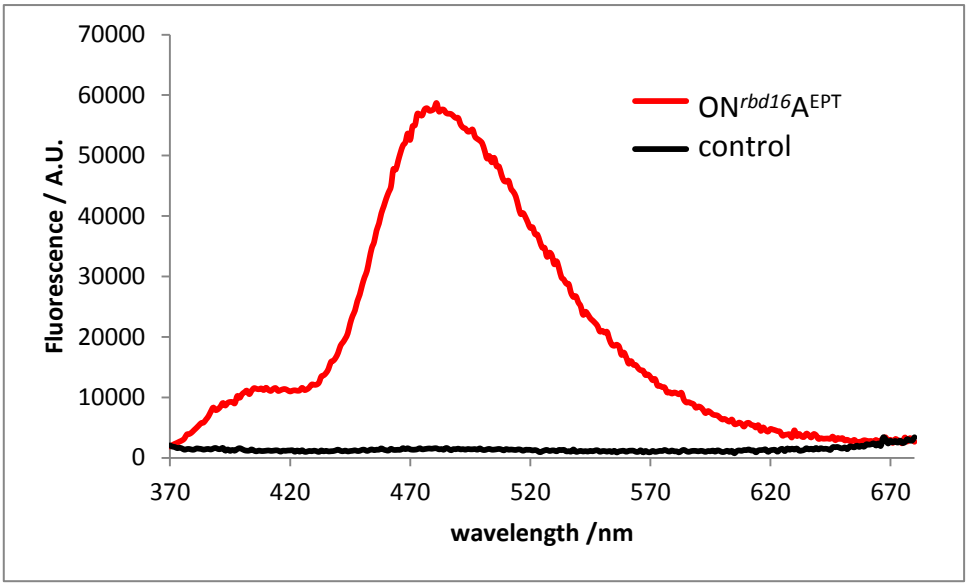


Figure 43. Fluorescent emission spectra.

Table 12. Spectroscopic properties of all new fluorescent EPT-compounds.

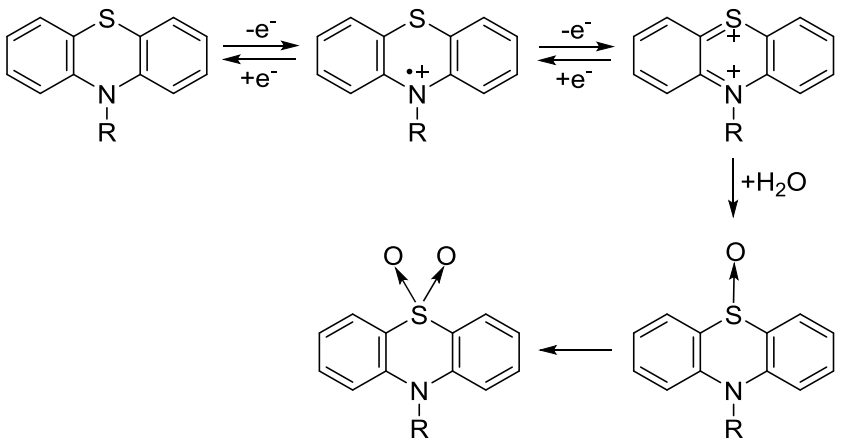
Compound	Solvent	λ_{abs} [nm] [(a)]	ε [10^3 M^{-1} cm^{-1}] [(b)]	λ_{em} [nm] [(c)]	Φ_f [(d)]
dA^{EPT}	EtOH	344 296 267	16.4 32.8 35.9	462	0.1889±0.0116
dC^{EPT}	EtOH	346 277	6.2 14.4	476	0.0101±0.0004
dA^{EPT}TP	H_2O	336 270	11.4 24.9	486	0.014±0.0003
dC^{EPT}TP	H_2O	340 275	8.4 19.8	489	0.0014±0.0001

[a] Position of the absorption maximum, ±1 nm; [b] confidence interval did not exceed ±0.2 10^3 M^{-1} cm^{-1}; [c] position of the emission maximum, ±1 nm; [d] quantum yield of fluorescence measured using quinine bisulfate in 0.5 M H_2SO_4 (Φ_f = 0.546 at 25 °C) as a standard.

3.2.5 Electrochemical studies of PT-modified nucleosides, dNTPs and DNA

Electrochemical behavior of phenothiazine-labeled nucleosides was analysed by cyclic voltammetry (CV) at the basal-plane pyrolytic graphite electrode (PGE).

Phenothiazine derivatives are known redox-active molecules and their electrochemical mechanism was extensively studied[144–149]. The oxidation of the phenothiazine moiety is the two electron process involving reversable formation of cation radical in the first oxidation step and sulfoxide product in the second irreversible electron oxidation (Scheme 31). Further oxidation of the phenothiazine sulfoxide leads to formation of sulfone.



Scheme 31. Mechanism of electrochemical oxidation of phenothiazine.

Electrochemical studies of PT-nucleosides were done in 0.2 M acetate buffer (pH 5.0) with 40 mM concentration of samples. CVs of modified nucleosides resulted in signals characteristic for the PT moiety (Figure 44, Table 13), only the peak of the first oxidation step (PT^{ox1}) was split into two distinct signals in experiments with **dA**^{PT} and **dC**^{PT}.

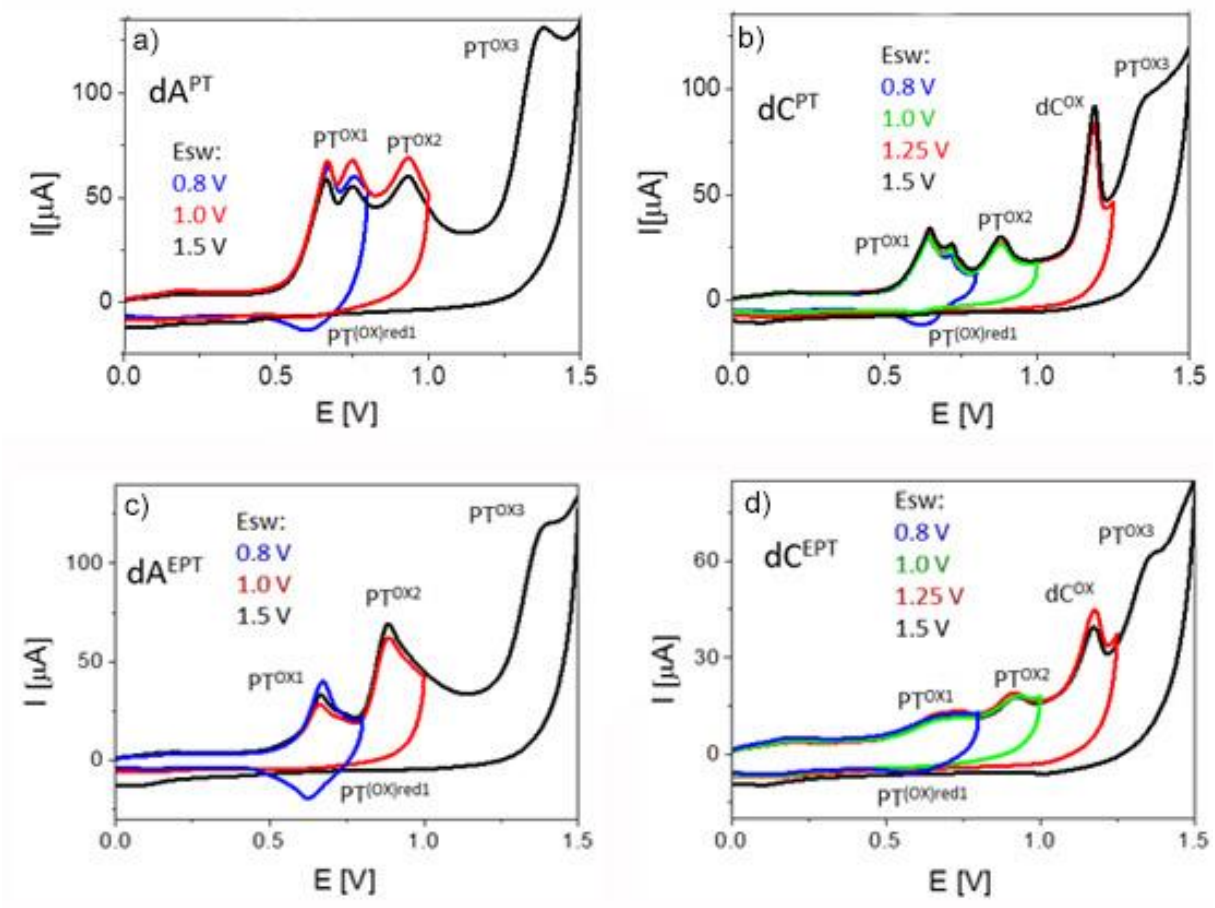


Figure 44. CV responses of (a) **dA**^{PT}, (b) **dC**^{PT}, (c) **dA**^{EPT}, and (d) **dC**^{EPT} at PGE.

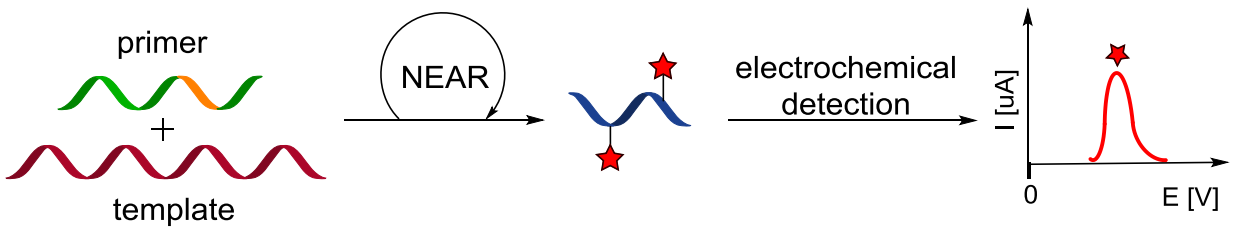
Reversibility of the first oxidation step oxidation was proved by turning the electrochemical stability window (ESW). When the CV scan was turned at +0.80 V the reversibility was still observed, while switching to +1.0 V led to an irreversible reaction with a formation of second oxidation peak (PT^{ox2}). After turning of the CV scan at +1.5 V third peak was observed around +1.33 - (+1.36) V, that corresponds to oxidation of PT sulfoxide moiety. The electrochemical oxidation of **dC^{PT}** and **C^{EPT}** conjugates was accompanied with a formation of a well-developed, irreversible peak close to +1.2 V (dC^{ox}), that could be explained by involvement of the cytosine moiety in the corresponding electrode processes. While oxidation of adenosine analogues (**dA^{PT}** and **dA^{EPT}**) showed the lack of separate peak of 7-deazaadenine oxidation (dA^{ox}) that could be explained by overlapping with a PT signal, possibly peak PT^{ox2}.

Table 13. CV peak potentials of PT-modified nucleosides.

Samples	Peaks				
	PT^{ox1}/mV	PT^{ox2}/mV	PT^{ox3}/mV	dC^{ox}/mV	A^{ox}/mV
dA^{PT}	655*	920[a]	1355	-	920 [a]
dA^{EPT}	655*	860[b]	1360	-	860 [b]
dC^{PT}	630*	855	1330	1160	-
dC^{EPT}	640/720#	880/985	1330	1160	-

[a], [b] overlapping signals, *the major SWV peak, #range of potentials spanning the double-peak envelope.

Next experiments involved electrochemical studies of short ONs bearing PT or EPT labels (Scheme 32). At first 10-mer or 16-mer ON-products of NEAR bearing one, two or four **dA^{PT}** conjugates were electrochemically analyzed. To distinguish oxidation signals of natural purine bases (peaks G^{ox} and A^{ox}) unmodified NEAR products were studied first. Well developed signals were identified at 1080 mV and 1360 mV for G^{ox} and A^{ox} respectively with intensities reflected their relative contents (Table 14). Then **dA^{PT}**-modified ONs (10-mer ON containing either one or two modifications or 16-mer ON containing four modifications) were examined for their electrochemical behavior.



Scheme 32. Electrochemical studies of short ON-products of NEAR experiments.

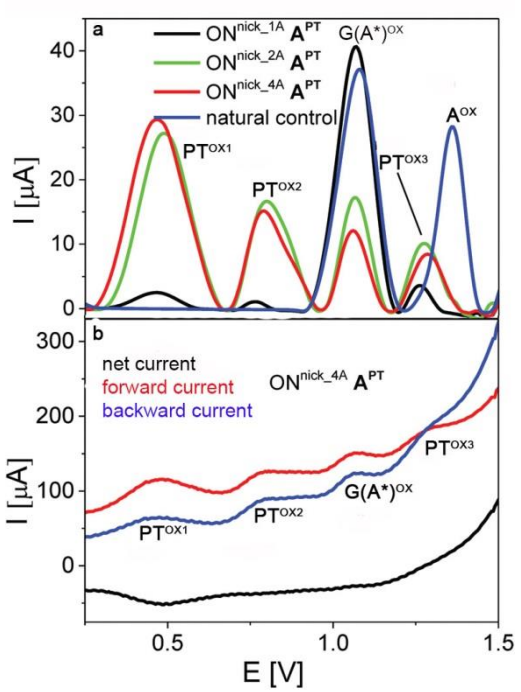


Figure 45. (a) Baseline-corrected AdTS SWV responses of NEAR products with different number of **PT**-modified nucleobases. (b) Components of the SWV current for $ON^{nick\_4A}\mathbf{A}^{\mathbf{PT}}$.

Voltametric responses were detected for each oxidation step of PT-moiety. Even when a 10-mer ONs containing one PT label (ON^{Nick_1A} $\mathbf{A}^{\mathbf{PT}}$) were analyzed, small but distinct peaks PT^{ox1}, PT^{ox2} and PT^{ox3} were observed (Figure 45a). Positions of first two peaks in electrochemical window are less positive than potentials of the purine oxidation signals, therefore they could be differentiated independently. Whereas the third oxidation peak (PT^{ox3}) was observed at the potential theoretically overlapped with a peak of adenine oxidation (A^{ox}), which was detected by electrochemical measurement of natural ON. The ability to detect the peak PT^{ox3} independently was possible because of the lack of natural adenine residues due to the choice of NEAR as a method for the synthesis of short ON (unlike the PEX products that always contained adanines). The oxidation peaks of guanine and 7-deazaadenine groups overlapped and were observed at 1060 mV. It was noticed that the intensities of the measured signals of NEAR products bearing different number of **PT** labels had a non-linear behavior. While the difference in intensity between ON with one or two **PT** moieties (black and green curve in Fig. 45a) was quite large, very similar peaks were observed for the samples with two and four **PT** labels (green and red curve in Fig. 45a). The differences in behavior could be explained by the fact that the relative content of **PT** tags in 16-mer ON is higher by a factor of 1.25 (instead of 2), compared to ON^{Nick_2A} $\mathbf{A}^{\mathbf{PT}}$, while differences between 10-mer ONs with one or two modifications could be the result of strong effect of the **PT** moieties on interactions of the ON with the PGE surface, such as preferential adsorption of the **PT** tags. The electrochemical measurement of **PT**-labeled ONs by square-wave voltammetry confirmed the reversibility of the first oxidation step (PT^{ox1})

and irreversibility of the more negative signals produced by either the **PT** or the nucleobases (PT^{ox2}, PT^{ox3} and $G(A^*)^{ox}$).

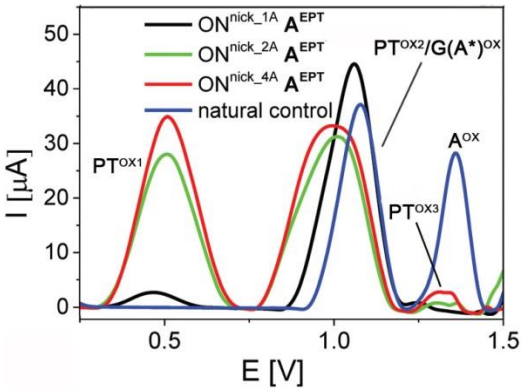


Figure 46. Baseline-corrected AdTS SWV responses of NEAR products with different number of **EPT**-modified nucleobases.

The analysis of electrochemical properties of oligonucleotides with **EPT** tags showed a different behavior of some oxidation peaks (Figure 46). The first oxidation peaks (PT^{ox1}) were detected at the potential around 510 mV and as in the case of ONs bearing $\mathbf{dA^{PT}}$ signals showed similar changes in peak intenses in dependence on the number of **EPT** conjugates incorporated to the ON. While second oxidation peak (PT^{ox2}) was observed in more positive potential overlapping with oxidation peak of guanine (G^{ox}). Therefore the measurement of their intensities was not possible because of the difficulty of differentiation between two overlapping signals.

Based on electrochemical behavior in described experiments, $\mathbf{dA^{PT}}$ conjugates were chosen for multipotential redox coding of DNA, because they produce individual **PT**-specific and natural base-specific signals without mutual overlaps.

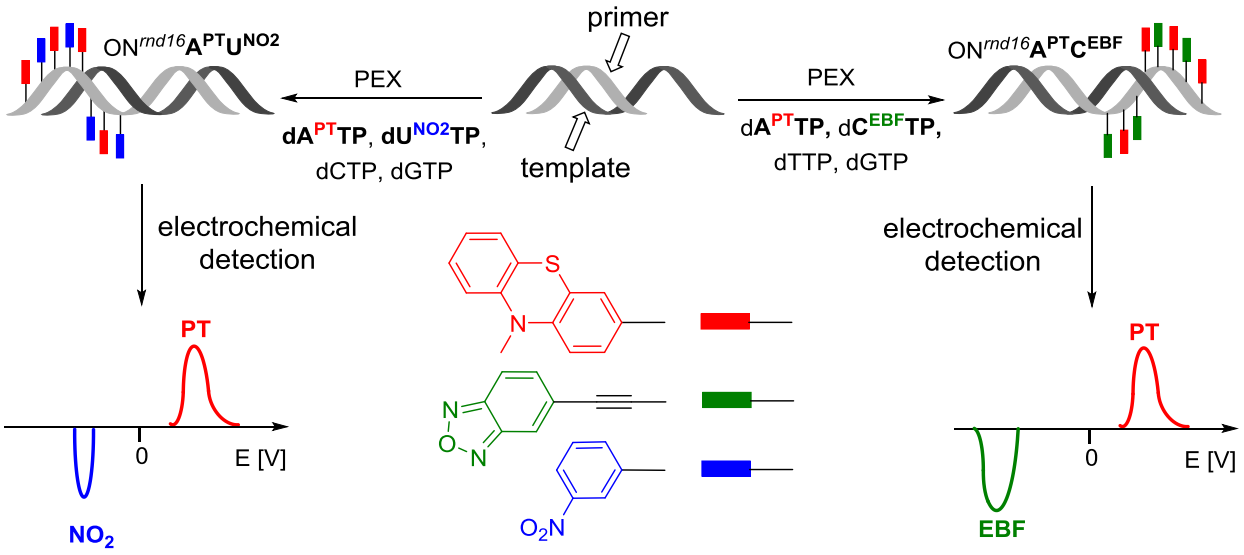
Table 14. SWV peak potentials of NEAR products.

Sample	Peaks					
	PT^{ox1}/mV	PT^{ox2}/mV	PT^{ox3}/mV	G^{ox}/mV	A^{*ox}	A^{ox}/mV
$ON^{nick\_4A}\,\mathbf{A^{PT}}$	460	790	1285	1060[a]	1060[a]	-
$ON^{nick\_4A}\,\mathbf{A^{EPT}}$	510	995[b]	1320	995[b]	995[b]	-
ON^{nick} natural	-	-	-	1080		1360

[a], [b] overlapping signals.

3.2.6 Multipotential redox coding of DNA (in collaboration with Doc. Fojta research group)

To examine the possibility of using **PT** moiety as a label for multipotential DNA coding by combining different tags for electrochemical analysis PEX products were prepared, where **dA^{PT}** label was combined either with benzofurazane group (**dC^{EBF}**) or with nitrophenyl moiety (**dU^{NO2}**) (Scheme 33).



Scheme 33. Electrochemical studies of ON-products of mixed PEX experiments.

Electrochemical measurements of modified PEX products showed characteristic peaks of **PT** oxidation (PT^{ox1}, PT^{ox2}) and purine-specific peaks without significant mutual interference on SWVs measured in anodic direction (Figure 47b). Benzofurazane and nitrophenyl moieties produced reduction signals around -0.95 V or -0.55 V respectively in the catodic scan as in previous studies (Figure 47a).

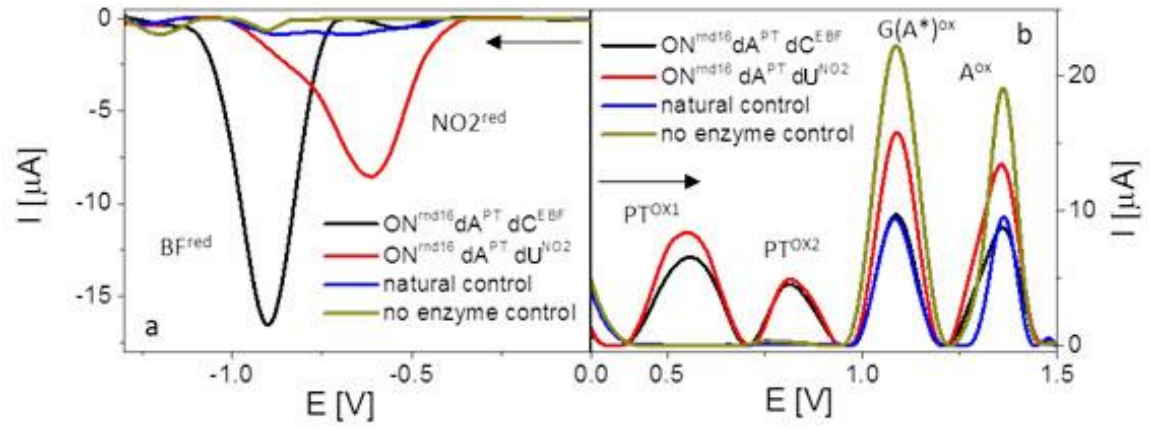


Figure 47 Baseline-corrected AdTS SWV responses of PEX products bearing **PT** labels combined with **EBF** or **NO$_2$** groups: (a) cathodic reduction of **BF** and **NO$_2$** moieties; (b) anodic oxidation of **PT** and nucleobase moieties (arrows indicate scan direction).

Initial potential (E_i) was set at 0.0 V both for anodic and cathodic scans without interference of **BF** or **NO_2** groups in oxidation process or **PT** label in reduction mechanism. Therefore, all three labels could be easily differentiated and determined independently by their peak potentials and/or by the direction of electron flow (Table 15).

Table 15. SWV peak potentials of PEX products.

Sample	Peaks					
	PT^{ox1}/mV	PT^{ox2}/mV	G^{ox}/mV	A^{ox}/mV	BF^{red}/mV	NO_2^{red}/mV
ON^{rnd16} **A^{PT} C^{EBF}**	550	815	1090	1360	-950	-
ON^{rnd16} **A^{PT} U^{NO2}**	550	815	1090	1360	-	-550
ON natural	-	-	1080	1360	-	-

3.2.7 Conclusion

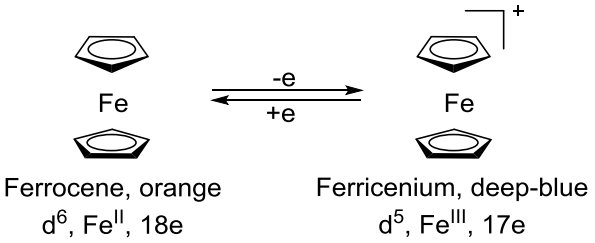
Modified nucleosides and dNTPs linked directly or through acetylene linker to phenothiazine moiety were synthesized by Suzuki or Sonogashira cross-coupling reactions with halogenated nucleosides or triphosphates. The obtained **$dN^{XPT}TPs$** were showen as good substrates for enzymatic synthesis of modified ON and DNA by PEX and NEAR experiments, but with limited activity in PCR amplification. Experiments with terminal deoxynucleotidyl transferase showed the posibility of using **$dA^{PT}TP$** and **$dC^{PT}TP$** for non-templated single-nucleotide incorporation. Interesting fluorescence properties were observed for **dA^{EPT}** and **dC^{EPT}** in EtOH, but the corresponding nucleotides and EPT-modified DNA showed low fluorescence in water. Electrochemical analysis of phenothiazine-labeled nucleosides and DNA showed that phenothiazine moiety is a useful redox label with two characteristic oxidation peaks where the first PT signal is reversibile and the second oxidation step is irreversibile, that can be used for identification of the PT label among other oxidizable moieties. The directly linked **A^{PT}** conjugate is more useful label for multipotential DNA coding, because it produces two distinct peaks, while in case of ethynyl linked **A^{EPT}** base the second oxidation peak overlaps with oxidation signals of natural purine bases. The electrochemical studies of PT-redox label showed the orthogonal behavior to previously reported benzofurazane and nitrophenyl labels, therefore it can be used for "multicolor" redox coding of DNA bases. From another side, the presence of two oxidation peaks limits the potential space for the last (fourth) redox label in the potential window of carbon electrod in prospective four-labels coding and therefore further studies of other alternative oxidizable labels are still needed.

3.3 Ferrocene-labeled nucleosides and dNTPs. Synthesis, enzymatic incorporation and electrochemical detection

3.3.1 Introduction

Ferrocene was first synthesized in 1951 by Kealy and Pauson[150] and in 1952 Wilkinson and Woodward disclosed and published the real sandwich structure $Fe(\eta^5\text{-}C_5H_5)_2$ based on its reactivity[151]. Idependently similar results were published by Fischer and Pfab in Munich[152]. In 1973 Fischer and Wilkinson shared a nobel prize for their contribution to development of organometallic chemistry.

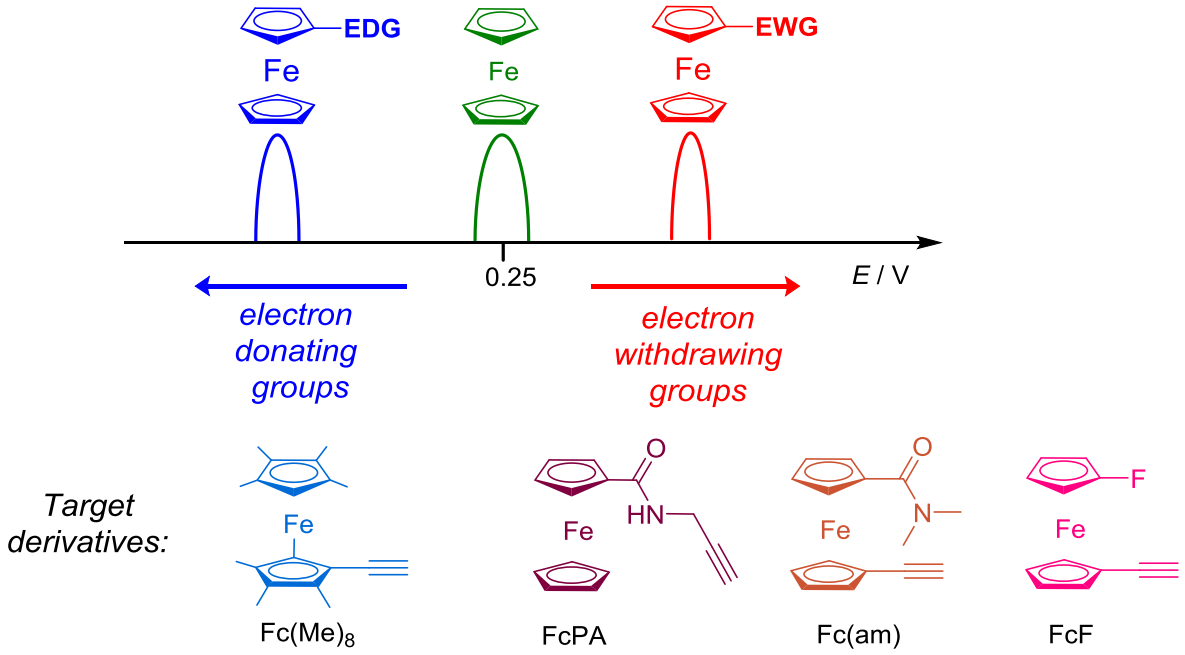
Sandwich structure of ferrocene compound led to high interest for its use in different fields such as nanomedicine, biological sensing, catalysis, battery and other materials, and other areas involving supramolecular, macromolecular, and optoelectronic property aspects[153]. Electrochemical studies have shown that ferrocene oxidation is a single-electron mechanism with reversible formation of ferricenium cation (Scheme 34). Therefore switching capability of ferrocene to ferrocenium was applied in many studies of surface functionalization[154].



Scheme 34. Mechanism of electrochemical oxidation of ferrocene.

The main interest of ferrocene-labeled oligonucleotide derivatives is in the field of electrochemical DNA sensors[155–157]. First synthesis of ferrocene derivatives of nucleosides was reported more then 25 years ago[158]. Later ferrocene-modified uridine phosphoramidites were synthesized for preparation of ferrocene-modified trinucleotides by solid-phase synthesis[159]. In Hocek group ferrocenylethynyl derivatives of nucleoside triphosphates were prepared by single-step cross-coupling reactions without the use of protective groups for incorporation in short ONs, which were detected by electrochemical technique[160]. Recently ferrocene-labeled trinucleotides were applied for electrochemical screening of single nucleotide polymorphisms[161]. Substituents on the cyclopentadienyl ring of ferrocene can change the redox potential in the following way: electron withdrawing groups shift the potential in the anodic direction[162] whereas electron donating groups shift the potential in the cathodic direction[163]. The wide use of ferrocene labels for electrochemical detection of DNA can be explained by favorable electrochemical properties, therefore a synthesis of new ferrocene derivatives with electron

donating and electron withdrawing groups has potential interest for shifting of oxidation peaks (Scheme 35).



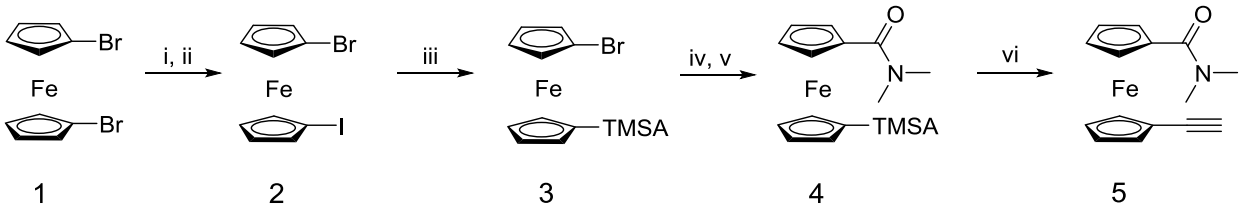
Scheme 35. Dependence of potential on the nature of the substituents on the cyclopentadienyl ring of ferrocene.

3.3.2 Synthesis of Fc labels

Based on the idea of tuning the electrochemical signal of ferrocene oxidation by adding electron withdrawing or electron donating groups, new ethynylferrocene labels were designed and synthesized for further study of their electrochemical behavior. Previous studies of electrochemical properties of ferrocene derivatives showed[160,164] that electron withdrawing groups reduce electron density at iron and thus shift the oxidation potential towards more positive values, whereas electron donating groups facilitate oxidation of ferrocene shifting the oxidation signal to less positive values. To examine electron withdrawing effects fluoro- and carbamoyl groups were attached to cyclopentadienyl rings of ferrocenes by lithiation reaction with following attack by electrophiles, N-fluorobenzenesulfonimide (NFSI) or dimethylcarbamoyl chloride respectively. Also modified ferrocene with propargylamide linker was synthesized following the published procedure. As an example of ferrocene with electron donating groups previously reported 1-ethynyl-1',2,2',3,3',4,4',5-octamethylferrocene was chosen for the synthesis of modified nucleosides and nucleoside triphosphates.

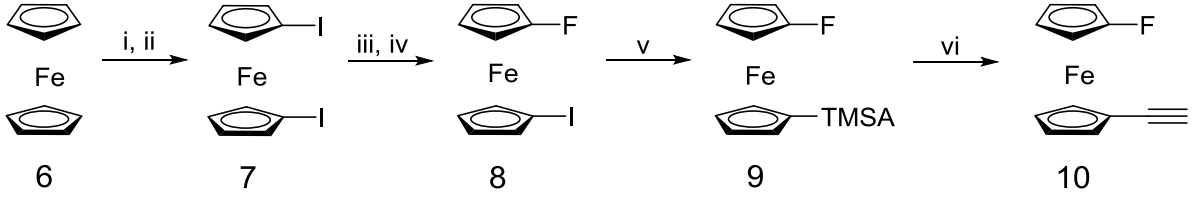
Synthesis of 1-(N,N-dimethylaminocarbonyl)-1'-ethynylferrocene (**5**) was performed from 1,1'-dibromoferrocene (**1**) in four steps (Scheme 36). First two steps proceeded under described conditions[165]. The dibromoferrocene (**1**) underwent a lithium-halogen exchange[166], followed

by quenching with iodine with producing an asymmetric 1-bromo-1'-iodoferrocene (**2**) in high yield. Next, Sonogashira cross-coupling reaction was applied for functionalizing of ferrocene with trimethylsilyl protected ethynyl group, where only Cp-ring with iodine reacted with trimethylsilylacetylene yielding 1-bromo-1'-(trimethylsilylethynyl)ferrocene (**3**), while bromide stayed inert under reaction conditions. Next step involved a second lithium-halogen exchange with following quenching by dimethylcarbamyl chloride with a formation 1-(N,N-dimethylaminocarbonyl)-1'-(trimethylsilylethynyl)ferrocene (**4**) with 64% yield. Deprotection with KF yielded desired product 1-(N,N-dimethylaminocarbonyl)-1'-ethynylferrocene (**5**) (98% yield).



Scheme 36. Reagents and conditions: i) n-BuLi (1.0 equiv.), THF, -78 °C, 3 h; ii) I_2, -78 °C to 25 °C (85%); iii) TMSA, CuI (1.0 mol%), $Pd(PPh_3)_2Cl_2$ (0.5 mol%), Et_3N (10 equiv.), THF, 60 °C, 16 h (60%); iv) n-BuLi (1.2 equiv.), THF, -78 °C, 1 h; v) $(CH3)_2NCOCl$, -78 °C to 25 °C over 3 h (64%); vi) KF (10 equiv), MeOH:dioxane (1:1) (98%).

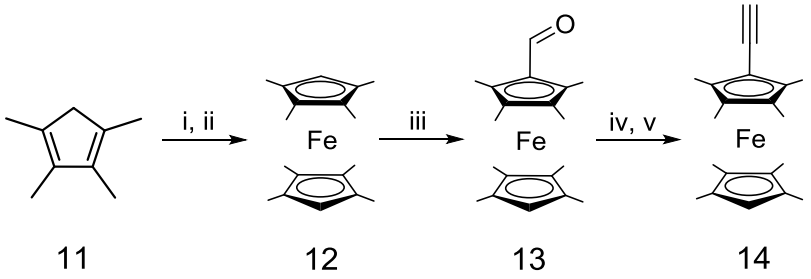
The synthesis of 1-fluoro-1'-ethynylferrocene (**10**) by analogous procedure was difficult because of the very low yield of fluorination step with 1-bromo-1'-(trimethylsilylethynyl)ferrocene (**3**), therefore fluoroderivative was obtained from 1,1'-diiodoferrocene (**7**) (Scheme 37). Starting diiodo derivative (**7**) was obtained from ferrocene (**6**) according to described conditions[167]. Next, 1-fluoro-1'-iodoferrocene (**8**) was synthesized by lithiation of metallocene and subsequent addition of the fluorinating agent N-fluorobenzenesulfonimide (NFSI) with 24 % yield. Following Sonogashira cross-coupling reaction with trimethylsilylacetylene yielded 1-fluoro-1'-(trimethylsilylethynyl)ferrocene (**9**) (40%) and deprotection of acetylene group led to the formation of desired 1-fluoro-1'-ethynylferrocene (**10**) (95%).



Scheme 37. Reagents and conditions: i) n-BuLi (2.5 equiv.), TMEDA (2.5 equiv.), hexane, 0 °C to 25 °C, 16 h; ii) I_2, THF, -78 °C to 25 °C (81%); iii) n-BuLi (1.1 equiv.), THF, -78 °C, 1 h; iv) NFSI, Et_2O, 25 °C (24 %); v) TMSA, CuI (1.0 mol%), $Pd(PPh_3)_2Cl_2$ (0.5 mol%), Et_3N (10 equiv.), THF, 60 °C, 16 h (40%); vi) KF (10 equiv), MeOH:dioxane (1:1) (95%).

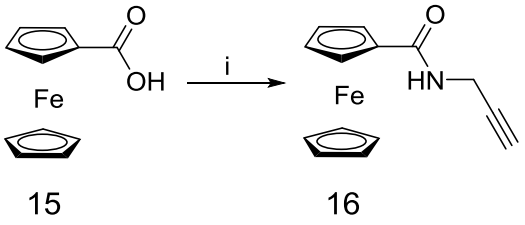
1-ethynyl-1',2,2',3,3',4,4',5-octamethylferrocene[168–170] (**14**) and propargylamideferrocene[171] (**16**) were obtained following published procedures.

The synthesis of 1-ethynyl-1',2,2',3,3',4,4',5-octamethylferrocene (**14**) started from the tetramethylcyclopentadiene (**11**) by lithiation reaction and subsequent addition of $FeCl_2$ with production of octamethylferrocene (**12**). The formylation of octamethylferrocene was achieved by a slight modification of the Vilsmeier reaction with DMF and phosphorus oxychloride and following quenching with water (**13**) (yield 79%). Desired octamethylethynylferrocene (**14**) was synthesized via Wittig olefination and subsequent dehydrohalogenation with 56% yield (Scheme 38).



Scheme 38. Reagents and conditions: i) (**11**) in benzene, CH_3Li in E_2O, 25 °C, reflux for 4 h, ii) $FeCl_2$ (0.7 equiv.), THF, 0 °C to 25 °C, 12h (48%); iii) $POCl_3$ (7.8 equiv.), DMF, 60 °C, 6 h (79%); iv) $[Ph_3PCH_2Cl]Cl$ (1.0 equiv.), THF, n-BuLi (2.5 equiv.), 25 °C, 3h, v) t-BuOK (2.0 equiv.), reflux for 1 day (56%).

Ferrocenoyl propargylamide (**16**) was synthesized by coupling ferrocene monocarboxylic acid (**15**) to propargylamine in the presence of dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) (Scheme 39).



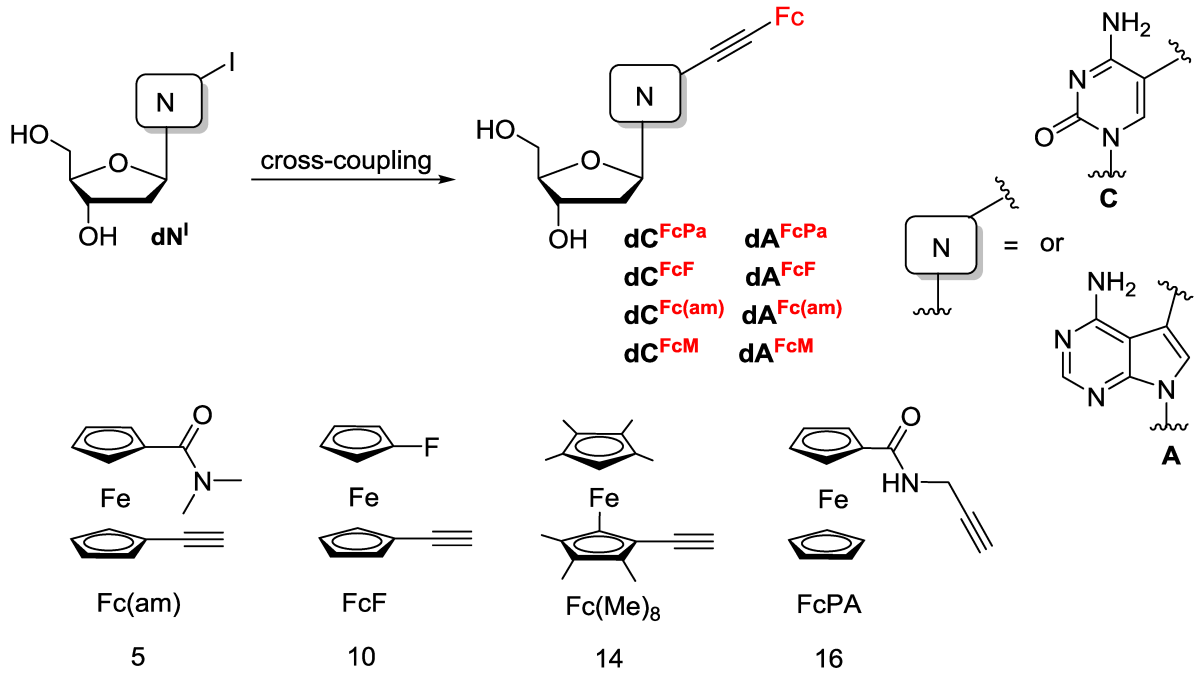
Scheme 39. Reagents and conditions: i) DCC (1.5 equiv.), DMAP (0.1 equiv.), propargylamine (1.0 equiv.), CH_2Cl_2, 25 °C, 24h (25%).

3.3.3 Synthesis of modified nucleosides and dNTPs

For the attachment of ferrocene labels linked through an acetylene the Sonogashira cross-coupling reactions of unprotected halogenated nucleosides (dA^I or dC^I) in the presence of Pd catalyst and CuI were applied to give labeled nucleosides $\mathbf{dC^{FcX}}$ and $\mathbf{dA^{FcX}}$ in high yields (Table 16, Sheme 40).

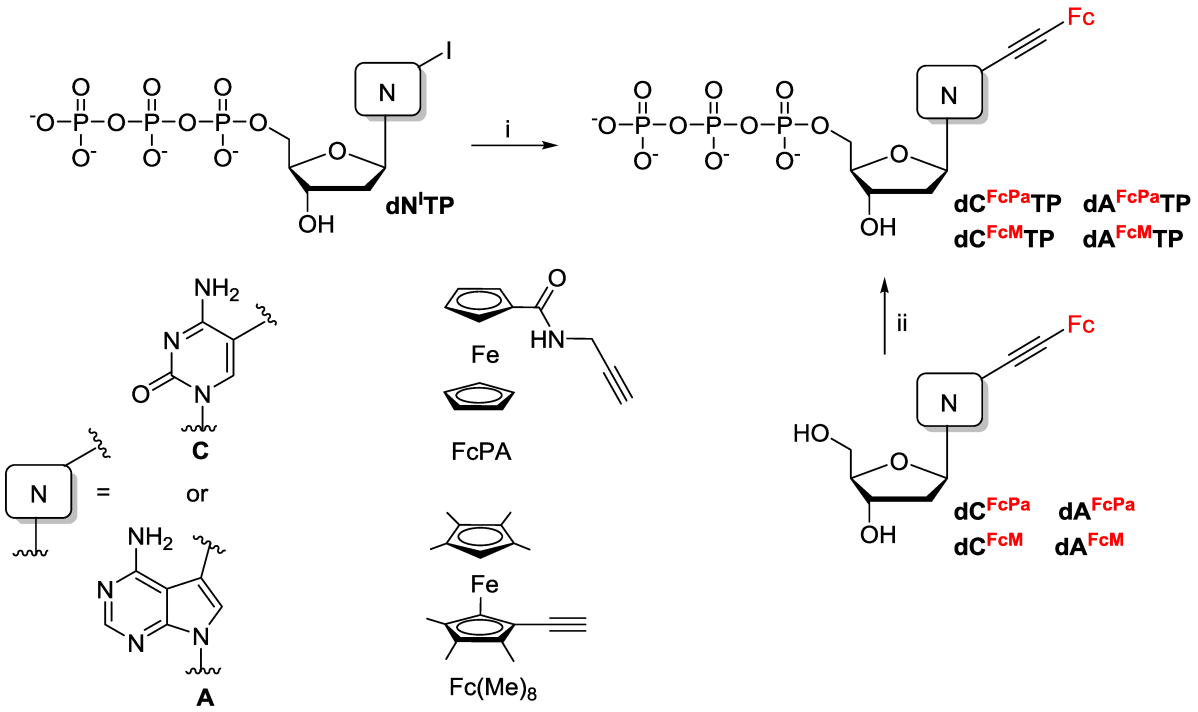
Table 16. Synthesis of nucleosides and nucleoside triphosphates bearing acetylene-linked ferrocene labels.

Starting compound	Reagent	Catalyst	Solvent	Base	Product	Yield (%)
dA^I	**Fc(am) (5)**	$Pd(PPh_3)_2Cl_2$, CuI, PPh_3	DMF	Et_3N	$\mathbf{dA^{Fc(am)}}$	98
dC^I					$\mathbf{dC^{Fc(am)}}$	98
dA^I	**FcF (10)**	$Pd(PPh_3)_2Cl_2$, CuI, PPh_3	DMF	Et_3N	$\mathbf{dA^{FcF}}$	90
dC^I					$\mathbf{dC^{FcF}}$	92
dA^I	**$Fc(Me)_8$ (14)**	$Pd(OAc)_2$, CuI, TPPTS	$MeCN/H_2O$ (1/1)	Et_3N	$\mathbf{dA^{FcM}}$	90
dC^I					$\mathbf{dC^{FcM}}$	86
dA^I	**FcPA (16)**	$Pd(PPh_3)_2Cl_2$, CuI PPh_3	DMF	Et_3N	$\mathbf{dA^{FcPA}}$	98
dC^I					$\mathbf{dC^{FcPA}}$	97



Scheme 40. Synthesis of ferrocene-modified nucleosides.

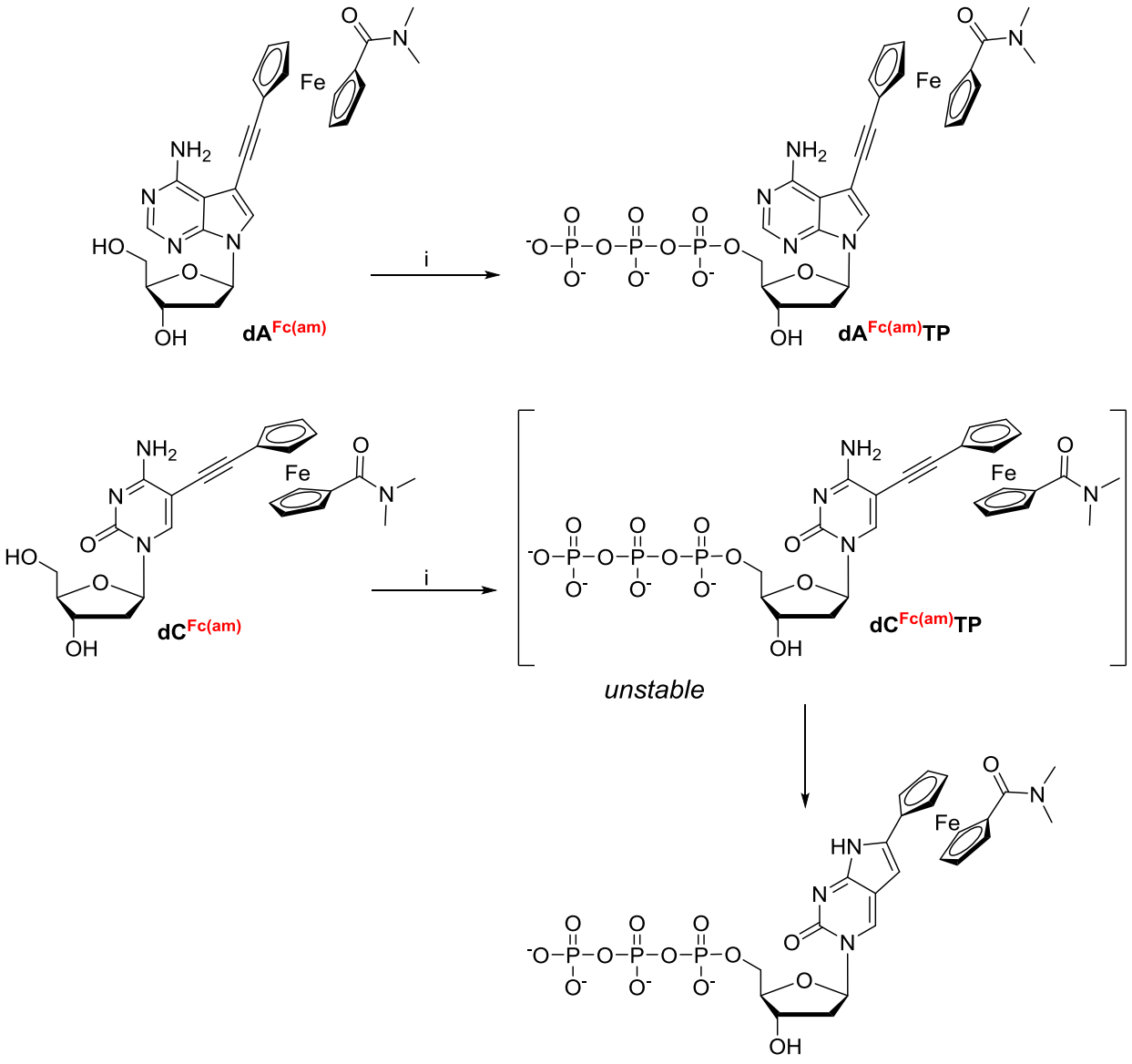
The aqueous Sonogashira cross-coupling reactions of dN^{I}TPs with **FcPA** and **Fc(Me)$_8$** resulted in moderate yields of desired Fc-acetylene-linked **dN^{FcX}TPs** (**dC^{FcPA}TP** in 16%, **dA^{FcPA}TP** in 13%, **dC^{FcM}TP** in 30%, **dA^{FcM}TP** in 38%; Sheme 41, Table 17). In order to synthesize a larger quantities of **dN^{FcPA}TPs** and **dN^{FcM}TPs,** the alternative approach was applied by triphosphorylation of the corresponding nucleosides in moderate yields. In all cases, the Fc-labeled nucleoside triphosphates were isolated by HPLC and **dN^{FcPA}TPs** were fully characterized. The characterization of compounds **dC^{FcM}TP** and **dA^{FcM}TP** was difficult because of partial oxidation of ferrocene during reaction/purification process. Nevertheless, ^{31}P spectra and MS analysis confirmed the obtaining of desired modified triphosphates, therefore they were used for further biochemical studies.



Scheme 41. Synthesis of modified nucleoside triphosphates bearing **FcPA** and **Fc(Me)$_8$** labels. Reagents and conditions: i) **FcPA** [or **Fc(Me)$_8$**], $Pd(PPh_3)_2Cl_2$, CuI, PPh_3, MeCN/H_2O (1:1), 60°C, 1 h; ii) 1. $POCl_3$, $PO(OMe)_3$, 0°C, 3 h; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0°C,1.5 h; 3. TEAB.

All attempts to synthesize **Fc(am)** derivatives under aqueous Sonogashira cross-coupling reactions with halogenated nucleoside triphosphates did not lead to the formation of modified triphosphates presumably due to the bad solubility of ferrocene labels in water conditions. **dA$^{Fc(am)}$TP** was synthesized by triphosphorylation of the corresponding nucleoside **dA$^{Fc(am)}$** in 16% yield. **dC$^{Fc(am)}$TP** synthesized by triphosphorylation of **dC$^{Fc(am)}$** appeared to be unstable and cyclized with a time (Sheme 42). The ^{1}H,^{13}C-HMBC-experiment of **dC$^{Fc(am)}$TP** sample in D_2O showed the absence of long-range correlation from proton H-6 of cytidine to carbons of

triple bond, that could be explained by hydrolysis or cyclization of triple bond. Such cyclization has been reported in numerous cases[172–175]. Attempted cross-couplings of **FcF** with halogenated triphosphates (dC^{I}TP and dA^{I}TP) under the aqueous conditions did not proceed neither in DMF:H_2O (4:1) (Table 17). The explanation could be in limited solubility of **FcF** in H_2O. Therefore the synthesis of **dA^{FcF}TP** and **dC^{FcF}TP** was carried out by triphosphorylation of the corresponding nucleoside **dA^{FcF}** and **dC^{FcF}**, but unfortunately modified triphosphates were not possible to isolate.



Scheme 42. Synthesis of modified nucleoside triphosphates bearing **Fc(am)** labels. Reagents and conditions: i) 1. $POCl_3$, $PO(OMe)_3$, 0°C, 3 h; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0°C,1.5 h; 3. TEAB.

Table 17. Synthesis of nucleoside triphosphates bearing acetylene-linked ferrocene labels.

Starting compound	Reagent	Catalyst	Solvent	Base	Product	Yield (%)
$dA^{I}TP$	**FcPA**	$Pd(PPh_3)_2Cl_2$, CuI PPh_3	$MeCN/H_2O$ (1/1)	Et_3N	**$dA^{FcPA}TP$**	13
$dC^{I}TP$					**$dC^{FcPA}TP$**	16
dA^{FcPA}	1) $PO(OMe)_3$, $POCl_3$, 0°C; 2) $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0°C; 3) TEAB (2M)				**$dA^{FcPA}TP$**	22
dC^{FcPA}					**$dC^{FcPA}TP$**	18
$dA^{I}TP$	**$Fc(Me)_8$**	$Pd(PPh_3)_2Cl_2$, CuI, PPh_3	$MeCN/H_2O$ (1/1)	Et_3N	**$dA^{FcM}TP$**	38[a]
$dC^{I}TP$					**$dC^{FcM}TP$**	30[a]
$dA^{Fc(Me)8}$	1) $PO(OMe)_3$, $POCl_3$, 0°C; 2) $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0°C; 3) TEAB (2M)				**$dA^{FcM}TP$**	15[a]
$dC^{Fc(Me)8}$					**$dC^{FcM}TP$**	20[a]
$dA^{I}TP$	**Fc(am)**	$Pd(PPh_3)_2Cl_2$, CuI, PPh_3	$MeCN/H_2O$ (1/1)	Et_3N	**$dA^{Fc(am)}TP$**	-
$dC^{I}TP$					**$dC^{Fc(am)}TP$**	-
$dC^{I}TP$			DMF/H_2O (1/1)		**$dC^{Fc(am)}TP$**	-
$dA^{Fc(am)}$	1) $PO(OMe)_3$, $POCl_3$, 0°C; 2) $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0°C; 3) TEAB (2M)				**$dA^{Fc(am)}TP$**	11
$dC^{Fc(am)}$					**$dC^{Fc(am)}TP$**	-[b]
$dA^{I}TP$	**FcF**	$Pd(PPh_3)_2Cl_2$, CuI	$MeCN/H_2O$ (1/1)	Et_3N	**$dA^{FcF}TP$**	-
$dC^{I}TP$					**$dC^{FcF}TP$**	-
$dA^{I}TP$			DMF/H_2O (4/1)		**$dA^{FcF}TP$**	-
dA^{FcF}	1) $PO(OMe)_3$, $POCl_3$, 0°C; 2) $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0°C; 3) TEAB (2M)				**$dA^{FcF}TP$**	-
dC^{FcF}					**$dC^{FcF}TP$**	-

a) isolated product was easily oxidized; b) isolated product cyclized with a time.

3.3.4 Enzymatic synthesis of modified DNA

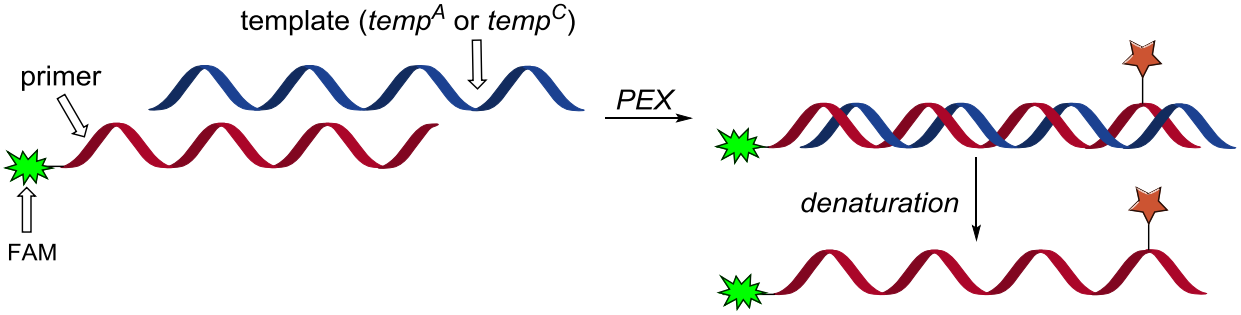
New **$dN^{FcX}TPs$** (**$dA^{FcPA}TP$**, **$dC^{FcPA}TP$**, **$dA^{FcM}TP$** and **$dC^{FcM}TP$**) then were applied as substrates for thermostable KOD XL polymerase in primer extension experiments (for sequences of primer, templates and products, see Table 18). The templates were chosen in order to analyze the PEX incorporation of compounds **$dN^{X}TPs$** forming products both with single modification and four modifications in the sequence. Each PEX experiment was analyzed by polyacrylamide gel electrophoresis using 6-carboxyfluorescein-(6-FAM-)-labeled primer.

Table 18. List of sequences of templates, primer, and products.

Name	Sequence
primer^{rnd}	5'-CATGGGCGGCATGGG-3'
temp^{rnd16}	5'-CTAGCATGAGCTCAGTCCCATGCCGCCCATG-3'
temp^{A}	5'-CCCTCCCATGCCGCCCATG-3'
temp^{C}	5'-CCCGCCCATGCCGCCCATG-3'
temp^{termA}	5'-TCCCATGCCGCCCATG-3'
temp^{termC}	5'-GCCCATGCCGCCCATG-3'
ON^{rnd16} **A**^{FcPA}	5'-CATGGGCGGCATGGGA^{FcPA}CTGA^{FcPA}GCTCA^{FcPA}TGCTA^{FcPA}G-3'
ON^{rnd16} **A**^{FcM}	5'-CATGGGCGGCATGGGA^{FcM}CTGA^{FcM}GCTCA^{FcM}TGCTA^{FcM}G-3'
ON^{rnd16} **C**^{FcPA}	5'-CATGGGCGGCATGGGAC^{FcPA}TGAGC^{FcPA}C^{FcPA}ATGC^{FcPA}TAG-3'
ON^{rnd16} **C**^{FcM}	5'-CATGGGCGGCATGGGAC^{FcM}TGAGC^{FcM}C^{FcM}ATGC^{FcM}TAG-3'
ON^{A} **A**^{FcPA}	5'-CATGGGCGGCATGGGA^{FcPA}GGG-3'
ON^{A} **A**^{FcM}	5'-CATGGGCGGCATGGGA^{FcM}GGG-3'
ON^{C} **C**^{FcPA}	5'-CATGGGCGGCATGGGC^{FcPA}GGG-3'
ON^{C} **C**^{FcM}	5'-CATGGGCGGCATGGGC^{FcM}GGG-3'
ON^{termA} **A**^{FcPA}	5'-CATGGGCGGCATGGGA^{FcPA}-3'
ON^{termA} **A**^{FcM}	5'-CATGGGCGGCATGGGA^{FcM}-3'
ON^{termC} **C**^{FcPA}	5'-CATGGGCGGCATGGGC^{FcPA}-3'
ON^{termC} **C**^{FcM}	5'-CATGGGCGGCATGGGC^{FcM}-3'

In the template ONs the segments forming duplex with the primer are underlined. Acronyms used in the text for primer extension products are analogous to those introduced for the templates.

At first, the single nucleotide incorporation was performed by extension of 15-nt primer with one of each **dN^{X}TPs** followed by three natural dGs using templates temp^{A} or temp^{C} (Scheme 43). The fully extended products were obtained in all cases like in previously reported **dC^{Fc}TP** and **dA^{Fc}TP**[160] with the exception of incorporation of **dA$^{Fc(am)}$TP** (Figure 48).



Scheme 43. Single nucleotide incorporation by primer extension experiments.

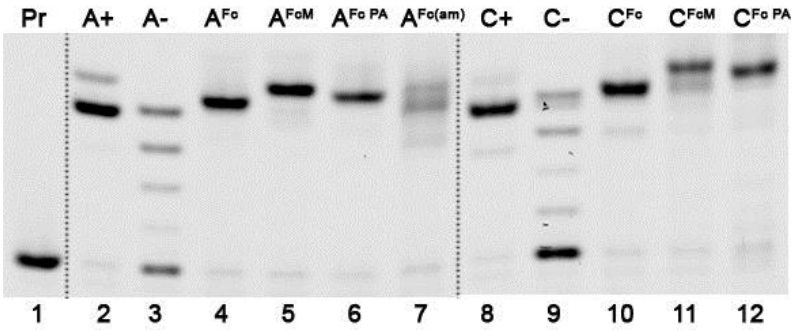


Figure 48. Primer extension with a KOD XL polymerase using prim^{rnd}, temp^{A}, and temp^{C}: (Pr) primer (5'-FAM-labeled); (A+) dATP, dGTP; (A-) dGTP; (A^{Fc}) **dA^{Fc}TP**, dGTP; (A^{FcM}) **dA^{FcM}TP**, dGTP; (A^{FcPA}) **dA^{FcPA}TP**, dGTP; (A$^{Fc(am)}$) **dA$^{Fc(am)}$TP**, dGTP (C+) dCTP, dGTP; (C-) dGTP; (C^{Fc}) **dC^{Fc}TP**, dGTP; (C^{FcM}) **dC^{FcM}TP**, dGTP; (C^{FcPA}) **dC^{FcPA}TP**, dGTP.

The kinetics studies in the presence of KOD XL polymerase were performed to examine the incorporation efficiency of modified **dN^{XFc}TP**s by PEX experiments in comparison with natural dNTPs (Figures 49-53). The rates of the PEX with natural dNTPs or modified nucleotides were revealed using templates temp^{termA} or temp^{termC} and primer^{rnd}. The incorporation of natural dATP or dCTP were complete in 2 minutes and ferrocenene-labeled nucleotides were fully incorporated within max. 1-2 minutes as well. The slowest extension was observed in the experiment with **dA^{FcM}TP** and incorporation of **dA $^{Fc(am)}$TP** was not detected even after 15min.

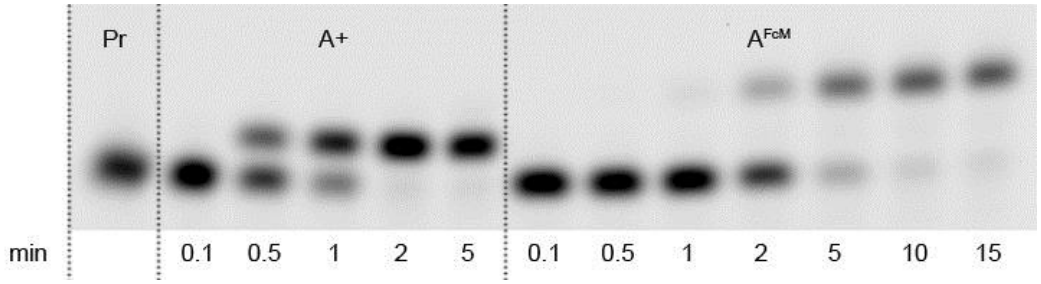


Figure 49. Comparison of incorporation of natural and modified dATPs to DNA with *temp^{termA}*: (**Pr**) primer; (**A+**) dATP; (**A^{FcM}**) **dA^{FcM}TP**.

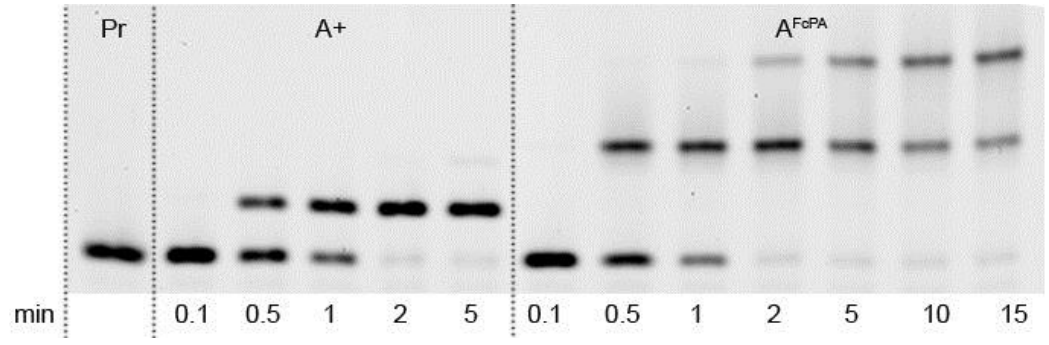


Figure 50. Comparison of incorporation of natural and modified dATPs to DNA with *temp^{termA}*: (**Pr**) primer; (**A+**) dATP; (**A^{FcPA}**) **dA^{FcPA}TP**.

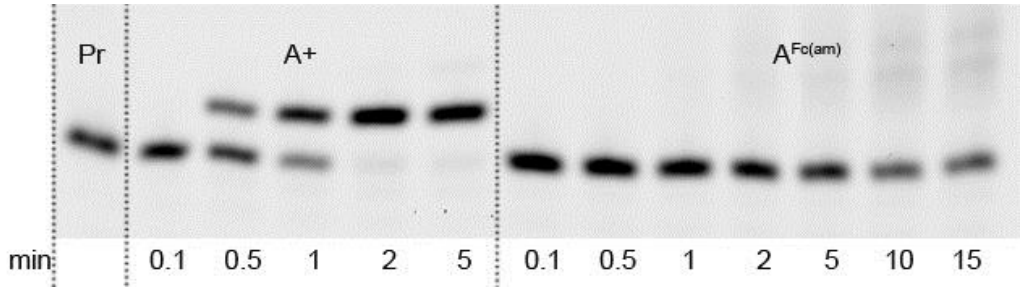


Figure 51. Comparison of incorporation of natural and modified dATPs to DNA with *temp*^{termA}: (**Pr**) primer; (**A+**) dATP; (**A** $^{\mathbf{Fc(am)}}$) **dA** $^{\mathbf{Fc(am)}}$**TP**.

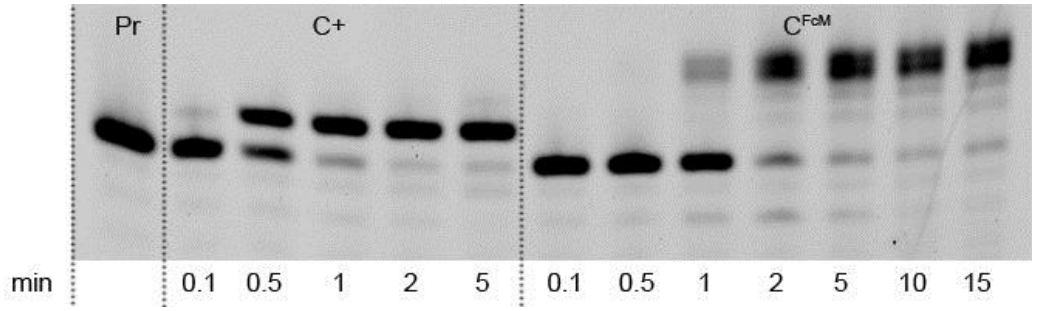


Figure 52. Comparison of incorporation of natural and modified dCTPs to DNA with *temp*^{termC}: (**Pr**) primer; (**C+**) dCTP; (**C**$^{\mathbf{FcM}}$) **dC**$^{\mathbf{FcM}}$**TP**.

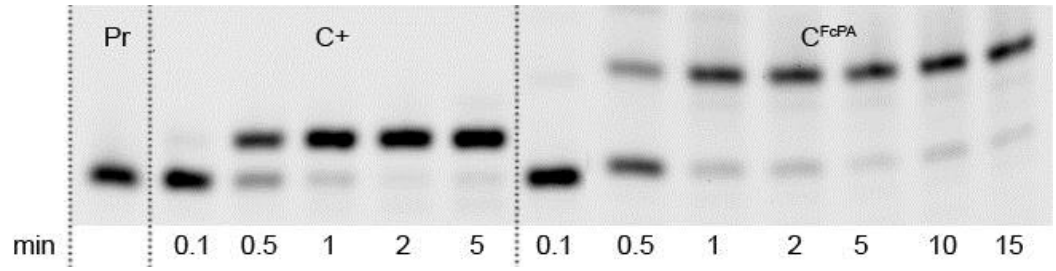
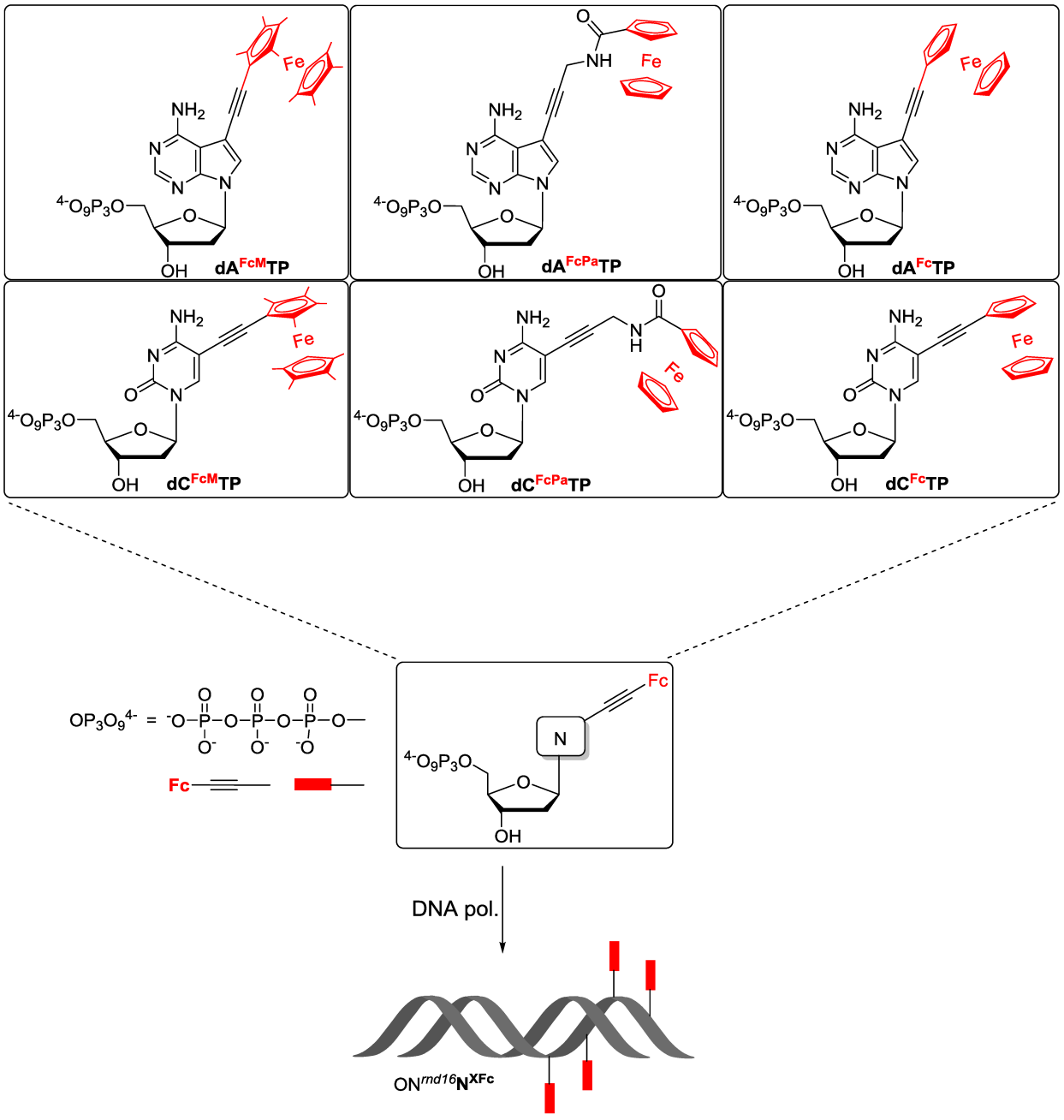


Figure 53. Comparison of incorporation of natural and modified dCTPs to DNA with *temp*^{termC}: (**Pr**) primer; (**C+**) dCTP; (**C**$^{\mathbf{FcPA}}$) **dC**$^{\mathbf{FcPA}}$**TP**.

Next, each of the modified **dN^{XFc}TPs** was used for a multiple incorporation into a longer 31-mer oligonucleotide (ON) by KOD XL polymerase using temp^{rnd16} (Scheme 44). The sequence of the template was designed to encode 4 modifications if one of **dN^{X}TPs** is modified (Figure 54).



Scheme 44. Multiple nucleotide incorporation of modified triphosphates dN^{XFc}**TPs** by primer extension experiments.

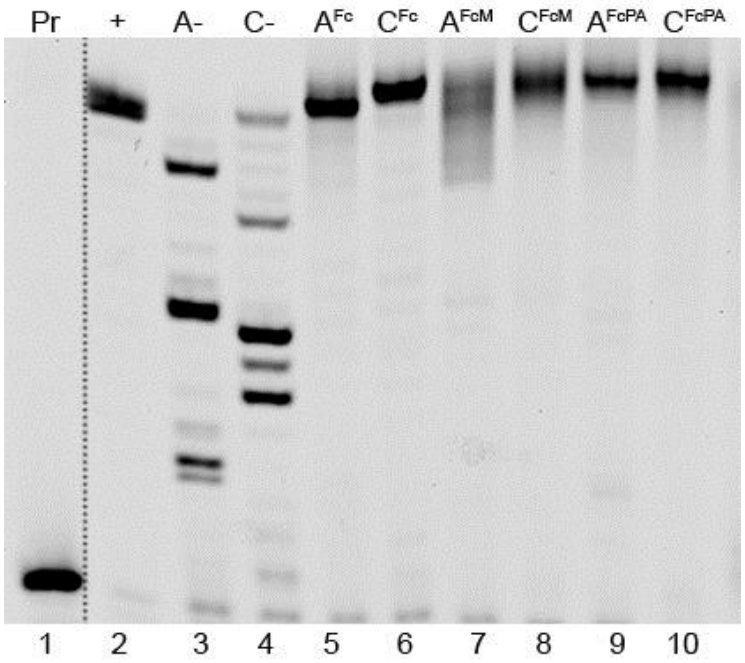


Figure 54. Primer extension with KOD XL polymerase using prim^{rnd}, temp^{rnd16}: (Pr) primer (5'-FAM-labeled); (+) natural dNTPs; (A-) dCTP, dTTP, dGTP; (C-) dATP, dTTP, dGTP; (A^{Fc}) **dA^{Fc}TP**, dCTP, dTTP, dGTP; (C^{Fc}) **dC^{Fc}TP**, dATP, dTTP, dGTP; (A^{FcM}) **dA^{FcM}TP**, dCTP, dTTP, dGTP; (C^{FcM}) **dC^{FcM}TP**, dATP, dTTP, dGTP; (A^{FcPA}) **dA^{FcPA}TP**, dCTP, dTTP, dGTP; (C^{FcPA}) **dC^{FcPA}TP**, dATP, dTTP, dGTP.

3.3.5 Electrochemical studies of modified nucleosides.

Square-wave voltammetric (SWV) responses of **dA^{FcM}** and **dC^{FcM}** at a pyrolytic graphite electrode (PGE) were compared with the response of previously reported **dA^{Fc}** and **dC^{Fc}** (Figure 55). Ethynylferrocene-labeled nucleosides yielded a well-developed peaks due to the reversible one-electron Fe^{II}/ Fe^{III} redox process around 0.45 V. Similar reversible signals were observed with both **dC^{FcM}** and **dA^{FcM}**, but their potentials exhibited remarkable negative shifts (around 380 mV). Such behavior can be explained by electron donating effects of methyl groups in ferrocene, which make the oxidation of the metal complexes easier.

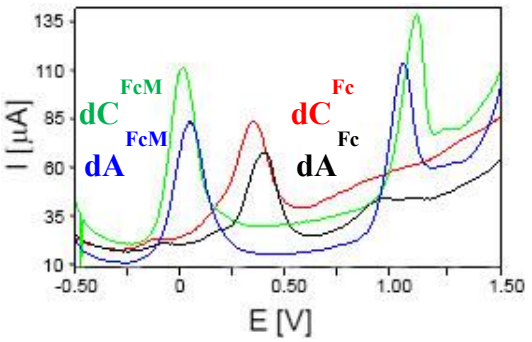


Figure 55. Square-wave voltammograms of **dC^{FcM}** and **dA^{FcM}** in comparison with previously reported **dA^{Fc}** and **dC^{Fc}**.

Next, modified nucleosides bearing ferrocene labels with electron withdrawing groups, were studied for their electrochemical behavior. As expected, oxidation of the metal complexes was more difficult compared to previously reported **dA^{Fc}** and **dC^{Fc}**, resulting with oxidation peaks in more positive shifts of redox potentials (around 550 mV for fluoroderivative and 670 mV for ferrocene-modified nucleosides with amide groups on Cp ring).

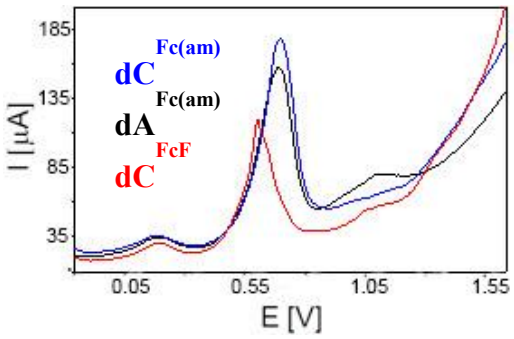


Figure 56. Square-wave voltammograms of **dC$^{Fc(am)}$**, **dA$^{Fc(am)}$** and **dC^{FcF}**.

Electrochemical studies of new ferrocene-modified nucleosides were conducted, where electron-withdrawing groups on the cyclopentadienyl ring of ferrocene shifted the potential in the anodic direction, whereas methyl groups shifted the potential in the cathodic direction. Tuning of the electrochemical signal of ferrocene oxidation has potential application in orthogonal labeling of short DNA fragments, therefore further electrochemical properties of Fc-modified dNTPs and DNA will be studied in near future in collaboration with Fojta group.

3.3.6 Conclusion

At first new alkynyl ferrocenes, 1-(N,N-dimethylaminocarbonyl)-1'-ethynylferrocene and 1-fluoro-1'-ethynylferrocene, were synthesized as new ferrocene labels with electron withdrawing groups. Propargylamideferrocene was obtained following published procedures. As an example of ferrocene label with electron donating group previously reported 1-ethynyl-1',2,2',3,3',4,4',5-octamethylferrocene was synthesized. Then modified nucleosides were obtained by Sonogashira cross-coupling reactions of unprotected halogenated nucleosides with alkynyl ferrocenes in high yields. Corresponding modified **dN^{X}TPs** were obtained with propargylamideferrocene linker in moderate yields, while the synthesis of nucleoside triphosphates with fluoroferrocene was not successful. Synthesis of modified **dN^{X}TPs** with dimethylcarbamoyl group on the cyclopentadienyl ring of ferrocene was performed via phosphorylation procedure, where **dA$^{Fc(am)}$TP** was obtained in moderate yield, while **dC$^{Fc(am)}$TP** was unstable and formed a cyclic side product. Synthesis and characterization of modified **dN^{X}TPs** with octamethylferrocene label was complicated because of the partial oxidation of ferrocene moiety that could be

explained by influence of methyl groups on oxidation of ferrocene. Modified **$dN^{FcX}TPs$** (**$dA^{FcPA}TP$**, **$dC^{FcPA}TP$**, **$dA^{FcM}TP$** and **$dC^{FcM}TP$**) were good substrates for DNA polymerase and served as building blocks in primer extension experiments for producing modified oligonucleotides with the exception of **$dA^{Fc(am)}TP$,** which was not incorporated into DNA. Electrochemical measurements of the labeled nucleosides showed distinct oxidation signals resulting from reversible one-electron Fe^{II}/ Fe^{III} redox process. Fluoro- and dimethylcarbamoyl groups on the cyclopentadienyl ring of ferrocene in modified nucleosides led to formation of oxidation peaks in more positive potential in comparison to nucleosides modified with ethynyl ferrocene. However use of these labels is limited because of the difficulties with the synthesis of corresponding triphosphates and their further use for enzymatic incorporation. Presence of eight methyl groups in ferrocene label resulted in oxidation peak in less positive potential for corresponding nucleosides, but application of **$dN^{X}TPs$** with octamethylferrocene label can be limited because of partial oxidation of ferrocene moiety during the synthesis. Further electrochemical measurements of Fc-modified dNTPs and DNA will be performed in near future in collaboration with Fojta group for studying the potential use of new nucleoside triphosphates in combination with other redox labels to form an orthogonal set of four tags for the electrochemical detection of short DNA sequences.

4 Conclusions

In the first part of my thesis, 2,3-dihydrobenzofuran and 2-methoxyphenol modified nucleosides and nucleoside triphosphates were synthesized by Suzuki-Miyaura cross-coupling reactions. Obtained modified dNTPs were incorporated into DNA by primer extension experiments using KOD XL or Pwo polymerases. MOP and DHB-labeled nucleoside triphosphates were also tested as substrates for PCR. Electrochemical behavior of MOP and DHB groups was studied on nucleosides, nucleotides and DNA using the square-wave voltammetry. Oxidation of DHB label results in formation of peak around 0.85 V close to oxidation of natural purine nucleobases, which makes it difficult to use in combination with other electrochemically active groups. MOP label, however, gives an analytically useful signal of oxidation at 0.5 V, therefore MOP moiety was used for for multipotential DNA coding with aminophenyl oxidizable label or reducible benzofurazane label. Electrochemical analysis of PEX product containing combination of two oxidizable MOP and aminophenyl labels gave two independently readable peaks at potentials of 550 mV and 830 mV that corresponded to oxidation of MOP group and aminophenyl moiety respectively. Electrochemical studies of modified DNAs bearing oxidizable MOP moiety in combination with irreversibly reducible benzofurazane label were performed using mercury electrode for detection of benzofurazane group and then graphite electrode for electrochemical oxidation of MOP label.

In the second part, phenothiazine was attached to nucleosides and dNTPs either directly or through acetylene linker by Suzuki-Miyaura or Sonogashira cross-coupling reactions. The incorporation of PT-modified dNTPs into DNA by primer extension experiment was performed with using of KOD XL, Pwo or Vent (*exo*-) polymerases. PCR reactions with PT-labeled nucleotides did not give a significant amount of product that can be explained by difficulty for enzyme to read through the PT-modified templates. Phenothiazine-labeled nucleoside triphosphates were also tested as substrates for nicking enzyme amplification reaction. Different templates were designed for the synthesis of short ON containing either one, two or four modifications. The enzymatic synthesis by terminal deoxynucleotidyl transferase yielded product of single nucleotide incorporation, that can be potentially used for specific elongation of 3'-end of ON with one PT redox label for diagnostics. Modified nucleosides and dNTPs containing PT group through the acetylene linker also exhibited fluorescence properties. Electrochemical measurements by cyclic (CV) and square-wave voltammetry at the pyrolytic graphite electrode showed that the phenothiazine moiety is a useful redox label for nucleosides and DNA giving two anodic peaks of PT oxidation, where the first PT signal in the region 660 mV is reversibile and the second oxidation step in the region 860 mV is irreversibile, that can be

used for identification of the PT label among other oxidizable moieties. PT moiety was also studied as a label for multipotential coding of DNA bases in combination with benzofurazane or nitrophenyl moiety. Electrochemical studies revealed that directly linked $\mathbf{A^{PT}}$ conjugate is more useful label for "multicolor" redox coding of DNA bases, because it produces two distinct peaks, while in case of ethynyl linked $\mathbf{A^{EPT}}$ base the second oxidation peak overlaps with oxidation signals of natural purine bases. Electrochemical measurements of PT redox label with previously reported benzofurazane and nitrophenyl groups showed the orthogonal behavior of PT moiety, therefore it can be used for "multicolor" redox coding of DNA bases. From another side, the presence of two anodic peaks of PT oxidation constrains the potential use of the last (fourth) redox label in the limited potential window of carbon electrode.

In the last part of my thesis, new substituted ferrocene (Fc) derivatives with electron donating and electron withdrawing groups were studied as new redox labels with tunable redox potential. At first, new ethynylferrocenes with electron withdrawing groups, 1-(N,N-dimethylaminocarbonyl)-1'-ethynylferrocene and 1-fluoro-1'-ethynylferrocene, were designed and synthesized for further use as building blocks for the synthesis of modified nucleosides and nucleoside triphosphates. These new derivatives, as well as known propargylamidoferrocene, were used for Sonogashira cross-coupling reactions with halogenated nucleosides. As an example of ferrocene label with electron donating group, previously reported 1-ethynyl-1',2,2',3,3',4,4',5-octamethylferrocene was also prepared for further synthesis of modified nucleosides and nucleoside triphosphates. Modified nucleosides bearing Fc-labels were obtained in high yields. Corresponding modified $\mathbf{dN^{X}TPs}$ were synthesized with octamethylferrocene and propargylamideferrocene labels in moderate yields, however partial oxidation of ferrocene moiety during the synthesis was observed in case of $\mathbf{dA^{FcM}TP}$ and $\mathbf{dC^{FcM}TP}$ because the influence of methyl groups on ferrocene label. At the same time synthesis of nucleoside triphosphates with fluoroferrocene and dimethylcarbamoylferrocene was not successful, that limits use of corresponding labels for orthogonal coding of DNA bases. The obtained nucleotides bearing modified Fc labels ($\mathbf{dA^{FcPA}TP}$, $\mathbf{dC^{FcPA}TP}$, $\mathbf{dA^{FcM}TP}$ and $\mathbf{dC^{FcM}TP}$) were revealed as good substrates for enzymatic synthesis of modified DNA by primer extension experiments. Electrochemical measurements of the labeled nucleosides were studied by SWV at the basal-plane pyrolytic graphite electrode. Oxidation signals resulting from reversible one-electron Fe^{II}/ Fe^{III} redox process were observed in more positive potential for modified nucleosides bearing ferrocenes with electron withdrawing groups, whereas electron donating groups tuned the oxidation signal to less positive values. Further electrochemical measurements

and applications of Fc-modified nucleoside triphosphates and DNA will be performed in near future in collaboration with Fojta group.

5 List of publications

1) A. Simonova, J. Balintová, R. Pohl, L. Havran, M. Fojta, M. Hocek: "Methoxyphenol and dihydrobenzofuran as new oxidizable labels for electrochemical detection of DNA", *ChemPlusChem* **2014**, *79*, 1703 – 1712.

2) A.Simonova, L. Havran, R. Pohl, M. Fojta, M. Hocek: "Phenothiazine-linked nucleosides and nucleotides for redox labelling of DNA", *Org. Biomol. Chem.* **2017**, *15*, 6984 – 6996.

3) J. Balintová, A. Simonova, M. Białek-Pietras, A. Olejniczak, Z. J. Lesnikowski, M. Hocek: "Carborane-linked 2'-deoxyuridine 5'-O-triphosphate as building block for polymerase synthesis of carborane-modified DNA", *Bioorg. Med. Chem.* Lett. **2017**, *27*, 4786 - 4788.

4) A Daňhel, Z. Trošanová, J. Balintová, A. Simonova, L. Pospíšil, J. Cvačka, M. Hocek,M. Fojta: "Electrochemical reduction of azidophenyl-deoxynucleoside conjugates at mercury surface", *Electrochim. Acta* **2018**, *259*, 377 - 385.

6 Experimental section

General chemistry:

All cross-coupling reactions were performed under argon atmosphere. Compounds **dC^{I}TP**[107], **dA^{NH2}TP**[107], **dU^{NO2}TP**[107], **dA^{BF}TP**[122], **dC^{EBF}TP**[122], **PT-Bpin**[140], **EPT**[141], **dA^{Fc}**160, **dC^{Fc}TP**[160], **dA^{Fc}TP**[160], **dA^{I}TP**[176] were prepared according to the literature procedures. Other chemicals were purchased from commercial suppliers and were used as received. NMR spectra were recorded on a 400 (400.0 MHz for ^{1}H, 162 MHz for ^{31}P, 100 MHz for ^{13}C) or a 500 (500 MHz for ^{1}H, 125.7 MHz for ^{13}C, 470.4 MHz for ^{19}F and 202.3 for ^{31}P) or a 600 (600.1 MHz for ^{1}H, 150.9 MHz for ^{13}C) spectrometers from sample solutions in D_2O, DMSO-*d_6*, CD_3CN or CD_3OD. Chemical shifts (in ppm, δ scale) were referenced as follows: D_2O (referenced to dioxane as internal standard; 3.75 ppm for ^{1}H NMR and 69.3 ppm for ^{13}C NMR); CD_3OD (referenced to solvent signal: 3.31 ppm for ^{1}H NMR and 49.00 ppm for ^{13}C NMR); DMSO-*d6* (referenced to solvent signal: 2.50 ppm for ^{1}H NMR and 39.7 ppm for ^{13}C NMR); CD_3CN (referenced to solvent signal: 1.94 ppm for ^{1}H NMR and 1.32 ppm for ^{13}C NMR). ^{31}P chemical shifts were referenced to H_3PO_4 as external reference or to phosphate buffer signal 2.35 ppm in the case of measurement in phosphate buffer (pH 7.1). Chemical shifts are given in ppm (δ scale), coupling constants (*J*) in Hz. Complete assignment of all NMR signals was achieved by using a combination of H,H-COSY, H,C-HSQC, and H,C-HMBC experiments. Mass spectra were measured on LCQ classic (Thermo-Finnigan) spectrometer using ESI or Q-Tof Micro (Waters, ESI source, internal calibration with lockspray). Preparative HPLC separations were performed on a column packed with 10 μm C18 reversed phase (Phenomenex, Luna C18(2)). IR spectra were measured either on Bruker Alpha FT-IR spectrometer using ATR technique or by using KBr tabletes. High resolution mass spectra were measured on a LTQ Orbitrap XL (Hermo Fischer Scientific) spectrometer using ESI ionization technique. Mass spectra of functionalized DNA were measured by Maldi-TOF, Reflex IV (Bruker) with nitrogen laser. UV-Vis spectra were measured on a Varian CARY 100. Fluorescence spectra were measured on a Fluoromax 4 spectrofluorimeter (HORIBA Scientific).

Materials for biochemistry:

Synthetic oligonucleotides (ONs) and unmodified nucleoside triphosphates (dATP, dTTP, dCTP, and dGTP) were purchased from Sigma; Dynabeads M-270 Streptavidin (DBStv) were obtained from Dynal A.S. (Norway); Pwo, Vent (exo-) polymerases, Nt.BstNBI restriction endonuclease and terminal transferase (TdT) were purchased from New England Biolabs (Great Britain); KOD XL DNA from Novagen; and γ-^{32}P-ATP from MP Empowered Discovery (USA). Other chemicals were of analytical grade.

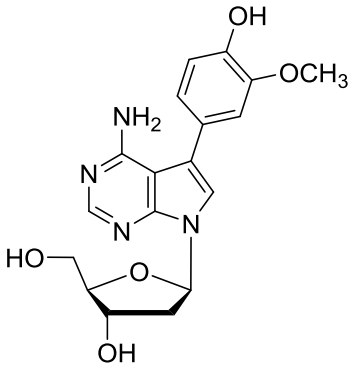
6.1 MOP and DHB-labeled nucleosides and dNTPs. Synthesis, enzymatic incorporation, and electrochemical detection

6.1.1 Synthesis of MOP and DHB-labeled nucleosides

Suzuki-Miyaura cross-coupling reaction for the synthesis of modified nucleosides:

Method A: A 2:1 mixture of H_2O-CH_3CN (2 mL) was added through a septum to an argon-purged flask containing halogenated nucleosides **dN^{I}** (1 equiv.), boronic acid/boronate (2 equiv.) and Cs_2CO_3 (3 equiv). In a separate flask $Pd(OAc)_2$ (10 mol-%), and TPPTS (2.5 equiv. with respect to Pd) were combined, the flask was evacuated and purged with argon, and then a 2:1 mixture of H_2O-CH_3CN (1 mL) was added. This catalyst solution was injected into the reaction mixture, which was then stirred at 75ºC for 1 h until complete consumption of the starting material and then evaporated in vacuo. The products were purified by silica gel column chromatography using chloroform/methanol (0 to 10%) as eluent.

7-(4-hydroxy-3-methoxyphenyl)-7-deaza-2'-deoxyadenosine (dA^{MOP})

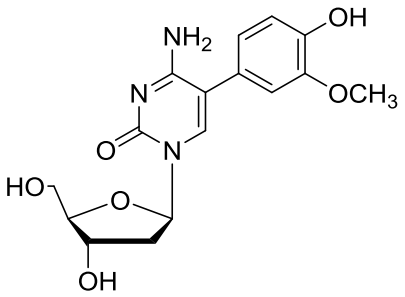


Compound **dA^{MOP}** was prepared from **dA^{I}** according to general procedure (Method A). The product was isolated as a yellow solid (38 mg, 77%); m.p. 90 ºC; ^{1}H NMR (500.0 MHz, DMSO-d_6): 2.18 (ddd, 1H, J_{gem} = 13.1, $J_{2'b,1'}$ = 6.0, $J_{2'b,3'}$ = 2.6, H-2'b); 2.56 (ddd, 1H, J_{gem} = 13.1, $J_{2'a,1'}$ = 8.3, $J_{2'a,3'}$ = 5.7, H-2'a); 3.50, 3.57 (2 × ddd, 2 × 1H, J_{gem} = 11.7, $J_{5',OH}$ = 5.3, $J_{5',4'}$ = 4.4, H-5'); 3.81 (s, 3H, CH_3O); 3.82 (td, 1H, $J_{4',5'}$ = 4.4, $J_{4',3'}$ = 2.5, H-4'); 4.35 (bm, 1H, H-3'); 5.07 (bt, 1H, $J_{OH,5'}$ = 5.3, OH-5'); 5.27 (bd, 1H, $J_{OH,3'}$ = 2.9, OH-3'); 6.12 (bs, 2H, NH_2); 6.57 (dd, 1H, $J_{1',2'}$ = 8.3, 6.0, H-1'); 6.84 (dd, 1H, $J_{6,5}$ = 8.0, $J_{6,2}$ = 1.9, H-6-C_6H_3OHOMe); 6.87 (d, 1H, $J_{5,6}$ = 8.0, H-5-C_6H_3OHOMe); 6.98 (d, 1H, $J_{2,6}$ = 1.9, H-2-C_6H_3OHOMe); 7.41 (s, 1H, H-6); 8.12 (s, 1H, H-2); 9.14 (bs, 1H, OH-4-C_6H_3OHOMe).

^{13}C NMR (125.7 MHz, DMSO-d_6): 39.53 (CH_2-2'); 55.76 (CH_3O); 62.20 (CH_2-5'); 71.25 (CH-3'); 83.06 (CH-1'); 87.49 (CH-4'); 100.79 (C-4a); 113.02 (CH-2-C_6H_3OHOMe); 116.04 (CH-5-C_6H_3OHOMe); 116.86 (C-5); 120.03 (CH-6); 121.08 (CH-6-C_6H_3OHOMe); 125.57 (C-1-C_6H_3OHOMe); 146.03 (C-4-C_6H_3OHOMe); 147.96 (C-3-C_6H_3OHOMe); 150.34 (C-7a); 151.77 (CH-2); 157.48 (C-4); ν(KBr)/cm^{-1}: 3497, 3385, 2968, 2865, 1735, 1630, 1470, 1291, 1097, 1052;

MS (ESI+): *m/z* (%): 373.2 (100) [M+H]; 395.2 (54) [M+Na]; HRMS (ESI+): calcd. 373.15060 for $C_{18}H_{21}N_4O_5$, found 373.15065; calcd 395.13254 for $C_{18}H_{20}N_4O_5Na$, found 395.3259

5-(4-hydroxy-3-methoxyphenyl)- 2'-deoxycytidine (dC^{MOP})

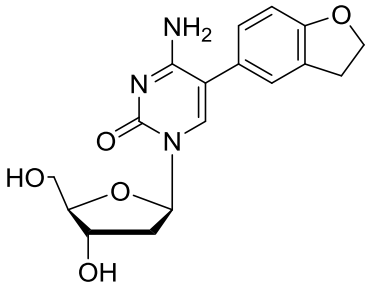


Compound **dC^{MOP}** was prepared from **dC^{I}** according to general procedure (Method A). The product was isolated as a yellow solid (48 mg, 83%); m.p. 204 ºC; ^{1}H NMR (499.8 MHz, DMSO-d_6): 2.12 (m, 2H, H-2'); 3.50, 3.57 (2 × ddd, 2 × 1H, J_{gem} = 11.7, $J_{5',OH}$ = 5.1, $J_{5',4'}$ = 3.4, H-5'); 3.77 (q, 1H, $J_{4',3'}$ = $J_{4',5'}$ = 3.4, H-4'); 3.78 (s, 3H, CH_3O); 4.22 (m, 1H, H-3'); 4.96 (t, 1H, $J_{OH,5'}$ = 5.1, OH-5'); 5.19 (d, 1H, $J_{OH,3'}$ = 4.2, OH-3'); 6.20 (dd, 1H, $J_{1',2'}$ = 7.1, 6.1, H-1'); 6.35 (bs, 1H, NH_aH_b); 6.70 (dd, 1H, $J_{6,5}$ = 8.0, $J_{6,2}$ = 2.0, H-6-C_6H_3OHOMe); 6.81 (d, 1H, $J_{5,6}$ = 8.0, H-5-C_6H_3OHOMe); 6.83 (d, 1H, $J_{2,6}$ = 2.0, H-2-C_6H_3OHOMe); 7.35 (bs, 1H, NH_aH_b); 7.79 (s, 1H, H-6); 9.10 (bs, 1H, OH-4-C_6H_3OHOMe);

^{13}C NMR (125.7 MHz, DMSO-d_6): 40.75 (CH_2-2'); 55.60 (CH_3O); 61.25 (CH_2-5'); 70.37 (CH-3'); 85.20 (CH-1'); 87.41 (CH-4'); 108.11 (C-5); 113.00 (CH-2-C_6H_3OHOMe); 115.98 (CH-5-C_6H_3OHOMe); 121.40 (CH-6-C_6H_3OHOMe); 124.75 (C-1-C_6H_3OHOMe); 139.64 (CH-6); 146.41 (C-4-C_6H_3OHOMe); 147.86 (C-3-C_6H_3OHOMe); 154.52 (C-2); 163.73 (C-4); ν(KBr)/cm^{-1}: 3305, 2928, 2859, 1744, 1642, 1601, 1473, 1280, 1092, 1059;

MS (ESI+): *m/z* (%): 350.2 (20) [M+H]; HRMS (ESI+): calcd. 350.13466 for $C_{16}H_{20}N_3O_6$, found 350.13466.

5-(2,3-dihydrobenzofuran-5-yl)- 2'-deoxycytidine (dC^{DHB})



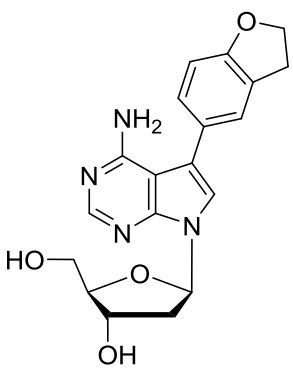
Compound **dC^{DHB}** was prepared from **dC^{I}** according to general procedure (Method A). The product was isolated as a dark purple solid (20 mg, 69%); m.p. 178 ºC; ^{1}H NMR (500.0 MHz, DMSO-d_6): 2.05 (ddd, 1H, J_{gem} = 13.1, $J_{2'b,1'}$ = 7.1, $J_{2'b,3'}$ = 6.1, H-2'b); 2.13 (ddd, 1H, J_{gem} = 13.1, $J_{2'a,1'}$ = 6.1, $J_{2'a,3'}$ = 3.5, H-2'a); 3.19 (t, 2H, $J_{3,2}$ = 8.7, H-3-dihydrobenzofuryl); 3.50, 3.55 (2 × ddd, 2 × 1H, J_{gem} = 11.8, $J_{5',OH}$ = 5.0, $J_{5',4'}$ = 3.5, H-5'); 3.76 (q, 1H, $J_{4',3'}$ = $J_{4',5'}$ = 3.5, H-4'); 4.21 (m, 1H, H-3'); 4.54 (t, 2H, $J_{2,3}$ = 8.7, H-2-dihydrobenzofuryl); 4.94 (t, 1H, $J_{OH,5'}$ = 5.0, OH-5'); 5.19 (d, 1H, $J_{OH,3'}$ = 4.2, OH-3'); 6.20 (dd, 1H, $J_{1',2'}$ = 7.1, 6.1, H-1'); 6.24 (bs, 1H, NH_aH_b); 6.79 (d, 1H,

$J_{7,6}$ = 8.2, H-7-dihydrobenzofuryl); 7.01 (dd, 1H, $J_{6,7}$ = 8.2, $J_{6,4}$ = 2.0, H-6-dihydrobenzofuryl); 7.16 (d, 1H, $J_{4,6}$ = 2.0, H-4-dihydrobenzofuryl); 7.34 (bs, 1H, NH_aH_b); 7.75 (s, 1H, H-6);

^{13}C NMR (125.7 MHz, DMSO-d_6): 29.31 (CH_2-3-dihydrobenzofuryl); 40.70 (CH_2-2'); 61.27 (CH_2-5'); 70.41 (CH-3'); 71.30 (CH_2-2-dihydrobenzofuryl); 85.12 (CH-1'); 87.36 (CH-4'); 108.04 (C-5); 109.43 (CH-7-dihydrobenzofuryl); 125.95 (C-5-dihydrobenzofuryl); 126.08 (CH-4-dihydrobenzofuryl); 128.24 (C-3a-dihydrobenzofuryl); 128.86 (CH-6-dihydrobenzofuryl); 139.58 (CH-6); 154.72 (C-2); 159.53 (C-7a-dihydrobenzofuryl); 163.94 (C-4);); ν(KBr)/cm^{-1}: 3410, 2929, 2860, 1649, 1601, 1475, 1233,1192, 1094, 1053;

MS (ESI+): *m/z* (%): 346.1 (35) [M+H]; 368.1 (100) [M+Na]; HRMS (ESI+): calcd. 346.13976 for $C_{17}H_{20}O_5N_3$, found 346.13975; calcd 368.12157 for $C_{17}H_{19}O_5N_3Na$, found 368.12169

7-(2,3-dihydrobenzofuran-5-yl)-7-deaza-2'-deoxyadenosine (dA^{DHB})



Compound **dA^{DHB}** was prepared from **dA^{I}** according to general procedure (Method A). The product was isolated as a weak yellow solid (38 mg, 78%); m.p. 107 ºC; ^{1}H NMR (499.8 MHz, DMSO-d_6): 2.18 (ddd, 1H, J_{gem} = 13.1, $J_{2'b,1'}$ = 6.0, $J_{2'b,3'}$ = 2.6, H-2'b); 2.55 (ddd, 1H, J_{gem} = 13.1, $J_{2'a,1'}$ = 8.4, $J_{2'a,3'}$ = 5.8, H-2'a); 3.23 (t, 2H, $J_{3,2}$ = 8.7, H-3-dihydrobenzofuryl); 3.50, 3.57 (2 × ddd, 2 × 1H, J_{gem} = 11.7, $J_{5',OH}$ = 5.5, $J_{5',4'}$ = 4.5, H-5'); 3.82 (td, 1H, $J_{4',5'}$ = 4.5, $J_{4',3'}$ = 2.4, H-4'); 4.35 (bm, 1H, H-3'); 4.57 (t, 2H, $J_{2,3}$ = 8.7, H-2-dihydrobenzofuryl); 5.05 (t, 1H, $J_{OH,5'}$ = 5.5, OH-5'); 5.25 (d, 1H, $J_{OH,3'}$ = 4.1, OH-3'); 6.10 (bs, 2H, NH_2); 6.57 (dd, 1H, $J_{1',2'}$ = 8.4, 6.0, H-1'); 6.86 (dd, 1H, $J_{7,6}$ = 8.1, $J_{7,4}$ = 0.5, H-7-dihydrobenzofuryl); 7.16 (ddt, 1H, $J_{6,7}$ = 8.1, $J_{6,4}$ = 2.0, $J_{6,3}$ = 0.7, H-6-dihydrobenzofuryl); 7.31 (dtd, 1H, $J_{4,6}$ = 2.0, $J_{4,3}$ = 1.1, $J_{4,7}$ = 0.5, H-4-dihydrobenzofuryl); 7.40 (s, 1H, H-6); 8.12 (s, 1H, H-2);

^{13}C NMR (125.7 MHz, DMSO-d_6): 29.33 (CH_2-3-dihydrobenzofuryl); 39.82 (CH_2-2'); 62.21 (CH_2-5'); 71.27 (CH_2-2-dihydrobenzofuryl); 71.28 (CH-3'); 83.06 (CH-1'); 87.50 (CH-4'); 100.77 (C-4a); 109.35 (CH-7-dihydrobenzofuryl); 116.70 (C-5); 120.11 (CH-6); 125.64 (CH-4-dihydrobenzofuryl); 126.65 (C-5-dihydrobenzofuryl); 128.34 (C-3a-dihydrobenzofuryl); 128.39 (CH-6-dihydrobenzofuryl); 150.35 (C-7a); 151.73 (CH-2); 157.44 (C-4); 159.17 (C-7a-dihydrobenzofuryl); ν(KBr)/cm^{-1}: 3332, 3191, 2929, 2860,1493, 1300, 1223, 1093, 1050;

MS (ESI+): *m/z* (%):369.2 (100) [M+H]; HRMS (ESI+): calcd. 369.15573 for $C_{19}H_{21}N_4O_4$; found 369.15575.

6.1.2 Synthesis of MOP- and DHB-labeled dNTPs

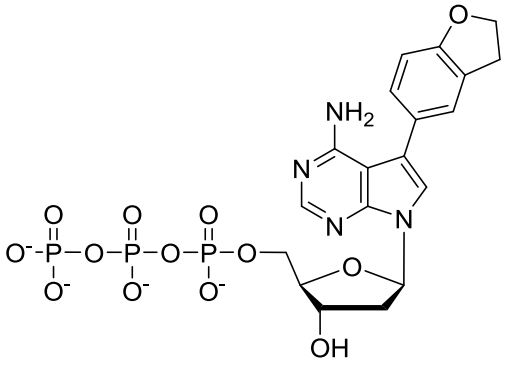
Suzuki-Miyaura cross-coupling reaction for the synthesis of modified nucleoside triphosphates:

Method A: A 2:1 mixture of H_2O-CH_3CN (1 mL) was added through a septum to an argon-purged flask containing halogenated nucleotides **dN^{I}TP** (1 equiv.), boronic acid/boronate (2 equiv.) and Cs_2CO_3 (3 equiv). In a separate flask $Pd(OAc)_2$ (10 mol-%), and TPPTS (2.5 equiv. with respect to Pd) were combined, the flask was evacuated and purged with argon, and then a 2:1 mixture of H_2O-CH_3CN (0.5 mL) was added. This catalyst solution was injected into the reaction mixture, which was then stirred at 50ºC for 40 min until complete consumption of the starting material and then evaporated in vacuo. Product was isolated from the crude reaction mixture by HPLC on a C18 column with the use of a linear gradient of 0.1 M TEAB (triethylammonium bicarbonate) in H_2O to 0.1 M TEAB in H_2O–MeOH (1 : 1) as eluent. Several co-distillations with water and conversion to sodium salt form (Dowex 50WX8 in Na^+ cycle) followed by freeze-drying from water gave solid product.

Synthesis of modified nucleoside triphosphates - triphosphorylation:

Method B: $POCl_3$ (1.2 equiv) in $PO(OMe)_3$ was added through a septum to an argon-purged flask containing modified nucleosides **dN^{X}** (1 equiv.). Reaction mixture was then stirred at 0ºC for 3 h until complete consumption of the starting material. Then an ice-cooled solution of $(NHBu_3)_2H_2P_2O_7$ (5 equiv) and Bu_3N (4.2 equiv) in dry DMF (2 ml) was added and the mixture was stirred at 0°C for another 1.5 h. The reaction was quenched by addition of 2 M aqueous TEAB (2 ml) and the solvents were evaporated in vacuo and the residue was co-distilled with water three times. The product was isolated by HPLC on a C18 column with the use of a linear gradient of 0.1 M TEAB (triethylammonium bicarbonate) in H_2O to 0.1 M TEAB in H_2O–MeOH (1 : 1) as eluent. Several co-distillations with water and conversion to sodium salt form (Dowex 50WX8 in Na^+ cycle) followed by freeze-drying from water gave solid product.

7-(2,3-dihydrobenzofuran-5-yl)-7-deaza-2'-deoxyadenosine 5'-*O*-triphosphate (dA^{DHB}TP)



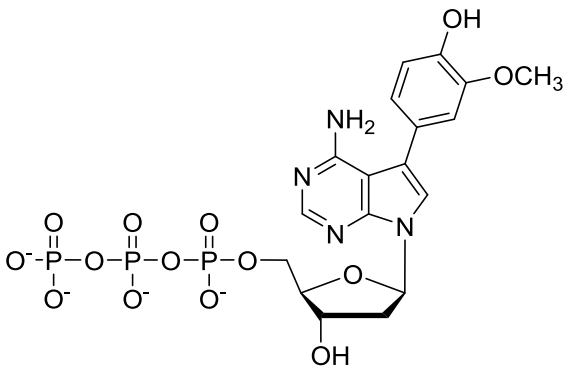
Compound **dA^{DHB}TP** was prepared from **dA^{I}TP** according to general procedure (Method A). The product was isolated as a purple solid (14 mg, 42%); ^{1}H NMR (499.8 MHz, D_2O, pD = 7.1, phosphate buffer, ref(dioxane) = 3.75 ppm): 2.46 (ddd, 1H, J_{gem} = 14.0, $J_{2'b,1'}$ = 6.2, $J_{2'b,3'}$ = 3.2, H-2'b); 2.72 (ddd, 1H, J_{gem} = 14.0, $J_{2'a,1'}$ = 7.8, $J_{2'a,3'}$ = 6.6, H-2'a); 3.76 (t, 2H, $J_{3,2}$ = 8.7, H-3-dihydrobenzofuryl); 4.11 (ddd, 1H, J_{gem} = 11.0, $J_{H,P}$ = 5.0, $J_{5'b,4'}$ = 4.4, H-5'b); 4.17 (ddd, 1H, J_{gem} = 11.0, $J_{H,P}$ = 6.4, $J_{5'a,4'}$ = 4.4, H-5'a); 4.24 (bq, 1H, $J_{4',5'}$ = $J_{4',3'}$ = 4.4, H-4'); 4.63 (t, 2H, $J_{2,3}$ = 8.7, H-2-dihydrobenzofuryl); 4.80 (m, 1H, H-3' overlapped with HDO signal); 6.66 (dd, 1H, $J_{1',2'}$ = 7.8, 6.2, H-1'); 6.90 (d, 1H, $J_{7,6}$ = 8.2, H-7-dihydrobenzofuryl); 7.24 (dd, 1H, $J_{6,7}$ = 8.2, $J_{6,4}$ = 1.7, H-6-dihydrobenzofuryl); 7.35 (d, 1H, $J_{4,6}$ = 2.0, H-4-dihydrobenzofuryl); 7.42 (s, 1H, H-6); 8.15 (s, 1H, H-2);

^{13}C NMR (125.7 MHz, D_2O, pD = 7.1, phosphate buffer, ref(dioxane) = 69.3 ppm): 31.67 (CH_2-3-dihydrobenzofuryl); 40.92 (CH_2-2'); 68.27 (d, $J_{C,P}$ = 5.5, CH_2-5'); 73.88 (CH-3'); 74.69 (CH_2-2-dihydrobenzofuryl); 85.42 (CH-1'); 87.76 (d, $J_{C,P}$ = 8.7, CH-4'); 103.88 (C-4a); 112.16 (CH-7-dihydrobenzofuryl); 121.05 (C-5); 122.29 (CH-6); 128.57 (CH-4-dihydrobenzofuryl); 128.96 (C-5-dihydrobenzofuryl); 131.27 (CH-6-dihydrobenzofuryl); 131.45 (C-3a-dihydrobenzofuryl); 152.37 (C-7a); 153.90 (CH-2); 159.88 (C-4); 161.24 (CH-7a-dihydrobenzofuryl);

^{31}P NMR (202.3 MHz, D_2O, pD = 7.1, phosphate buffer, ref(phosphate buffer) = 2.35 ppm): -21.53 (t, J = 19.6, P_b); -10.55 (d, J = 19.6, P_a); -6.48 (d, J = 19.6, P_γ).

MS (ESI+): *m/z* (%): 629.0 (8) [M+Na]; 651.0 (20) [M+2Na]; HRMS (ESI+): calcd. 607.04017 for $C_{19}H_{22}O_{13}N_4P_3$, found 607.04022; 629.02211 for $C_{19}H_{21}O_{13}N_4NaP_3$, found 629.02208

7-(4-hydroxy-3-methoxyphenyl)-7-deaza-2'-deoxyadenosine 5'-*O*-triphosphate (dA^{MOP}TP)



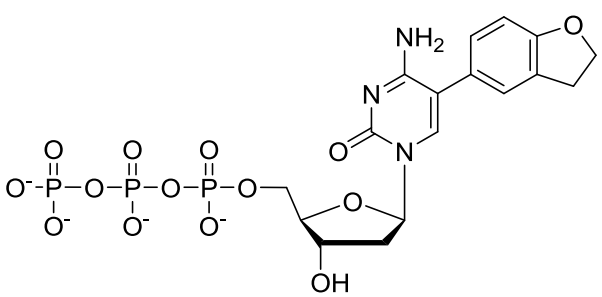
Compound **dA^{MOP}TP** was prepared from **dA^{I}TP** according to general procedure (Method A). The product was isolated as a purple solid (21 mg, 42%); ^{1}H NMR (500.0 MHz, D_2O, pD = 7.1, phosphate buffer, ref(dioxane) = 3.75 ppm): 2.46 (ddd, 1H, J_{gem} = 14.0, $J_{2'b,1'}$ = 6.2, $J_{2'b,3'}$ = 3.0, H-2'b); 2.73 (ddd, 1H, J_{gem} = 14.0, $J_{2'a,1'}$ = 8.1, $J_{2'a,3'}$ = 6.4, H-2'a); 3.88 (s, 3H, CH_3O); 4.11 (ddd, 1H, J_{gem} = 10.7, $J_{H,P}$ = 5.6, $J_{5'b,4'}$ = 4.4, H-5'b); 4.17 (ddd, 1H, J_{gem} = 10.7, $J_{H,P}$ = 6.3, $J_{5'a,4'}$ = 4.4, H-5'a); 4.24 (td, 1H, $J_{4',5'}$ = 4.4, $J_{4',3'}$ = 3.0, H-4'); 4.76 (dt, 1H, $J_{3',2'}$ = 6.4,. 3.0, $J_{3',4'}$ = 3.0, H-3'); 6.67 (dd, 1H, $J_{1',2'}$ = 8.1, 6.2, H-1'); 6.98 (dd, 1H, $J_{6,5}$ = 8.0, $J_{6,2}$ = 1.7, H-6-C_6H_3OHOMe); 7.01 (d, 1H, $J_{5,6}$ = 8.0, H-5-C_6H_3OHOMe); 7.10 (d, 1H, $J_{2,6}$ = 1.7, H-2-C_6H_3OHOMe); 7.45 (s, 1H, H-6); 8.16 (s, 1H, H-2);

^{13}C NMR (125.7 MHz, D_2O, pD = 7.1, phosphate buffer, ref(dioxane) = 69.3 ppm): 40.93 (CH_2-2'); 58.73 (CH_3O); 68.31 (d, $J_{C,P}$ = 5.5, CH_2-5'); 73.95 (CH-3'); 85.46 (CH-1'); 87.79 (d, $J_{C,P}$ = 8.9, CH-4'); 103.82 (C-4a); 115.81 (CH-2-C_6H_3OHOMe); 118.68 (CH-5-C_6H_3OHOMe); 120.89 (C-5); 122.36 (CH-6); 124.55 (CH-6-C_6H_3OHOMe); 128.93 (C-1-C_6H_3OHOMe); 147.14 (C-4-C_6H_3OHOMe); 150.38 (C-3-C_6H_3OHOMe); 152.40 (C-7a); 153.70 (CH-2); 159.86 (C-4);

^{31}P NMR (202.3 MHz, D_2O, pD = 7.1, ref(phosphate buffer) = 2.35 ppm): -21.49 (dd, J = 19.8, 19.2, P_b); -10.44 (d, J = 19.2, P_a); -6.65 (d, J = 19.8, P_γ);

MS (ESI+): *m/z* (%): 611.1 (8) [M+H]; 633.0 (20) [M+Na]; HRMS (ESI+): calcd. 611.03508 for $C_{18}H_{22}O_{14}N_4P_3$, found 611.03483; calcd 633.01703 for $C_{18}H_{21}O_{14}N_4NaP_3$, found 633.01664.

5-(2,3-dihydrobenzofuran-5-yl)- 2'-deoxycytidine 5'-*O*-triphosphate (dC^{DHB}TP)



Compound **dC^{DHB}TP** was prepared from **dC^{I}TP** according to general procedure (Method A). The product was isolated as a white solid (17 mg, 41%); ^{1}H NMR (500.0 MHz, D_2O, pD = 7.1, phosphate buffer, ref(dioxane) = 3.75 ppm): 2.35 (ddd, 1H, J_{gem} = 14.1, $J_{2'b,1'}$ = 7.5, $J_{2'b,3'}$ = 6.4, H-2'b); 2.42 (ddd, 1H, J_{gem} = 14.1, $J_{2'a,1'}$ = 6.3, $J_{2'a,3'}$ = 3.6, H-2'a); 3.28 (t, 2H, $J_{3,2}$ = 8.7, H-3-dihydrobenzofuryl); 4.15 (m, 2H, H-5'); 4.21 (m, 1H, H-4'); 4.61 (dt, 1H, $J_{3',2'}$ = 6.4,.

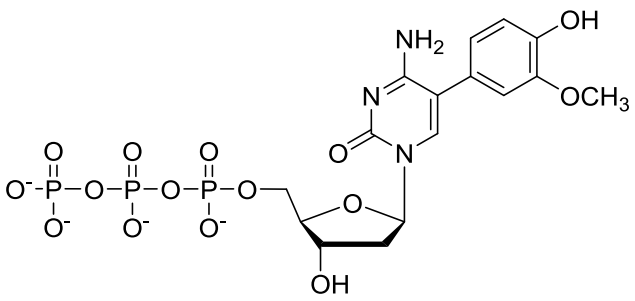
3.6, $J_{3',4'}$ = 3.6, H-3'); 4.64 (t, 2H, $J_{2,3}$ = 8.7, H-2-dihydrobenzofuryl); 6.35 (dd, 1H, $J_{1',2'}$ = 7.5, 6.3, H-1'); 6.91 (d, 1H, $J_{7,6}$ = 8.2, H-7-dihydrobenzofuryl); 7.18 (dd, 1H, $J_{6,7}$ = 8.2, $J_{6,4}$ = 2.0, H-6-dihydrobenzofuryl); 7.31 (d, 1H, $J_{4,6}$ = 2.0, H-4-dihydrobenzofuryl); 7.70 (s, 1H, H-6);

^{13}C NMR (125.7 MHz, D_2O, pD = 7.1, phosphate buffer, ref(dioxane) = 69.3 ppm): 31.62 (CH_2-3-dihydrobenzofuryl); 41.59 (CH_2-2'); 68.03 (d, $J_{C,P}$ = 5.4, CH_2-5'); 73.37 (CH-3'); 74.76 (CH_2-2-dihydrobenzofuryl); 88.16 (d, $J_{C,P}$ = 8.8, CH-4'); 88.62 (CH-1'); 112.38 (CH-7-dihydrobenzofuryl); 113.56 (C-5); 127.38 (C-5-dihydrobenzofuryl); 129.13 (CH-4-dihydrobenzofuryl); 131.68 (C-3a-dihydrobenzofuryl); 131.96 (CH-6-dihydrobenzofuryl); 142.03 (CH-6); 159.88 (C-2); 161.99 (CH-7a-dihydrobenzofuryl); 167.79 (C-4);

^{31}P NMR (202.3 MHz, D_2O, pD = 7.1, ref(phosphate buffer) = 2.35 ppm): -21.60 (t, J = 19.7, P_b); -10.74 (d, J = 19.7, P_a); -6.63 (d, J = 19.7, P_γ);

MS (ESI+): *m/z* (%): 628.0 (15) [M+2Na]; HRMS (ESI+): calcd 584.02418 for $C_{17}H_{21}O_{14}N_3P_3$, found 584.02419; calcd 606.00613 for $C_{17}H_{20}O_{14}N_3NaP_3$, found 606.00623.

5-(4-hydroxy-3-methoxyphenyl)- 2'-deoxycytidine 5'-*O*-triphosphate (dC^{MOP}TP)



Compound **dC^{MOP}TP** was prepared from **dC^{MOP}** according to general procedure (Method B). The product was isolated as a white solid (11 mg, 28%); ^{1}H NMR (500.0 MHz, D_2O, pD = 7.1, phosphate buffer, ref(dioxane) = 3.75 ppm): 2.36 (ddd, 1H, J_{gem} = 14.0, $J_{2'b,1'}$ = 7.4, $J_{2'b,3'}$ = 6.4, H-2'b); 2.42 (ddd, 1H, J_{gem} = 14.0, $J_{2'a,1'}$ = 6.4, $J_{2'a,3'}$ = 3.5, H-2'a); 3.89 (s, 3H, CH_3O); 4.15 (m, 2H, H-5'); 4.22 (td, 1H, $J_{4',5'}$ = 4.4, $J_{4',3'}$ = 3.5, H-4'); 4.60 (dt, 1H, $J_{3',2'}$ = 6.4,. 3.5, $J_{3',4'}$ = 3.5, H-3'); 6.35 (dd, 1H, $J_{1',2'}$ = 7.4, 6.4, H-1'); 6.93 (dd, 1H, $J_{6,5}$ = 8.1, $J_{6,2}$ = 2.0, H-6-C_6H_3OHOMe); 7.02 (d, 1H, $J_{5,6}$ = 8.1, H-5-C_6H_3OHOMe); 7.03 (d, 1H, $J_{2,6}$ = 2.0, H-2-C_6H_3OHOMe); 7.71 (s, 1H, H-6);

^{13}C NMR (125.7 MHz, D_2O, pD = 7.1, phosphate buffer, ref(dioxane) = 69.3 ppm): 41.59 (CH_2-2'); 58.72 (CH_3O); 68.05 (d, $J_{C,P}$ = 5.5, CH_2-5'); 73.41 (CH-3'); 88.18 (d, $J_{C,P}$ = 8.7, CH-4'); 88.70 (CH-1'); 113.36 (C-5); 116.11 (CH-2-C_6H_3OHOMe); 118.90 (CH-5-C_6H_3OHOMe); 125.22 (CH-6-C_6H_3OHOMe); 127.33 (C-1-C_6H_3OHOMe); 142.12 (CH-6); 147.97 (C-4-C_6H_3OHOMe); 150.42 (C-3-C_6H_3OHOMe); 159.88 (C-2); 167.73 (C-4);

^{31}P NMR (202.3 MHz, D_2O, pD = 7.1, ref(phosphate buffer) = 2.35 ppm): -21.57 (t, *J* = 19.6, P_b); -10.72 (d, *J* = 19.6, P_a); -6.60 (d, *J* = 19.6, P_γ).

MS (ESI+): *m/z* (%): 610.0 (15) [M+H]; 653.9 (10) [M+Na]; HRMS (ESI+): calcd. 610.00104 for $C_{16}H_{20}O_{15}N_3P_3$, found 610.00062; calcd 653.96493 for $C_{16}H_{18}O_{15}N_3Na_2P_3$, found 653.96465.

6.1.3 Analysis and isolation of PEX products

Primer extension experiment:

Single incorporation:

Method A: reaction mixture (20 µL) contained temp^A (3 µM, 1 µl), 5'-^{32}P-labeled primer^{rnd} (3 µM, 1.5 µl), dGTP (4 mM, 0.1µl), either dATP or **dA^{DHB}TP** or **dA^{MOP}TP** (4 mM, 1 µl), DNA polymerase (0.125 U KOD XL or 0.25U Pwo) and reaction buffer (2 µl) supplied by manufacturer. Primer was labeled at its 5'-end by use of [g-32P]-ATP according to standard techniques. The reaction mixture was incubated for 10 minutes at 60 °C. The PEX reaction was stopped by the addition of PAGE stop solution [40 µL, formamide (80%, v/v), ethylenediaminetetraacetic acid (EDTA, 20 mM), bromophenol blue (0.025%, w/v), xylene cyanol (0.025%,w/v) and Milli-Q water] and heated for 2 minutes at 95 °C. Samples were analyzed by use of a 12.5 % denaturizing polyacrylamide gel electrophoresis (PAGE:1 h, 50 °C) and visualized by phosphorimager (Typhoon 9410, Amersham Biosciences).

Method B: PEX reactions with temp^C were performed in the same way as described for temp^A exept either dCTP, **dC^{DHB}TP** or **dC^{MOP}TP** (4 mM, 1µl) were used.

Multiple incorporation:

Method A: the reaction mixture (20 µL) contained template (temp^{rnd16} or temp^{4A1C} or temp^{4A}, 3 µM, 1µl), 5'-^{32}P-labeled primer^{rnd} (3 µM, 1.5 µl), dNTP (either natural or modified, 4 mM, 1µl), DNA polymerase (0.125 U KOD XL, 0.25 U PWO) and reaction buffer (2 µl) supplied by manufacturer. Primer was labeled at its 5'-end by use of [g-32P]-ATP according to standard techniques. The reaction mixture was incubated for 20 minutes at 60 °C. The PEX reaction was stopped by the addition of PAGE stop solution [40 µL, formamide (80%, v/v), EDTA (20 mM), bromophenol blue (0.025%, w/v), xylene cyanol (0.025%,w/v) and Milli-Q water] and heated for 2 minutes at 95 °C. Samples were analyzed by use of a 12.5 % denaturizing polyacrylamide gel electrophoresis (1h, 50 °C) and visualized by phosphorimager (Typhoon 9410, Amersham Biosciences).

Method B: PEX reactions with temp^{4C} were performed in the same way as described for template^{rnd16} exept either 0.5 U KOD XL or 0.5 U PWO were used.

Kinetics of PEX:

Rate of incorporation was compared by preparation of samples with natural and modified dNTPs with various time periods.

Single incorporation:

PEX reaction mixture (20 µl) was performed with 5′-(FAM)-labeled $prim^{rnd}$ (3 µM, 1 µl), $temp^{termA}$ (3 µM, 1.5µl) or $temp^{termC}$ (3 µM, 1.5 µl), dNTPs (4 mM, 1 µl) with KOD XL polymerase (0.125 U) in enzyme reaction buffer (1 µL) supplied by the manufacturer by followed stopping of reaction by using of PAGE stop solution [20µL, formamide (80%, v/v), EDTA (20 mM), bromophenol blue (0.025%, w/v), xylene cyanol (0.025%,w/v) and Milli-Q water] and immidiate heating for 2 minutes at 95 °C. Samples were analyzed by use of a 12.5 % denaturizing polyacrylamide gel electrophoresis (1h, 50 °C) and visualized by a phosphorimager (Typhoon 9410, Amersham Biosciences).

Multiple incorporation:

Method A: PEX reactions with $temp^{4A1C}$ were performed in the same way as described for $temp^{termA}$ exept either 0.125 U KOD XL or 0.2 U PWO were used.

Method B: PEX reactions with $temp^{4C}$ were performed in the same way as described for $temp^{termC}$ exept either 0.25 U KOD XL or 0.5 U PWO were used.

PCR:

Method A: The PCR reaction mixture (20 µL) contained KOD XL (1,25 U), natural dNTPs (4 mM, 0.5µl), modified dNTPs (4 mM, 2µl), primers ($prim^{LT25TH}$ and $prim^{L20}$ 10 µM, 2 µL each), and a 98-mer template (1 µM, 0.5 µL, $temp^{FVL\text{-}A}$) in reaction buffer (2 µL) supplied by the manufacturer. Thirty PCR cycles were run under the following conditions: denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C, extension for 1min at 72 °C, followed by final extension step of 5 min at 72 °C. Reaction mixtures were than separated by use of a 2% agarose gel in 0.5x TBE buffer with GelRed as an intercalator. Visualization was performed by an electronic dual wave transilluminator equipped with GBox iChemi XRQ Bio imaging system (Syngene).

Method B: The PCR reaction mixture (20 µL) contained KOD XL (3,75 U), combination of modified dNTPs (**$dA^{DHB}TP$** and **$dC^{MOP}TP$** or **$dC^{DHB}TP$** and **$dA^{MOP}TP$,** 4 mM, 2µl), natural dNTPs (dTTP and dGTP, 4 mM, 1µl) primers ($prim^{LT25TH}$ and $prim^{L20}$ 10 µM, 2 µL each), and a 98-mer template (1 µM, 0.5 µL, $temp^{FVL\text{-}A}$) in reaction buffer (2 µL) supplied by the manufacturer. Thirty PCR cycles were run under the following conditions: denaturation for 1

min at 94 °C, annealing for 1,5 min at 55 °C, extension for 3min at 72 °C, followed by final extension step of 5 min at 72 °C. Reaction mixtures were than separated by use of a 2% agarose gel in 0.5x TBE buffer with GelRed as an intercalator. Visualization was performed by an electronic dual wave transilluminator equipped with GBox iChemi XRQ Bio imaging system (Syngene).

MALDI-TOF:

The MALDI-TOF spectra were measured with 1 kHz smartbeam II laser technology. The measurements were done in reflectron mode by droplet technique, with the mass range up to 30 kDa. The matrix consisted of 3-hydroxypicolinic acid (HPA)/picolinic acid (PA)/ ammonium tartrate in ratio 9/1/1. The matrix (1 μL) was applied on the target (ground steel) and dried down at room temperature. The sample (1 μL) and matrix (1 μL) were mixed and added on the top of the dried matrix preparation spot and dried at room temperature.

The reaction mixture (50 μL) contained biotinylated $temp^{rnd16}$ ($temp^{A}$ or $temp^{C}$) (100 μM, 1.6 μL), $primer^{rnd}$ (100 μM, 1.6 μL), dNTPs (4 mM, 2.6 μL), KOD XL polymerase (1.25 U, in the case of multiple incorporation of two different modified **$dN^{X}TPs$** 2.5U of KOD XL polymerase were used) in enzyme reaction buffer (5 μL) supplied by the manufacturer. The reaction mixture was incubated for 40 min at 60 °C in a thermal cycler. The reaction was stopped by cooling to 4°C.

Isolation of single-strand oligonucleotides by the DBStv magnetoseparation procedure:

Streptavidin Magnetic Particles (Roche, 50 μL) were washed with Binding buffer TEN100 (10mM Tris, 1mM EDTA, 100mM NaCl, pH 7.5; 3 × 200 μL). The reaction mixture after PEX was diluted with the Binding buffer TEN100 (50 μL), the solution was added to the prewashed magnetic beads and incubated for 30 min at 15 °C and 1400 rpm. After the incubation, the magnetic beads were collected on a magnet (DynaMag-2, Invitrogen) and the solution was discarded. The beads were washed successively with Wash buffer TEN 500 (10 mM Tris, 1mM EDTA, 500 mM NaCl, pH 7.5; 3 × 200 μL), and water (3 × 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. The product was analyzed by MALDI-TOF mass spectrometry.

6.1.4 Electrochemical analysis

Nucleosides, **$dN^{X}TPs$**, and boronic acids were analyzed by conventional in situ square-wave voltammetry (SWV). Purified PEX products were analyzed by *ex situ* (adsorptive transfer stripping) SWV. The PEX products were accumulated for 60 s from aliquots (5 μL) containing

0.2m NaCl at the surface of the working electrode (hanging mercury drop electrode or basal-plane pyrolytic graphite). The electrode was then rinsed with deionized water and placed in the electrochemical cell. SWV settings: initial potential 0.0 V, end potential +1.5 V (for oxidation) or -1.5 V (for reduction), frequency 200 Hz, amplitude 50 mV. Background electrolyte: 0.2M sodium acetate pH 5.0. All measurements were performed at room temperature using an Autolab analyzer (Eco Chemie, The Netherlands) in connection with VA-stand 663 apparatus (Metrohm, Herisau, Switzerland). The three-electrode system was used with an Ag/AgCl/3M KCl electrode as a reference and platinum wire as an auxiliary electrode. Measurements at HMDE were done after deaeration of the solution by argon purging.

6.2 PT-labeled nucleosides and dNTPs. Synthesis, enzymatic incorporation and electrochemical detection

6.2.1 Synthesis of PT-labeled nucleosides

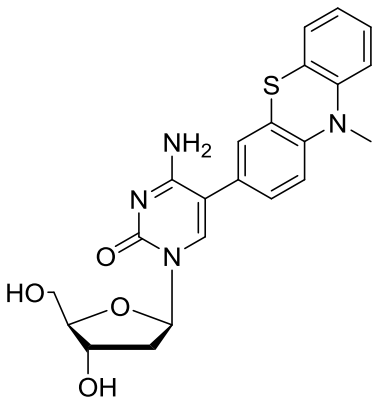
Synthesis of modified nucleosides - Suzuki-Miyaura cross-coupling:

Method A: A 1:1 mixture of H_2O-CH_3CN (2 mL) was added through a septum to an argon-purged flask containing a halogenated nucleoside **dN^{I}** (1 equiv.), **PT-Bpin** (2 equiv.) and Cs_2CO_3 (3 equiv). In a separate flask, $Pd(OAc)_2$ (10 mol-%), and TPPTS (2.5 equiv. with respect to Pd) were combined, the flask was evacuated and purged with argon, and then a 1:1 mixture of H_2O-CH_3CN (1 mL) was added. This catalyst solution was injected into the reaction mixture, which was then stirred at 50ºC for 40 min until complete consumption of the starting material and then evaporated in vacuo. The products were purified by silica gel column chromatography using chloroform/methanol (0 to 10%) as eluent.

Synthesis of modified nucleosides - Sonogashira cross-coupling:

Metod B: Dry DMF (3 mL) was added to an argon-purged flask containing **PTE** (1.5 equiv.), a nucleoside analogue **dN^{I}** (1 equiv.), CuI (10 mol-%) and $[Pd(PPh_3)_2Cl_2]$ (5 mol-%) followed by Et_3N (10 equiv.). The reaction mixture was stirred at 75°C for 1 h until complete consumption of the starting material and then evaporated in vacuo. The products were purified by silica gel column chromatography using chloroform/methanol (0 to 10%) as eluent.

5-(10-methyl-10H-phenothiazine-3-yl)-2'-deoxycytidine (dC^{PT})

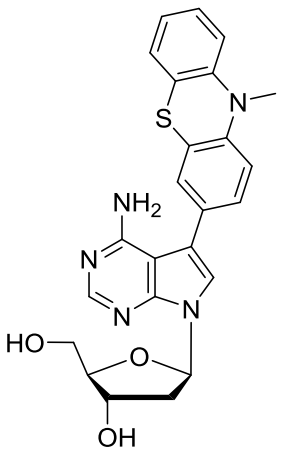


Compound **dC^{PT}** was prepared from **dC^{I}** according to general procedure (Method A). The product was isolated as a white solid (59 mg, 75%); M.p. 145°C; ^{1}H NMR (600.1 MHz, DMSO-d_6): 2.06 (ddd, 1H, J_{gem} = 13.2, $J_{2'b,1'}$ = 7.2, $J_{2'b,3'}$ = 6.1, H-2′b); 2.13 (ddd, 1H, J_{gem} = 13.2, $J_{2'a,1'}$ = 6.1, $J_{2'a,3'}$ = 3.7, H-2′a); 3.33 (s, 3H, CH_3N); 3.49, 3.54 (2 × ddd, 2 × 1H, J_{gem} = 11.8, $J_{5',OH}$ = 5.1, $J_{5',4'}$ = 3.7, H-5′); 3.76 (q, 1H, $J_{4',3'}$ = $J_{4',5'}$ = 3.7, H-4′); 4.23 (ddt, 1H, $J_{3',2'}$ = 6.1, 3.7, $J_{3',OH}$ = 4.3, $J_{3',4'}$ = 3.7, H-3′); 4.91 (t, 1H, $J_{OH,5'}$ = 5.1, OH-5′); 5.17 (d, 1H, $J_{OH,3'}$ = 4.3, OH-3′); 6.20 (dd, 1H, $J_{1',2'}$ = 7.2, 6.1, H-1′); 6.37 (bs, 1H, $NH_a\mathbf{H_b}$); 6.94 – 7.01 (m, 3H, H-1,7,9-phenothiazine); 7.09 (d, 1H, $J_{4,2}$ = 2.1, H-4-phenothiazne); 7.14 (dd, 1H, $J_{2,1}$ = 8.3, $J_{2,4}$ = 2.1, H-2-phenothiazne); 7.17 (m, 1H, H-6-phenothiazine); 7.23 (m, 1H, H-8-phenothiazne); 7.31 (bs, 1H, $N\mathbf{H_a}H_b$); 7.77 (s, 1H, H-6).

^{13}C NMR (150.9 MHz, DMSO-d_6): 35.34 (CH_3N); 40.66 (CH_2-2′); 61.23 (CH_2-5′); 70.36 (CH-3′); 85.18 (CH-1′); 87.39 (CH-4′); 106.93 (C-5); 114.77 (CH-9-phenothiazine); 114.96 (CH-1-phenothiazine); 122.17 (C-5a-phenothiazine); 122.72 (CH-7-phenothiazine); 122.77 (C-4a-phenothiazine); 127.00 (CH-6-phenothiazine); 127.46 (CH-4-phenothiazine); 127.97 (CH-8-phenothiazine); 128.26 (C-3-phenothiazine); 128.60 (CH-2-phenothiazine); 139.83 (CH-6); 144.95 (C-10a-phenothiazine); 145.29 (C-9a-phenothiazine); 154.60 (C-2); 163.66 (C-4).

MS (ESI+): *m/z* (%): 461.1 (100) [M+Na]; HRMS (ESI+): calcd. 439.14345 for $C_{22}H_{23}N_4O_4S$, found 439.14365; calcd. 461.12540 for $C_{22}H_{22}N_4O_4NaS$, found 461.12549.

7-(10-methyl-10H-phenothiazine-3-yl)-7-deaza-2'-deoxyadenosine (dA^{PT})

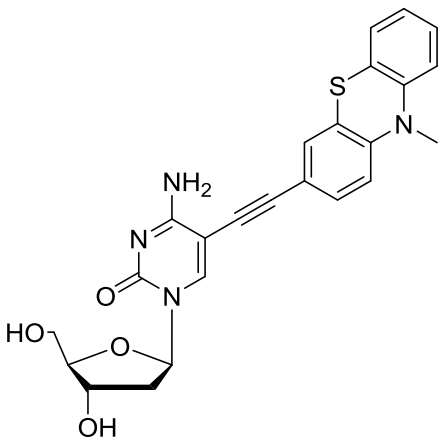


Compound **dA^{PT}** was prepared from **dA^{I}** according to general procedure (Method A). The product was isolated as a white solid (52 mg, 96%); M.p. 204°C; ^{1}H NMR (500.0 MHz, DMSO-d_6): 2.18 (ddd, 1H, J_{gem} = 13.1, $J_{2'b,1'}$ = 6.0, $J_{2'b,3'}$ = 2.6, H-2′b); 2.55 (ddd, 1H, J_{gem} = 13.1, $J_{2'a,1'}$ = 8.3, $J_{2'a,3'}$ = 5.8, H-2′a); 3.35 (s, 3H, CH_3N); 3.50 (dd, 1H, J_{gem} = 11.7, $J_{5'b,4'}$ = 4.4, H-5′b); 3.57 (dd, 1H, J_{gem} = 11.7, $J_{5'a,4'}$ = 4.7, H-5′a); 3.84 (ddd, 1H, $J_{4',5'}$ = 4.7, 4.4, $J_{4',3'}$ = 2.6, H-4′); 4.35 (dt, 1H, $J_{3',2'}$ = 5.8, 2.6, $J_{3',4'}$ = 2.6, H-3′); 5.05 (bs, 1H, OH-5′); 5.27 (bs, 1H, OH-3′); 6.17 (bs, 2H, NH_2); 6.57 (dd, 1H, $J_{1',2'}$ = 8.3, 6.0, H-1′); 6.94 – 7.02 (m, 2H, H-7,9-phenothiazine); 7.06 (d, 1H, $J_{1,2}$ = 8.3, H-1-phenothiazine); 7.18 (dd, 1H, $J_{6,7}$ = 7.5, $J_{6,8}$ = 1.5, H-6-phenothiazine); 7.24 (ddd, 1H, $J_{8,9}$ = 8.2, $J_{8,7}$ = 7.3, $J_{8,6}$ = 1.5, H-8-phenothiazne); 7.26 (d, 1H, $J_{4,2}$ = 2.0, H-4-phenothiazne); 7.28 (dd, 1H, $J_{2,1}$ = 8.3, $J_{2,4}$ = 2.0, H-2-phenothiazne); 7.50 (s, 1H, H-6); 8.14 (bs, 1H, H-2).

^{13}C NMR (125.7 MHz, DMSO-d_6): 35.39 (CH_3N); 39.81 (CH_2-2′); 62.20 (CH_2-5′); 71.25 (CH-3′); 83.06 (CH-1′); 87.54 (CH-4′); 100.55 (C-4a); 114.84 (CH-9-phenothiazine); 115.11 (CH-1-phenothiazine); 115.56 (C-5); 120.59 (CH-6); 121.96 (C-5a-phenothiazine); 122.73 (CH-7-phenothiazine); 122.92 (C-4a-phenothiazine); 126.77 (CH-2-phenothiazine); 127.06 (CH-6-phenothiazine); 127.93 (CH-4-phenothiazine); 128.05 (CH-8-phenothiazine); 128.90 (C-3-phenothiazine); 144.32 (C-10a-phenothiazine); 145.42 (C-9a-phenothiazine); 150.57 (C-7a); 151.80 (CH-2); 157.49 (C-4).

MS (ESI+): *m/z* (%): 462.1 (40) [M+H]; 484.1 (100) [M+Na]; HRMS (ESI+): calcd. 462.15944 for $C_{24}H_{24}N_5O_3S$, found 462.15934; calcd. 484.14138 for $C_{24}H_{23}O_3N_5NaS$, found 484.14141.

5-[(10-methyl-10H-phenothiazine-3-yl)ethynyl]-2'-deoxycytidine (dC^{EPT})

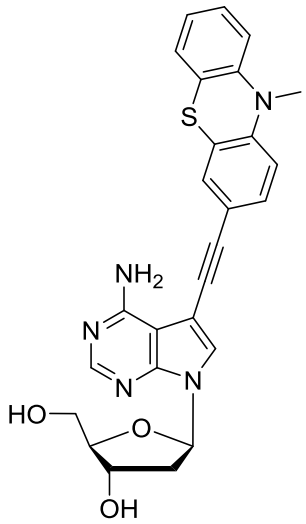


Compound **dC^{EPT}** was prepared from dC^{I} according to general procedure (Method B). The product was isolated as a yellow solid (54 mg, 76%); M.p. 143°C; ^{1}H NMR (600.1 MHz, DMSO-d_6): 2.02 (ddd, 1H, J_{gem} = 13.1, $J_{2'b,1'}$ = 7.0, $J_{2'b,3'}$ = 6.2, H-2′b); 2.17 (ddd, 1H, J_{gem} = 13.1, $J_{2'a,1'}$ = 6.0, $J_{2'a,3'}$ = 3.6, H-2′a); 3.33 (s, 3H, CH_3N); 3.58, 3.65 (2 × ddd, 2 × 1H, J_{gem} = 11.8, $J_{5',OH}$ = 5.0, $J_{5',4'}$ = 3.6, H-5′); 3.80 (q, 1H, $J_{4',3'}$ = $J_{4',5'}$ = 3.6, H-4′); 4.23 (ddt, 1H, $J_{3',2'}$ = 6.2, 3.6, $J_{3',OH}$ = 4.3, $J_{3',4'}$ = 3.6, H-3′); 5.11 (t, 1H, $J_{OH,5'}$ = 5.0, OH-5′); 5.21 (d, 1H, $J_{OH,3'}$ = 4.3, OH-3′); 6.13 (dd, 1H, $J_{1',2'}$ = 7.0, 6.0, H-1′); 6.95 (d, 1H, $J_{1,2}$ = 8.5, H-1-phenothiazine); 6.97 – 7.00 (m, 2H, H-7,9-phenothiazine); 7.01 (bs, 1H, NH_a**H_b**); 7.17 (dd, 1H, $J_{6,7}$ = 7.8, $J_{6,8}$ = 1.5, H-6-phenothiazine); 7.23 (ddd, 1H, $J_{8,9}$ = 8.2, $J_{8,7}$ = 7.4, $J_{8,6}$ = 1.5, H-8-phenothiazne); 7.41 (dd, 1H, $J_{2,1}$ = 8.5, $J_{2,4}$ = 2.0, H-2-phenothiazne); 7.44 (d, 1H, $J_{4,2}$ = 2.0, H-4-phenothiazne); 7.76 (bs, 1H, N**H_a**H_b); 8.27 (s, 1H, H-6).

^{13}C NMR (150.9 MHz, DMSO-d_6): 35.44 (CH_3N); 41.00 (CH_2-2′); 61.08 (CH_2-5′); 70.13 (CH-3′); 81.64 (C5-**C**≡C-phenothiazine); 85.55 (CH-1′); 87.58 (CH-4′); 89.90 (C-5); 93.30 (C5-C≡**C**-phenothiazine); 114.63 (CH-1-phenothiazine); 115.08 (CH-9-phenothiazine); 116.50 (C-3-phenothiazine); 121.54 (C-5a-phenothiazine); 122.28 (C-4a-phenothiazine); 123.04 (CH-7-phenothiazine); 127.02 (CH-6-phenothiazine); 128.11 (CH-8-phenothiazine); 129.32 (CH-4-phenothiazine); 130.99 (CH-2-phenothiazine); 144.59 (CH-6); 144.82 (C-9a-phenothiazine); 145.46 (C-10a-phenothiazine); 153.53 (C-2); 163.91 (C-4).

MS (ESI-): *m/z* (%):461.1 (100) [M-H]; HRMS (ESI-): calcd. 461.12890 for $C_{24}H_{21}N_4O_4S$, found 461.12835.

7-[(10-methyl-10H-phenothiazine-3-yl)ethynyl]-7-deaza-2'-deoxyadenosine (dA^{EPT})



Compound **dA^{EPT}** was prepared from **dA^{I}** according to general procedure (Method B). The product was isolated as a yellow solid (50 mg, 93%); M.p. 208°C; ^{1}H NMR (600.1 MHz, DMSO-d_6): 2.20 (ddd, 1H, J_{gem} = 13.1, $J_{2'b,1'}$ = 6.0, $J_{2'b,3'}$ = 2.8, H-2′b); 2.49 (ddd, 1H, J_{gem} = 13.1, $J_{2'a,1'}$ = 8.0, $J_{2'a,3'}$ = 5.7, H-2′a); 3.34 (s, 3H, CH_3N); 3.53 (ddd, 1H, J_{gem} = 11.8, $J_{5'b,OH}$ = 5.9, $J_{5'b,4'}$ = 4.4, H-5′b); 3.59 (ddd, 1H, J_{gem} = 11.8, $J_{5'a,OH}$ = 5.3, $J_{5'a,4'}$ = 4.4, H-5′a); 3.84 (td, 1H, $J_{4',5'}$ = 4.4, $J_{4',3'}$ = 2.5, H-4′); 4.35 (dddd, 1H, $J_{3',2'}$ = 5.7, 2.8, $J_{3',OH}$ = 4.1, $J_{3',4'}$ = 2.5, H-3′); 5.06 (dd, 1H, $J_{OH,5'}$ = 5.9, 5.3, OH-5′); 5.27 (d, 1H, $J_{OH,3'}$ = 4.1, OH-3′); 6.51 (dd, 1H, $J_{1',2'}$ = 8.0, 6.0, H-1′); 6.69 (bs, 2H, NH_2); 6.95 – 7.01 (m, 3H, H-1,7,9-phenothiazine); 7.17 (dd, 1H, $J_{6,7}$ = 7.8, $J_{6,8}$ = 1.5, H-6-phenothiazine); 7.24 (ddd, 1H, $J_{8,9}$ = 8.2, $J_{8,7}$ = 7.3, $J_{8,6}$ = 1.5, H-8-phenothiazne); 7.40 (d, 1H, $J_{4,2}$ = 2.0, H-4-phenothiazne); 7.42 (dd, 1H, $J_{2,1}$ = 8.4, $J_{2,4}$ = 2.0, H-2-phenothiazne); 7.83 (s, 1H, H-6); 8.20 (bs, 1H, H-2).

^{13}C NMR (150.9 MHz, DMSO-d_6): 35.43 (CH_3N); 40.04 (CH_2-2′); 62.05 (CH_2-5′); 71.12 (CH-3′); 82.97 (C5-**C**≡C-phenothiazine); 83.35 (CH-1′); 87.71 (CH-4′); 90.57 (C5-C≡**C**-phenothiazine); 95.18 (C-5); 102.50 (C-4a); 114.79 (CH-1-phenothiazine); 115.07 (CH-9-phenothiazine); 116.50 (C-3-phenothiazine); 121.56 (C-5a-phenothiazine); 122.54 (C-4a-phenothiazine); 123.05 (CH-7-phenothiazine); 126.64 (CH-6); 127.03 (CH-6-phenothiazine); 128.11 (CH-8-phenothiazine); 129.16 (CH-4-phenothiazine); 131.06 (CH-2-phenothiazine); 144.82 (C-9a-phenothiazine); 145.53 (C-10a-phenothiazine); 149.54 (C-7a); 152.83 (CH-2); 157.81 (C-4).

MS (ESI-): *m/z* (%): 484.1 (30) [M-H]; HRMS (ESI-): calcd. 484.14488 for $C_{26}H_{22}N_5O_3S$, found 484.14429.

6.2.2 Synthesis of PT-labeled dNTPs

Synthesis of modified nucleoside triphosphates - Suzuki-Miyaura cross-coupling:

Method A: 1:1 mixture of H_2O-CH_3CN (1 mL) was added through a septum to an argon-purged flask containing a halogenated nucleotide **dN^{I}TP** (1 equiv.), **PT-Bpin** (2 equiv.) and Cs_2CO_3 (3 equiv). In a separate flask, $Pd(OAc)_2$ (10 mol-%), and TPPTS (2.5 equiv. with respect to Pd) were combined, the flask was evacuated and purged with argon, and then a 1:1 mixture of H_2O-CH_3CN (0.5 mL) was added. This catalyst solution was injected into the reaction mixture,

which was then stirred at 50ºC for 40 min until complete consumption of the starting material and then evaporated in vacuo. Product was isolated from the crude reaction mixture by HPLC on a C18 column with the use of a linear gradient of 0.1 M TEAB (triethylammonium bicarbonate) in H_2O to 0.1 M TEAB in H_2O–MeOH (1 : 1) as eluent. Several co-distillations with water and conversion to sodium salt form (Dowex 50WX8 in Na^+ cycle) followed by freeze-drying from water gave solid product.

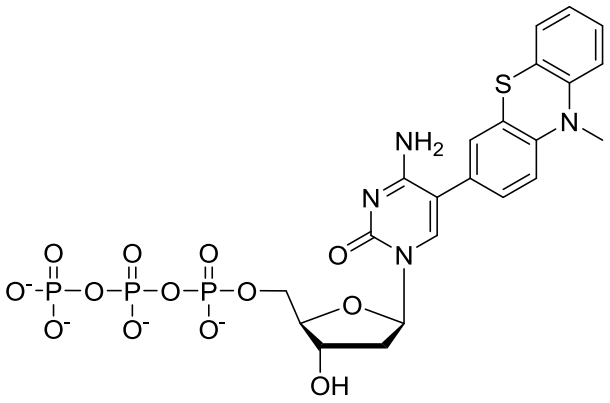
Synthesis of modified nucleoside triphosphates - Sonogashira cross-coupling:

Method B: A 1:1 mixture of H_2O-CH_3CN (2 mL) was added through a septum to an argon-purged flask containing a halogenated nucleotide **dN^{I}TP** (1 equiv.), **PTE** (1.5 equiv.), CuI (10 mol-%), Et_3N (10 equiv.). In a separate flask, $Pd(OAc)_2$ (5 mol-%), and TPPTS (2.5 equiv. with respect to Pd) were combined, the flask was evacuated and purged with argon, and then a 1:1 mixture of H_2O-CH_3CN (0.5 mL) was added. This catalyst solution was injected into the reaction mixture, which was then stirred at 75ºC for 1 h until complete consumption of the starting material and then evaporated in vacuo. Product was isolated from the crude reaction mixture by HPLC on a C18 column with the use of a linear gradient of 0.1 M TEAB (triethylammonium bicarbonate) in H_2O to 0.1 M TEAB in H_2O–MeOH (1 : 1) as eluent. Several co-distillations with water and conversion to sodium salt form (Dowex 50WX8 in Na^+ cycle) followed by freeze-drying from water gave solid product.

Synthesis of modified nucleoside triphosphates - Triphosphorylation:

Method C: $POCl_3$(1.2 equiv) in $PO(OMe)_3$ (1 ml) was added through a septum to an argon-purged flask containing modified nucleosides **dN^{EPT}** (1 equiv.). Reaction mixture was then stirred at 0ºC for 3 h until complete consumption of the starting material. Then an ice-cooled solution of $(NHBu_3)_2H_2P_2O_7$ (5 equiv) and Bu_3N (4.2 equiv) in dry DMF (2 ml) was added and the mixture was stirred at 0°C for another 1.5 h. The reaction was quenched by addition of 2 M aqueous TEAB (2 ml) and the solvents were evaporated in vacuo and the residue was co-distilled with water three times. The product was isolated by HPLC on a C18 column with the use of a linear gradient of 0.1 M TEAB (triethylammonium bicarbonate) in H_2O to 0.1 M TEAB in H_2O–MeOH (1 : 1) as eluent. Several co-distillations with water and conversion to sodium salt form (Dowex 50WX8 in Na^+ cycle) followed by freeze-drying from water gave solid product.

5-(10-methyl-10H-phenothiazine-3-yl)-2'-deoxycytidine 5'-*O*-triphosphate (dC^{PT}TP)



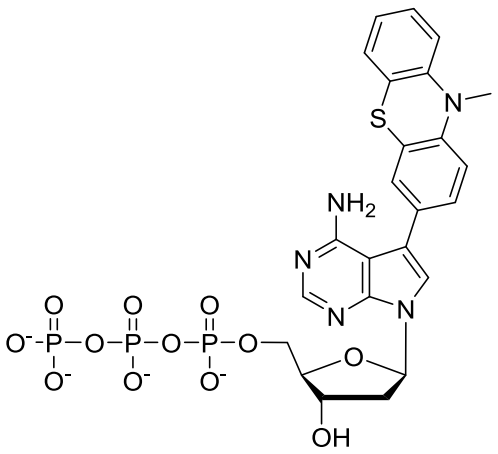
Compound **dC^{PT}** was prepared from **dC^{I}TP** according to general procedure (Method A). The product was isolated as a white solid (30 mg, 53%); ^{1}H NMR (500.0 MHz, D_2O, ref(dioxane) = 3.75 ppm): 2.35 (ddd, 1H, J_{gem} = 14.0, $J_{2'b,1'}$ = 7.5, $J_{2'b,3'}$ = 6.6, H-2′b); 2.42 (ddd, 1H, J_{gem} = 14.0, $J_{2'a,1'}$ = 6.3, $J_{2'a,3'}$ = 3.6, H-2′a); 3.38 (s, 3H, CH_3N); 4.11 –4.19 (m, 2H, H-5′); 4.22 (tdd, 1H, $J_{4',5'}$ = 4.2, $J_{4',3'}$ = 3.6, $J_{H,P}$ = 1.2, H-4′); 4.60 (dt, 1H, $J_{3',2'}$ = 6.6, 3.6, $J_{3',4'}$ = 3.6, H-3′); 6.31 (dd, 1H, $J_{1',2'}$ = 7.5, 6.3, H-1′); 7.01 (dd, 1H, $J_{9,8}$ = 8.2, $J_{9,7}$ = 1.2, H-9-phenothiazine); 7.02 – 7.09 (m, 2H, H-1,7-phenothiazine); 7.11 (d, 1H, $J_{4,2}$ = 2.1, H-4-phenothiazne); 7.23 (dd, 1H, $J_{2,1}$ = 8.4, $J_{2,4}$ = 2.1, H-2-phenothiazne); 7.24 – 7.33 (m, 2H, H-6,8-phenothiazine); 7.61 (s, 1H, H-6).

^{13}C NMR (125.7 MHz, D_2O, ref(dioxane) = 69.3 ppm): 37.47 (CH_3N); 41.52 (CH_2-2′); 68.02 (d, $J_{C,P}$ = 5.5, CH_2-5′); 73.31 (CH-3′); 88.20 (d, $J_{C,P}$ = 8.5, CH-4′); 88.77 (CH-1′); 112.32 (C-5); 117.48 (CH-9-phenothiazine); 117.95 (CH-1-phenothiazine); 124.97 (C-5a-phenothiazine); 125.85 (CH-7-phenothiazine); 126.16 (C-4a-phenothiazine); 129.16 (C-3-phenothiazine); 129.96 (CH-6-phenothiazine); 130.09 (CH-4-phenothiazine); 130.88 (CH-8-phenothiazine); 131.73 (CH-2-phenothiazine); 142.14 (CH-6); 148.12 (C-9a-phenothiazine); 148.65 (C-10a-phenothiazine); 159.73 (C-2); 167.20 (C-4).

^{31}P{^{1}H} NMR (202.3 MHz, D_2O): -22.04 (dd, J = 20.0, 19.6, P_β); -11.22 (d, J = 19.6, P_α); -6.39 (d, J = 20.0, P_γ).

MS (ESI-): *m/z* (%): 597.1 (100) [M-H-H_2PO_3]; HRMS (ESI-): calcd. 677.02789 for $C_{22}H_{24}O_{13}N_4P_3S$, found 677.02661.

7-(10-methyl-10H-phenothiazine-3-yl)-7-deaza-2'-deoxyadenosine 5'-*O*-triphosphate (dA^{PT}TP)



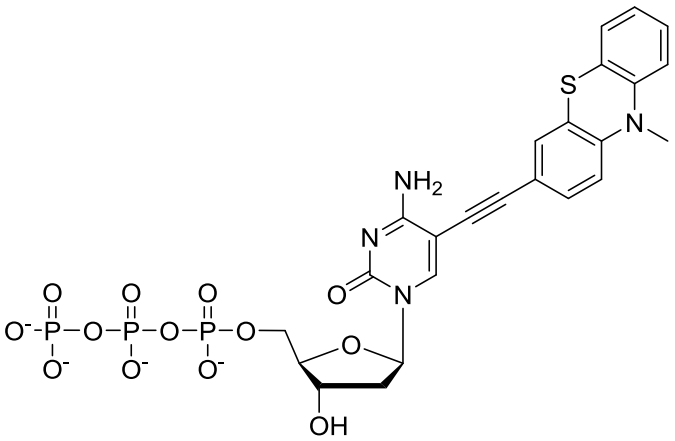
Compound **dA^{PT}TP** was prepared from **dA^{I}TP** according to general procedure (Method A). The product was isolated as a white solid (38 mg, 68%); ^{1}H NMR (500.0 MHz, D_2O): 2.55 (ddd, 1H, J_{gem} = 13.9, $J_{2'b,1'}$ = 6.2, $J_{2'b,3'}$ = 3.4, H-2′b); 2.81 (ddd, 1H, J_{gem} = 13.9, $J_{2'a,1'}$ = 7.8, $J_{2'a,3'}$ = 7.0, H-2′a); 3.18 (bs, 3H, CH_3N); 4.11 – 4.24 (m, 2H, H-5′); 4.19 (td, 1H, $J_{4',5'}$ = 4.4, $J_{4',3'}$ = 3.5, H-4′); 4.79 (m, 1H, H-3′); 6.59 (dd, 1H, $J_{1',2'}$ = 7.8, 6.2, H-1′); 6.74 – 6.83 (m, 3H, H-1,4,9-phenothiazine); 7.00 (t, 1H, $J_{7,6}$ = $J_{7,8}$ = 7.5, H-7-phenothiazine); 7.18 – 7.27 (m, 3H, H-2,6,8-phenothiazine); 7.41 (s, 1H, H-6); 8.06 (bs, 1H, H-2).

^{13}C NMR (150.9 MHz, D_2O): 37.27 (CH_3N); 40.72 (CH_2-2′); 68.30 (d, $J_{C,P}$ = 6.0, CH_2-5′); 73.75 (CH-3′); 85.27 (CH-1′); 87.74 (d, $J_{C,P}$ = 8.6, CH-4′); 103.39 (C-4a); 117.22 (CH-9-phenothiazine); 117.57 (CH-1-phenothiazine); 119.88 (C-5); 122.21 (CH-6); 124.72 (C-5a-phenothiazine); 125.55 (CH-7-phenothiazine); 125.66 (C-4a-phenothiazine); 129.27 (CH-4-phenothiazine); 129.84 (CH-6-phenothiazine); 130.45 (CH-2-phenothiazine); 130.49 (C-3-phenothiazine); 130.64 (CH-8-phenothiazine); 147.29 (C-10a-phenothiazine); 147.94 (C-9a-phenothiazine); 152.48 (C-7a); 153.94 (CH-2); 159.68 (C-4).

^{31}P{^{1}H} NMR (202.3 MHz, D_2O): -21.75 (dd, J = 20.1, 19.2, P_β); -10.88 (d, J = 19.2, P_α); -5.83 (d, J = 20.1, P_γ).

MS (ESI-): *m/z* (%): 620.1 (100) [M-H-H_2PO_3]; HRMS (ESI-): calcd. 700.04387 for $C_{24}H_{25}O_{12}N_5P_3S$, found 700.04255.

5-[(10-methyl-10H-phenothiazine-3-yl)ethynyl]-2'-deoxycytidine 5'-*O*-triphosphate (dC^{EPT}TP)



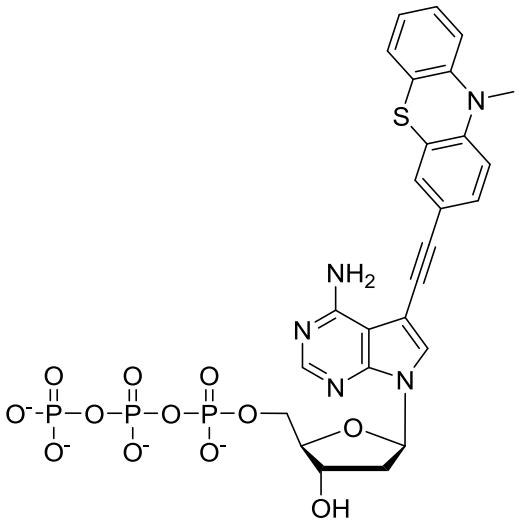
Compound **dC^{EPT}** was prepared from **dC^{I}TP** according to the Method B in 48% yield or from **dC^{EPT}** according to the Method C in 45% yield. The product was isolated as a yellow solid. ^{1}H NMR (500.0 MHz, D_2O, ref(dioxane) = 3.75 ppm): 2.23 (bm, 1H, H-2′b); 2.36 (bm, 1H, H-2′a); 3.28 (s, 3H, CH_3N); 4.12-4.25 (m, 3H, H-4′,5′); 4.54 (bm, 1H, H-3′); 6.11 (bt, 1H, $J_{1',2'}$ = 6.7, H-1′); 6.85 (bd, 1H, $J_{1,2}$ = 8.0, H-1-phenothiazine); 6.91 (bd, 1H, $J_{9,8}$ = 7.9, H-9-phenothiazine); 6.99 (bt, 1H, $J_{7,6}$ = $J_{7,8}$ = 7.9, H-7-phenothiazine); 7.16 (bd, 1H, $J_{6,7}$ = 7.9, H-6-phenothiazine); 7.21-7.26 (bm, 2H, H-4,8-phenothiazne); 7.37 (bd, 1H, $J_{2,1}$ = 8.0, H-2-phenothiazne); 7.96 (s, 1H, H-6).

^{13}C NMR (125.7 MHz, D_2O, ref(dioxane) = 69.3 ppm): 37.50 (CH_3N); 41.62 (CH_2-2′); 67.91 (d, $J_{C,P}$ = 5.0, CH_2-5′); 72.88 (CH-3′); 81.98 (C5-**C**≡C-phenothiazine); 88.09 (d, $J_{C,P}$ = 8.4, CH-4′); 89.10 (CH-1′); 95.59 (C-5); 97.96 (C5-C≡**C**-phenothiazine); 117.14 (CH-1-phenothiazine); 117.50 (CH-9-phenothiazine); 118.32 (C-3-phenothiazine); 124.36 (C-5a-phenothiazine); 125.18 (C-4a-phenothiazine); 125.86 (CH-7-phenothiazine); 129.80 (CH-6-phenothiazine); 130.82 (CH-8-phenothiazine); 132.15 (CH-4-phenothiazine); 134.24 (CH-2-phenothiazine); 146.45 (CH-6); 147.55 (C-9a-phenothiazine); 148.77 (C-10a-phenothiazine); 158.49 (C-2); 167.22 (C-4).

^{31}P{^{1}H} NMR (202.3 MHz, D_2O): -21.55 (bs, P_β); -10.91 (bs, P_α); -5.70 (bs, P_γ).

MS (ESI-): *m/z* (%): 621.1 (100) [M-H-H_2PO_3]; HRMS (ESI-): calcd. 701.02789 for $C_{24}H_{24}O_{13}N_4P_3S$, found 701.02649.

7-[(10-methyl-10H-phenothiazine-3-yl)ethynyl]-7-deaza-2'-deoxyadenosine 5'-*O*-triphosphate (dA^{EPT}TP)



Compound **dA^{EPT}** was prepared from **dA^{I}TP** according to the Method B in 49% yield or from **dA^{EPT}** according to the Method C in 43% yield. The product was isolated as a yellow solid. ^{1}H NMR (500.0 MHz, D_2O): 2.35, 2.50 (2 × bm, 2 × 1H, H-2′); 3.02 (bs, 3H, CH_3N); 4.03 – 4.17 (bm, 2H, H-5′); 4.19 (btd, 1H, $J_{4',5'}$ = 4.4, $J_{4',3'}$ = 3.5, H-4′); 4.61 (bm, 1H, H-3′); 6.20 (bt, 1H, $J_{1',2'}$ = 6.2, H-1′); 6.38 (bm, 1H, H-1-phenothiazine); 6.68 (bm, 1H, H-9-phenothiazine); 6.82 – 6.92 (bm, 2H, H-4,7-phenothiazine); 6.98 (bm, 1H, H-6-phenothiazine); 7.00 (bm, 1H, H-2-phenothiazine); 7.12 (bm, 1H, H-8-phenothiazne); 7.35 (s, 1H, H-6); 7.69 (bs, 1H, H-2).

^{13}C NMR (150.9 MHz, D_2O): 37.38 (CH_3N); 41.11 (CH_2-2′); 68.40 (d, $J_{C,P}$ = 4.8, CH_2-5′); 73.70 (CH-3′); 84.32 (C5-**C**≡C-phenothiazine); 85.49 (CH-1′); 87.61 (d, $J_{C,P}$ = 8.2, CH-4′); 94.95 (C5-C≡**C**-phenothiazine); 100.04 (C-5); 105.32 (C-4a); 116.45 (CH-1-phenothiazine); 117.29 (CH-9-phenothiazine); 118.22 (C-3-phenothiazine); 124.09 (C-5a-phenothiazine); 124.62 (C-4a-phenothiazine); 125.52 (CH-7-phenothiazine); 127.64 (CH-6); 129.56 (CH-6-phenothiazine); 130.57 (CH-8-phenothiazine); 131.22 (CH-4-phenothiazine); 133.32 (CH-2-phenothiazine); 147.15 (C-9a-phenothiazine); 147.65 (C-10a-phenothiazine); 150.49 (C-7a); 153.51 (CH-2); 159.05 (C-4).

^{31}P{^{1}H} NMR (202.3 MHz, D_2O): -22.21 (t, J = 19.2, P_β); -11.00 (d, J = 19.2, P_α); -8.41 (d, J = 19.2, P_γ).

MS (ESI-): *m/z* (%): 644.1 (100) [M-H-H_2PO_3]; HRMS (ESI-): calcd. 724.04387 for $C_{26}H_{25}O_{12}N_5P_3S$, found 724.04273.

6.2.3 Analysis and isolation of PEX products

Primer extension experiment:

Single incorporation:

Method A: reaction mixture (20 µL) contained temp^{A} (3 µM, 1 µl), 5′-(FAM)-labeled primer^{rnd} (3 µM, 1.5 µl), dGTP (4 mM, 0.1µl), either dATP or dA^{XTP}TP (4 mM, 1 µl) DNA polymerase (0.125 U KOD XL or 0.2 U Vent(exo-)) and reaction buffer (2 µl) supplied by manufacturer. In

the case of Pwo polymerase, 1.25U of the enzyme, 0.25 µl of dGTP (4 mM) and 2 µl of either dATP or **dA^{XTP}TP** (4 mM) were used. The reaction mixture was incubated for 40 minutes at 60 °C. The PEX reaction was stopped by the addition of PAGE stop solution [20 µL, formamide (80%, v/v), EDTA (20 mM), bromophenol blue (0.025%, w/v), xylene cyanol (0.025%,w/v) and Milli-Q water] and heated for 2 minutes at 95 °C. Samples were analyzed by use of a 12.5 % denaturizing polyacrylamide gel electrophoresis (1 h, 50 °C) and visualized by fluorescence imaging.

Method B: PEX reactions with temp^{C} were performed in the same way as described for temp^{A} exept either dCTP or **dC^{XTP}TP** (4 mM, 1µl for KOD XL and VENT(exo-) polymerases and 2µl for Pwo polymerase) were used.

Multiple incorporation:

The reaction mixture (20 µL) contained template^{rnd16} (3 µM, 1µl), 5′-(FAM)-labeled primer^{rnd} (3 µM, 1.5 µl), dNTP (either natural or modified, 4 mM, 1µl for KOD XL and VENT(exo-) polymerases and 2µl for Pwo polymerase), DNA polymerase (0.25 U KOD XL, 0.5 U Vent(exo-) or 12.5 U PWO) and reaction buffer (2 µl) supplied by manufacturer. The reaction mixture was incubated for 40 minutes at 60 °C. The PEX reaction was stopped by the addition of PAGE stop solution [20 µL, formamide (80%, v/v), EDTA (20 mM), bromophenol blue (0.025%, w/v), xylene cyanol (0.025%,w/v) and Milli-Q water] and heated for 2 minutes at 95 °C. Samples were analyzed by use of a 12.5 % denaturizing polyacrylamide gel electrophoresis (1h, 50 °C) and visualized by a fluorescence imaging.

Kinetics of incorporation of modified dNTPs:

Rate of incorporation was compared by preparation of samples with natural and modified dNTPs with various time periods. PEX reaction mixture (20 µl) was performed with 5′-(FAM)-labeled *prim*^{rnd} (3 µM, 1 µl), *temp*^{termA} (3 µM, 1.5µl) or *temp*^{termC} (3 µM, 1.5 µl), dNTPs (4 mM, 1 µl) with KOD XL polymerase (0.125 U) in enzyme reaction buffer (1 µL) supplied by the manufacturer by followed stopping of reaction by using of PAGE stop solution [20µL, formamide (80%, v/v), EDTA (20 mM), bromophenol blue (0.025%, w/v), xylene cyanol (0.025%,w/v) and Milli-Q water] and immidiate heating for 2 minutes at 95 °C. Samples were analyzed by use of a 12.5 % denaturizing polyacrylamide gel electrophoresis (1h, 50 °C) and visualized by a fluorescence imaging.

PCR:

A) The PCR reaction mixture (20 µL) contained KOD XL (3U), natural dNTPs (4 mM, 0.15µl), modified dNTPs (4 mM, 2µl), primers (prim^{LT25TH} and prim^{L20} 10 µM, 2 µL each), and a 98-mer

template (1 μM, 0.5 μL, temp$^{FVL\text{-}A}$) in reaction buffer (2 μL) supplied by the manufacturer. Thirty PCR cycles were run under the following conditions: denaturation for 1 min at 95 °C, annealing for 1 min at 53 °C, extension for 1,5 min at 72 °C, followed by final extension step of 2 min at 72 °C. Reaction mixtures were than separated by use of a 2% agarose gel in 0.5x TBE buffer with GelRed as an intercalator. Visualization was performed by an electronic dual wave transilluminator equipped with GBox iChemi XRQ Bio imaging system (Syngene).

B) The PCR reaction mixture (20 μL) contained KOD XL (3 U), natural dNTPs (4 mM, 0.15μl), primers (prim^{LT25TH} and prim^{L20} 10 μM, 2 μL each), and a 98-mer template (1 μM, 0.5 μL, temp$^{FVL\text{-}A}$) in reaction buffer (2 μL) supplied by the manufacturer. Modified dNTPs (either **dA^{PT}TP** or **dA^{EPT}TP**) were added in combination with natural dATP (100%: **dA^{XPT}TP** (4 mM, 2μl); 90%: **dA^{XPT}TP** (4 mM, 1.8μl), dATP (4 mM, 0.2 μl); 80%: **dA^{XPT}TP** (4 mM, 1.6μl), dATP (4 mM, 0.4μl); 50%: **dA^{XPT}TP** (4 mM, 1.0 μl), dATP (4 mM, 1.0 μl)) Thirty PCR cycles were run under the following conditions: denaturation for 1 min at 95 °C, annealing for 1 min at 53 °C, extension for 1,5 min at 72 °C, followed by final extension step of 2 min at 72 °C. Reaction mixtures were than separated by use of a 2% agarose gel with GelRed as an intercalator. Visualization was performed by an electronic dual wave transilluminator equipped with GBox iChemi XRQ Bio imaging system (Syngene).

C) PCR reactions were performed in the same way as described above exept 5′-(FAM)-labeled primers (10 μM, 4 μL, prim^{LT25TH} and prim^{L20}) were used and visualization was performed by a fluorescence imaging.

TdT Elongation:

The reaction mixture (10 μL) contained TdT (12 U), dATP, **dA^{PT}TP**, or **dA^{EPT}TP** (4 mM, 1μl) and 5′-FAM labeled primer^{rnd} (3 μM, 0.5 μl) in enzyme reaction buffer (1 μL) supplied by the manufacturer. The reaction mixture was incubated for 1h or 4 h at 37 °C in a thermal cycler. Before gel loading samples were denatured by the addition 10 μL of stop solution (80% [v/v] formamide, 20 mM EDTA, 0.025%, [w/v] bromophenol blue, 0.025% [w/v] xylene cyanol, and Milli-Q water) and heated for 10 min at 95 °C. Reaction mixtures were separated by using 12.5% denaturing PAGE. Visualization was performed by fluorescence imaging.

NEAR:

NEAR General Procedure. The reaction mixture contained the template (0.125 μM), primer (0.125 μM), modified **dN^{X}TP** (160 μM), natural dNTPs (125 μM), 1×ThermoPol buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl/pH 8.8/, 0.1% Triton-X-100, and 2 mM $MgSO_4$; 5 μL for analytical scale and 50 μL for preparative scale), and 0.5× NEBuffer 3 (50 mM NaCl, 25

mM Tris-HCl/pH 7.9/, 5 mM $MgCl_2$, and 0.5 mM DTT; 2.5 µL for analytical scale and 25 µL for preparative scale), Vent(exo-) (5U for analytical scale and 80U for preparative scale), Nt.BstNBI (30U for analytical scale and 150U for preparative scale). The reaction mixture was incubated at 55 °C for 3 h. The reaction was stopped by cooling to 4 °C.

NEAR on Analytical Scale. The analytical reactions were performed according to the general procedure in a volume of 50 µL. The products were analyzed by agarose gel electrophoresis using 4% agarose gels stained with GelRed (Lab Mark). Samples were prepared by mixing 1.6 µL of 6× DNA Loading Dye (Thermo Scientific) and 8 µL of the reaction mixture or ss DNA ladder. The gel was run for 70 min at 120 V and imaged using an electronic dual wave transilluminator equipped with GBox iChemi XRQ Bio imaging system (Syngene).

NEAR on Preparative Scale. The preparative reactions were performed according to the general procedure in a volume of 500 µL. After the reaction was stopped, the solution was concentrated on a vacuum concentrator to approximately 100 µL. The viscous concentrate was injected on an HPLC XBridge OST C18 Column (Waters; 2.5 µm particle size, 4.6 mm × 50 mm) and separated using a gradient of triethylammonium acetate (TEAA) and acetonitrile at a flow rate of 1 mL/min. 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 fractions containing the product were evaporated on a vacuum concentrator. The products were analyzed by MALDI-TOF mass spectrometry.

MALDI-TOF:

The MALDI-TOF spectra were measured with 1 kHz smartbeam II laser technology. The measurements were done in reflectron mode by droplet technique, with the mass range up to 30 kDa. The matrix consisted of 3-hydroxypicolinic acid (HPA)/picolinic acid (PA)/ ammonium tartrate in ratio 9/1/1. The matrix (1 µL) was applied on the target (ground steel) and dried down at room temperature. The sample (1 µL) and matrix (1 µL) were mixed and added on the top of the dried matrix preparation spot and dried at room temperature.

The reaction mixture (50 µL) contained biotinylated $temp^{rnd16}$ ($temp^{A}$ or $temp^{C}$) (100 µM, 1.6 µL), $primer^{rnd}$ (100 µM, 1.6 µL), dNTPs (4 mM, 2.6 µL), KOD XL polymerase (1.25 U, in the case of multiple incorporation of two different modified $dN^{X}TPs$ 2.5U of KOD XL polymerase were used) in enzyme reaction buffer (5 µL) supplied by the manufacturer. The reaction mixture was incubated for 40 min at 60 °C in a thermal cycler. The reaction was stopped by cooling to 4°C.

Isolation of single-strand oligonucleotides by the DBStv magnetoseparation procedure:
Streptavidin Magnetic Particles (Roche, 50 μL) were washed with Binding buffer TEN100 (10mM Tris, 1mM EDTA, 100mM NaCl, pH 7.5; 3 × 200 μL). The reaction mixture after PEX was diluted with the Binding buffer TEN100 (50 μL), the solution was added to the prewashed magnetic beads and incubated for 30 min at 15 °C and 1400 rpm. After the incubation, the magnetic beads were collected on a magnet (DynaMag-2, Invitrogen) and the solution was discarded. The beads were washed successively with Wash buffer TEN 500 (10 mM Tris, 1mM EDTA, 500 mM NaCl, pH 7.5; 3 × 200 μL), and water (3 × 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. The product was analyzed by MALDI-TOF mass spectrometry.

6.2.4 Fluorescence measurements of PT-modified nucleosides, triphosphates and DNA

Determination of extinction coefficients

Extinction coefficients were measured using 1 ml quartz cuvettes on a Cary 100 UV-VIS spectrometer (Agilent Technologies). The absorption coefficients were calculated according to the following The Beer-Lambert Law equation:

$$A = \varepsilon \times c \times l$$

where ε is the absorption coefficient, c is the exact concentration of the sample in the cuvette, l is the length of the path that the light travels through the cuvette and A is the absorbance of the sample. Measurements were triplicated.

Determination of fluorescence quantum yields

Measurements were performed in 1 ml quartz fluorescence cuvettes (Hellma Analytics) on a Fluoromax 4 spectrofluorimeter equipped with a thermostated cuvette holder set to 25 °C. The solvents used were of spectroscopy or HPLC grade. The excitation wavelength was 350 nm and the recorded spectral range was 370 – 680 nm. Relative determination of the fluorescence quantum yields was performed using quinine bisulfate in 0.5 M H_2SO_4 ($\Phi_f = 0.546$ at 25 °C) as a standard[177]. The absorbance of sample solutions at the excitation wavelength were kept below 0.08 to avoid inner filter effect. The quantum yields were calculated using following equation:

$$\Phi_{f,x} = \Phi_{f,st} \frac{F_x}{F_{st}} \frac{1 - 10^{-Abs_{st}}}{1 - 10^{-Abs_x}} \frac{n_x^2}{n_{st}^2}$$

where Φ_f is the quantum yield, F is the integrated fluorescence intensity, Abs is the absorbance of the solution at the excitation wavelength, n is the refractive index of the solvent. The

subscripts *x* and *st* stand for the sample and standard, respectively. Measurements were triplicated.

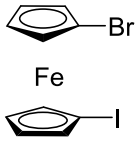
6.2.5 Electrochemical analysis

Nucleosides were analyzed by conventional *in situ* cyclic voltammetry (CV). Purified NEAR and PEX products were analyzed by *ex situ* (adsorptive transfer stripping) SWV. The NEAR and PEX products were accumulated at the surface of the working basal-plane pyrolytic graphite electrode for 60 s from 5 μL aliquots containing 0.2 M NaCl. The electrode was then rinsed with deionized water and placed in the electrochemical cell. CV settings: initial potential (Ei) 0.0 V, scan rate 1V/s, SWV settings: initial potential 0.0 V, end potential 1.5 V, frequency 200 Hz, amplitude 50 mV. Background electrolyte: 0.2 M sodium acetate pH 5.0. All measurements were performed at room temperature using an Autolab analyzer (Eco Chemie, The Netherlands) in connection with VA-stand 663 (Metrohm, Herisau, Switzerland). The three-electrode system was used with Ag/AgCl/3M KCl electrode as a reference and platinum wire as an auxiliary electrode. Measurements at HMDE were done after deaeration of the solution by argon purging.

6.3 Ferrocene-labeled nucleosides and dNTPs. Synthesis, enzymatic incorporation and electrochemical detection

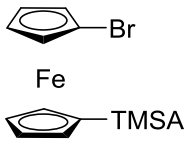
6.3.1 Synthesis of ferrocene labels

1-bromo-1'-iodoferrocene (**2**)



yield: 85 %. Spectral data were in accordance with literature[165].

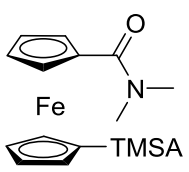
1-bromo-1'-(trimethylsilylethynyl)ferrocene (**3**)



yield: 60 %. Spectral data were in accordance with literature[165].

1-(N,N-dimethylaminocarbonyl)-1'-(trimethylsilylethynyl)ferrocene (**4**):

n-BuLi (2.5 M in hexane, 1 mL, 1.1 equiv.) was added dropwise to a solution of 1-bromo-1'-(trimethylsilylethynyl)ferrocene (**3**) (0.52 g, 1.0 equiv.) in anhydrous THF (50 mL) at -78 °C under argon. The reaction was allowed to stir for 1 h at -78 °C and carbomoyl chloride (0.4 mL, 3.0 equiv.) was added slowly, while maintaining the temperature. After the addition was complete the reaction was left to stir at -78 °C for an additional hour followed by slow warming to r.t., and a further 2 h of stirring. The reaction mixture was hydrolysed with water (20 mL) and the product was extracted with Et_2O (3× 50mL). The extracts were combined, dried over $MgSO_4$ concentrated in vacuo. The product was obtained following purification by flash column chromatography (10% EA in PE) as a dark brown oil (0.32g, 64%).



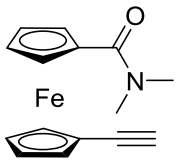
^{1}H NMR (500.0 MHz, CD_3CN): 0.20 (s, 9H, CH_3Si); 2.98, 3.24 (2 × bs, 2 × 3H, CH_3N); 4.28 – 4.31 (m, 2H, H-3′,4′); 4.34 – 4.37 (m, 2H, H-2,5); 4.40 – 4.43 (m, 2H, H-2′,5′); 4.58 – 4.61 (m, 2H, H-3,4).

^{13}C NMR (125.7 MHz, CD_3CN): 0.09 (CH_3Si); 36.68, 39.37 (CH_3N); 66.78 (C-1′); 71.51 (CH-3′,4′); 72.54 (CH-2,5); 73.40 (CH-3,4); 73.86 (CH-2′,5′); 80.73 (C-1); 92.02 (TMS-**C**≡C); 104.27 (TMS-C≡**C**); 169.77 (CO).

MS (ESI-): *m/z* (%):353.1 (100) [M-H]; HRMS (ESI-): calcd. 353.0898 for $C_{18}H_{23}NOSiFe$, found 353.0900.

1-(N,N-dimethylaminocarbonyl)-1'-ethynylferrocene Fc(am) (5):

The mixture of 1-(N,N-dimethylaminocarbonyl)-1'-(trimethylsilylethynyl)ferrocene (**4**) (0.32g, 1.0 equiv.) and KF (0.52g, 10.0 equiv.) in MeOH:dioxane (1:1, 10 ml) was stirred at 25 °C for 2 h until complete consumption of the starting material and then evaporated in vacuo. The product was obtained following purification by flash column chromatography (10% EA in PE) as dark brown solid (0.25g, 98%).

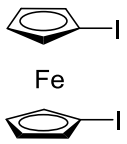


^{1}H NMR (500.0 MHz, CD_3CN): 2.97 (bs, 3H, CH_3N); 3.01 (s, 1H, HC≡C); 3.23 (bs, 3H, CH_3N); 4.27 – 4.30 (m, 2H, H-3′,4′); 4.35 – 4.38 (m, 2H, H-2,5); 4.45 – 4.49 (m, 2H, H-2′,5′); 4.62 – 4.66 (m, 2H, H-3,4).

^{13}C NMR (125.7 MHz, CD_3CN): 36.65, 39.30 (CH_3N); 66.19 (C-1′); 71.39 (CH-3′,4′); 72.44 (CH-2,5); 73.19 (CH-3,4); 73.88 (CH-2′,5′); 75.94 (H**C**≡C); 80.86 (C-1); 82.08 (**C**≡CH); 169.63 (CO).

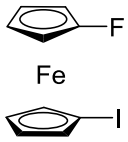
MS (ESI-): *m/z* (%):281.1 (100) [M]; HRMS (ESI-): calcd. 281.0503 for $C_{15}H_{15}NOFe$, found 281.0502.

1,1'-diiodoferrocene (7)



Yield: 81 %. Spectral data were in accordance with literature[167].

1-fluoro-1'-iodoferrocene (8)

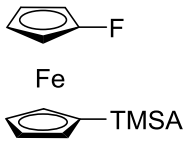


n-BuLi (2.5 M in hexane, 1.8 ml, 1.1 equiv.) was added dropwise to a solution of 1,1'-diiodoferrocene (**7**) (2g, 1.0 equiv.) in anhydrous THF (50 mL) at -78 °C under argon. The resulting suspension was stirred for 2 h at -78 °C. In a second flask, a suspension of NFSI (2.88 g, 2 equiv., dried for 3 h in vacuo) in diethylether (40 ml) was prepared. After 30 min the reaction mixture was transferred into the NFSI solution via cannula. Directly after the addition the solution was quenched with $NaBH_4$ and 50 ml of 0.1M $Ca(OH)_2$, and the resulting slurry was diluted with hexane (100 ml). The two phase system was stirred for 1 h, the organic phase was separated and washed three times with water. The extracts were combined, dried over $MgSO_4$ concentrated in vacuo. The product was purified by flash column chromatography (100% PE)

and then by HPLC (CH_3CN/H_2O (70:30); isocratic). The HPLC fractions were extracted with hexane (4 × 20 ml). The organic phase was dried with $MgSO_4$ and evaporated in vacuo, leaving the product as a dark red oil (0.36g, 24%). Spectral data were in accordance with literature[178].

1-fluoro-1'-(trimethylsilylethynyl)ferrocene (**9**):

1-fluoro-1'-iodoferrocene (**8**) (0.5g, 1.0 equiv.), CuI (2.8mg, 0.01 equiv.), $Pd(PPh_3)Cl_2$ (0.5 mg, 0.005 equiv.) and PPh_3 (7.8 mg, 0.02 equiv.) were dissolved in anhydrous THF (4mL) and placed under argon. Et_3N (1.0 ml, 10 equiv.) and trimethylsilylacetylene (1.1 mL, 10 equiv.) were and the reaction mixture was heated at 60 °C for 16 h. The reaction was cooled to r.t., diluted with Et_2O (30 mL) and filtered. The filtrate was concentrated in vacuo. The product (**9**) was obtained following purification by flash column chromatography (100% PE) as a brown-red oil (0.18g, 40%):



^{1}H NMR (500.0 MHz, CD_3CN): 0.19 (s, 9H, $(CH_3)_3Si$); 3.88 (td, 2H, $J_{2'\&5',3'\&4'}$ = 2.1, $J_{H,F}$ = 1.4, H-2′,5′); 4.34 (t, 2H, $J_{3\&4,2\&5}$ = 1.9, H-3,4); 4.35 (dt, 2H, $J_{H,F}$ = 2.7, $J_{3'\&4',2'\&5'}$ = 2.1, H-3′,4′); 4.50 (t, 2H, $J_{2\&5,3\&4}$ = 1.9, H-2,5).

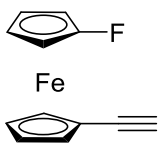
^{13}C NMR (125.7 MHz, CD_3CN): 0.05 ($(CH_3)_3Si$); 58.74 (d, $J_{C,F}$ = 15.4, CH-3′,4′); 64.42 (d, $J_{C,F}$ = 4.0, CH-2′,5′); 67.07 (C-1); 71.18 (CH-3,4); 73.64 (CH-2,5); 91.71 (TMS-**C**≡C); 104.24 (**C**≡C-TMS); 136.51 (d, $J_{C,F}$ = 267.7, C-1′).

^{19}F NMR (470.4 MHz, CD_3CN): -186.69.

MS (ESI-): *m/z* (%): 300.0 (100) [M]; HRMS (ESI-): calcd. 300.0433 for $C_{15}H_{17}FSiFe$ found 300.0434.

1-fluoro-1'-ethynylferrocene FcF (**10**):

The mixture of 1-fluoro-1'-(trimethylsilylethynyl)ferrocene (**9**) (0.18g, 1.0 equiv.) and KF (0.52g, 10.0 equiv.) in MeOH:dioxane (1:1, 10 ml) was stirred at 25 °C for 4 h until complete consumption of the starting material and then evaporated in vacuo. The product was obtained following purification by flash column chromatography (10% EA in PE) as dark red oil (0.13g, 95%)



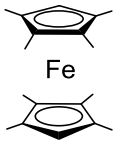
^{1}H NMR (500.0 MHz, CD_3CN): 3.01 (s, 1H, HC≡C); 3.90 (td, 2H, $J_{2'\&5',3'\&4'}$ = 2.1, $J_{H,F}$ = 1.3, H-2′,5′); 4.34 (t, 2H, $J_{3\&4,2\&5}$ = 1.9, H-3,4); 4.38 (dt, 2H, $J_{H,F}$ = 2.7, $J_{3'\&4',2'\&5'}$ = 2.1, H-3′,4′); 4.55 (t, 2H, $J_{2\&5,3\&4}$ = 1.9, H-2,5).

^{13}C NMR (125.7 MHz, CD_3CN): 58.61 (d, $J_{C,F}$ = 15.4, CH-3′,4′); 64.30 (d, $J_{C,F}$ = 4.0, CH-2′,5′); 66.44 (C-1); 71.06 (CH-3,4); 73.74 (CH-2,5); 75.68 (H**C**≡C); 82.18 (**C**≡CH); 136.51 (d, $J_{C,F}$ = 267.5, C-1′).

^{19}F NMR (470.4 MHz, CD_3CN): -186.71.

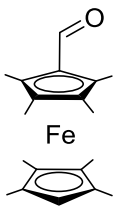
MS (ESI-): *m/z* (%): 228.0 (100) [M]; HRMS (ESI-): calcd. 228.0038 for $C_{12}H_9FFe$ found 228.0037.

1,1',2,2',3,3',4,4'-octamethylferrocene (**12**)



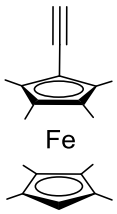
Yield: 48 %. Spectral data were in accordance with literature[168].

1-formyl-1',2,2',3,3',4,4',5-octamethylferrocene (**13**)



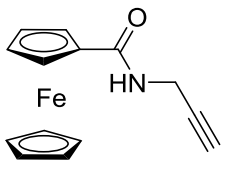
Yield: 79 %. Spectral data were in accordance with literature[169].

1-ethynyl-1',2,2',3,3',4,4',5-octamethylferrocene Fc(Me)$_8$ (**14**)



Yield: 56 %. Spectral data were in accordance with literature[170].

Ferrocenoyl propargylamide FcPA (**16**)



Yield: 25 %. Spectral data were in accordance with literature[171].

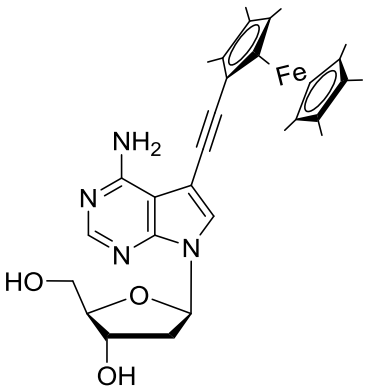
6.3.2 Synthesis of ferrocene-labeled nucleosides

Synthesis of modified nucleosides - Sonogashira cross-coupling:

Method A: A 1:1 mixture of H_2O-CH_3CN (2 mL) was added through a septum to an argon-purged flask containing a halogenated nucleoside **dN^{I}** (1 equiv.), **FcX** (1.5 equiv.) CuI (10 mol-%), TPPTS (2 mol-%) and $Pd(OAc)_2$ (5 mol-%) followed by Et_3N (10 equiv.). The reaction mixture was stirred at 50 ºC for 40 min until complete consumption of the starting material and then evaporated in vacuo. The products were purified by silica gel column chromatography using chloroform/methanol (0 to 10%) as eluent.

Metod B: Dry DMF (3 mL) was added to an argon-purged flask containing **FcX** (1.5 equiv.), a nucleoside analogue **dN^{I}** (1 equiv.), CuI (10 mol-%), PPh_3 (2 mol-%) and $[Pd(PPh_3)_2Cl_2]$ (5 mol-%) followed by Et_3N (10 equiv.). The reaction mixture was stirred at 75°C for 1 h until complete consumption of the starting material and then evaporated in vacuo. The products were purified by silica gel column chromatography using chloroform/methanol (0 to 10%) as eluent.

7-(1',2,2',3,3',4,4',5-octamethylferrocene-1-yl-ethynyl)-7-deaza-2'-deoxyadenosine (dA^{FcM})

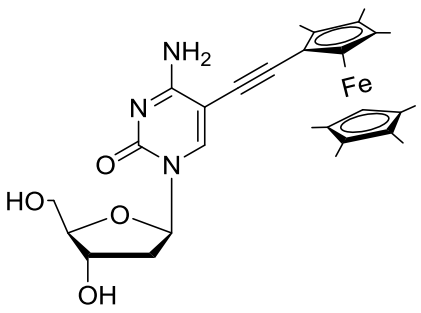


Compound **dA^{FcM}** was prepared from **dA^{I}** according to general procedure (Method A). The product was isolated as a yellow solid (45 mg, 90%); ^{1}H NMR (500.0 MHz, CD_3OD): 1.38, 1.41, 1.53, 1.56 (4 × bs, 24H, CH_3-ferrocene); 2.32 (ddd, 1H, J_{gem} = 13.6, $J_{2'b,1'}$ = 6.0, $J_{2'b,3'}$ = 2.6 H-2′b); 2.66 (ddd, 1H, J_{gem} = 13.6, $J_{2'a,1'}$ = 8.3, $J_{2'a,3'}$ = 5.9, H-2′a); 3.58 (bs, 1H, H-ferrocene); 3.73 (dd, 1H, J_{gem} = 12.1, $J_{5'b,4'}$ = 3.5, H-5′b); 3.81 (dd, 1H, J_{gem} = 12.1, $J_{5'a,4'}$ = 3.2, H-5′a); 4.00 (ddd, 1H, $J_{4',5'}$ = 3.5, 3.2, $J_{4',3'}$ = 2.6, H-4′); 4.52 (dt, H, $J_{3',2'}$ = 5.9, 2.6, $J_{3',4'}$ = 2.6, H-3′); 6.49 (dd, 1H, $J_{1',2'}$ = 8.3, 6.0, H-1′); 7.56 (s, 1H, H-6); 8.10 (s, 1H, H-2).

^{13}C NMR (125.7 MHz, CD_3OD): 9.15, 9.97, 10.89, 11.02 (CH_3-ferrocene); 41.56 (CH_2-2′); 63.61 (CH_2-5′); 73.00 (CH-3′); 74.28 (CH-ferrocene); 82.03 (C5-**C**≡C-ferrocene); 83.85, 84.13 (C-ferrocene); 86.69 (CH-1′); 89.19 (CH-4′); 92.58 (C5-C≡**C**-ferrocene); 97.87 (C-5); 104.43 (C-4a); 126.38 (CH-6); 149.81 (C-7a); 153.22 (CH-2); 159.39 (C-4).

MS (ESI-): *m/z* (%):571.2 (100) [M]; HRMS (ESI-): calcd. 571.2366 for $C_{31}H_{39}O_3N_4Fe$, found 571.2362.

5-(1',2,2',3,3',4,4',5-octamethylferrocene-1-yl-ethynyl)-2'-deoxycytidine (**dC^{FcM}**)

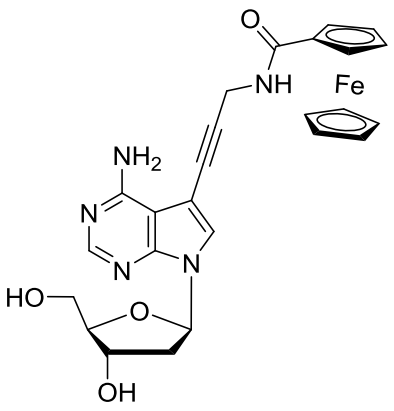


Compound **dC^{FcM}** was prepared from **dC^{I}** according to general procedure (Method A). The product was isolated as a yellow solid (40 mg, 86%); ^{1}H NMR (500.0 MHz, CD_3OD): 0.35 – 1.24 (bm, 24H, CH_3-ferrocene); 2.10 (dt, 1H, J_{gem} = 13.6, $J_{2'b,1'} = J_{2'b,3'}$ = 6.2, H-2′b); 2.37 (ddd, 1H, J_{gem} = 13.6, $J_{2'a,1'}$ = 6.2, $J_{2'a,3'}$ = 4.0, H-2′a); 3.68 (dd, 1H, J_{gem} = 12.1, $J_{5'b,4'}$ = 3.5, H-5′b); 3.74 (dd, 1H, J_{gem} = 12.1, $J_{5'a,4'}$ = 3.2, H-5′a); 3.92 (ddd, 1H, $J_{4',3'}$ = 4.0, $J_{4',5'}$ = 3.5, 3.2, H-4′); 4.31 (dt, 1H, $J_{3',2'}$ = 6.2, 4.0, $J_{3',4'}$ = 4.0, H-3′); 6.19 (t, 1H, $J_{1',2'}$ = 6.2, H-1′); 8.31 (bs, 1H, H-6); (CH-ferrocene not detected).

^{13}C NMR (125.7 MHz, CD_3OD): 8.77, 9.41, 10.28, 10.59 (CH_3-ferrocene); 42.39 (CH_2-2′); 62.39 (CH_2-5′); 71.77 (CH-3′); 76.70 (C5-**C**≡C-ferrocene); 87.96 (CH-1′); 89.12 (CH-4′); 94.68 (C-5); 97.24 (C5-C≡**C**-ferrocene); 143.46 (CH-6); 156.57 (C-2); 165.49 (C-4); (C,CH-ferrocene not detected)

MS (ESI-): *m/z* (%):547.2 (100) [M]; HRMS (ESI-): calcd. 547.2328 for $C_{29}H_{37}O_4N_3Fe$, found 547.2131.

7-[3-(ferrocene-1-carboxamido)prop-1-yn-1-yl]-7-deaza-2'-deoxyadenosine (**dA^{FcPA}**)

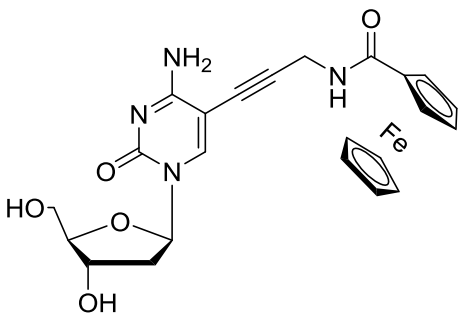


Compound **dA^{FcPA}** was prepared from **dA^{I}** according to general procedure (Method B). The product was isolated as a yellow solid (52 mg, 98%); ^{1}H NMR (500.0 MHz, CD_3OD): 2.31 (ddd, 1H, J_{gem} = 13.7, $J_{2'b,1'}$ = 6.0, $J_{2'b,3'}$ = 2.7 H-2′b); 2.61 (ddd, 1H, J_{gem} = 13.7, $J_{2'a,1'}$ = 8.0, $J_{2'a,3'}$ = 6.0, H-2′a); 3.70 (dd, 1H, J_{gem} = 12.1, $J_{5'b,4'}$ = 3.7,H-5′b); 3.77 (dd, 1H, J_{gem} = 12.1, $J_{5'a,4'}$ = 3.3,H-5′a); 3.99 (ddd, 1H, $J_{4',5'}$ = 3.7, 3.3, $J_{4',3'}$ = 2.7, H-4′); 4.21 (bs, 5H, H-cp); 4.25 (bs, 2H, CH_2N); 4.37 – 4.45 (m, 2H, H-2″,5″); 4.49 (dt, H, $J_{3',2'}$ = 6.0, 2.7, $J_{3',4'}$ = 2.7, H-3′); 4.83 – 4.85 (m, 2H, H-3″,4″); 6.46 (dd, 1H, $J_{1',2'}$ = 8.0, 6.0, H-1′); 7.58 (s, 1H, H-6); 8.15 (bs, 1H, H-2).

^{13}C NMR (125.7 MHz, CD_3OD): 30.90 (CH_2N); 41.55 (CH_2-2′); 63.61 (CH_2-5′); 69.52, 69.54 (CH-3″,4″); 70.82 (CH-cp); 72.04 (CH-2″,5″); 72.96 (CH-3′); 75.74, 75.86 (C-1″, C5-**C**≡C-CH_2); 86.63 (CH-1′); 89.18 (CH-4′); 90.43 (C5-C≡**C**-CH_2); 97.16 (C-5); 104.77 (C-4a); 127.78 (CH-6); 149.83 (C-7a); 153.49 (CH-2); 159.00 (C-4); 174.14 (CONH).

MS (ESI-): *m/z* (%):516.1 (90) [M]; 538.1 (100) [M+Na]; HRMS (ESI-): calcd. 516.1329 for $C_{25}H_{26}O_4N_5Fe$, found 516.1327; calcd. 538.1148 for $C_{25}H_{25}O_4N_5FeNa$, found 538.1148 .

5-[3-(ferrocene-1-carboxamido)prop-1-yn-1-yl]-2'-deoxycytidine (dC^{FcPA})

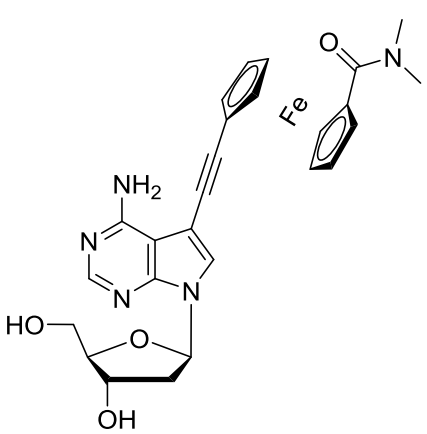


Compound **dC^{FcPA}** was prepared from **dC^{I}** according to general procedure (Method B). The product was isolated as a yellow solid (48 mg, 97%); ^{1}H NMR (600.1 MHz, DMSO-d_6): 1.95 (ddd, 1H, J_{gem} = 13.2, $J_{2'b,1'}$ = 7.2, $J_{2'b,3'}$ = 6.0, H-2'b); 2.12 (ddd, 1H, J_{gem} = 13.2, $J_{2'a,1'}$ = 5.9, $J_{2'a,3'}$ = 3.3, H-2'a); 3.52, 3.57 (2 × ddd, 2 × 1H, J_{gem} = 11.9, $J_{5',OH}$ = 5.2, $J_{5',4'}$ = 3.5, H-5'); 3.77 (q, 1H, $J_{4',3'}$ = $J_{4',5'}$ = 3.5, H-4'); 4.18 (m, 8H, H-3', CH_2N, H-cp); 4.38 (m, 2H, H-2",5"); 4.81 (m, 2H, H-3",4"); 5.07 (bt, 1H, $J_{OH,5'}$ = 5.2, OH-5'); 5.21 (bd, 1H, $J_{OH,3'}$ = 4.2, OH-3'); 6.09 (dd, 1H, $J_{1',2'}$ = 7.2, 5.9, H-1'); 6.84, 7.88 (2 × bs, 2 × 1H, NH_2); 8.12 (s, 1H, H-6); 8.29 (t, 1H, J = 5.4, NH).

^{13}C NMR (150.9 MHz, DMSO-d_6): 29.68 (CH_2N); 40.98 (CH_2-2'); 61.29 (CH_2-5'); 68.47 (CH-3",4"); 69.57 (CH-cp); 70.45 (CH-3', CH-2",5"); 74.23 (C5-**C**≡C-CH_2); 75.83 (C-1"); 85.56 (CH-1'); 87.68 (CH-4'); 89.71 (C-5); 93.58 (C5-C≡**C**-CH_2); 143.72 (CH-6); 153.67 (C-2); 164.69 (C-4); 169.67 (CONH).

MS (ESI-): *m/z* (%):493.1 (10) [M]; 515.1 (100) [M+Na]; HRMS (ESI-): calcd. 493.1169 for $C_{23}H_{25}O_5N_4Fe$, found 493.1164; calcd. 515.0988 for $C_{23}H_{24}O_5N_4FeNa$, found 515.0983.

7-[1'-(N,N-dimethylaminocarbonyl)-ferrocene-1-yl-ethynyl]-7-deaza-2'-deoxyadenosine (dA$^{Fc(am)}$)



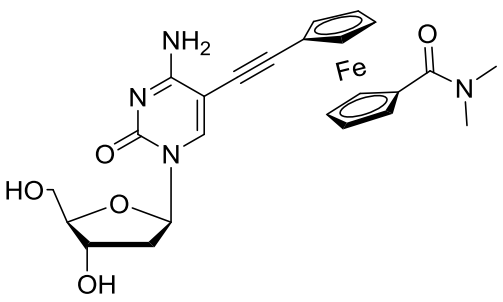
Compound **dA$^{Fc(am)}$** was prepared from **dA^{I}** according to general procedure (Method B). The product was isolated as a yellow solid (51 mg, 98%); ^{1}H NMR (500.0 MHz, CD_3OD): 2.35 (ddd, 1H, J_{gem} = 13.7, $J_{2'b,1'}$ = 6.0, $J_{2'b,3'}$ = 2.7, H-2′b); 2.65 (ddd, 1H, J_{gem} = 13.7, $J_{2'a,1'}$ = 8.0, $J_{2'a,3'}$ = 5.8, H-2′a); 2.95, 3.29 (2 × bs, 2 × 3H, $(CH_3)_2N$); 3.74 (dd, 1H, J_{gem} = 12.1, $J_{5'b,4'}$ = 3.6, H-5′b); 3.81 (dd, 1H, J_{gem} = 12.1, $J_{5'a,4'}$ = 3.3, H-5′a); 4.02 (ddd, 1H, $J_{4',5'}$ = 3.6, 3.3, $J_{4',3'}$ = 2.7, H-4′);

4.38 – 4.40 (m, 2H, H-3,4-cp); 4.48 – 4.50 (m, 2H, H-2,5-cpCONMe$_2$); 4.53 (dt, 1H, $J_{3',2'}$ = 5.8, 2.7, $J_{3',4'}$ = 2.7, H-3′); 4.57 – 4.59 (m, 2H, H-2,5-cp); 4.77 – 4.79 (m, 2H, H-3,4-cpCONMe$_2$); 6.52 (dd, 1H, $J_{1',2'}$ = 8.0, 6.0, H-1′); 7.68 (s, 1H, H-6); 8.13 (bs, 1H, H-2).

^{13}C NMR (125.7 MHz, CD_3OD): 37.16, 39.78 ((CH$_3$)$_2$N); 41.66 (CH$_2$-2′); 63.64 (CH$_2$-5′); 68.17 (C-1-cp); 71.71 (CH-3,4-cp); 72.69 (CH-2,5-cpCONMe$_2$); 73.03 (CH-3′); 73.63, 73.64 (CH-3,4-cpCONMe$_2$); 73.93 (CH-2,5-cp); 80.11, 80.12 (C5-**C**≡C-cp, C-1-cpCONMe$_2$); 86.61 (CH-1′); 89.24 (CH-4′); 90.38 (C5-C≡**C**-cp); 97.85 (C-5); 104.54 (C-4a); 127.76 (CH-6); 150.00 (C-7a); 153.30 (CH-2); 159.31 (C-4); 172.13 (CONH$_2$).

MS (ESI-): *m/z* (%):530.153 (100) [M]; HRMS (ESI-): calcd. 530.1485 for $C_{26}H_{28}O_4N_5Fe$, found 530.1486.

5-[1'-(N,N-dimethylaminocarbonyl)-ferrocene-1-yl-ethynyl[-2'-deoxycytidine (dC$^{Fc(am)}$)

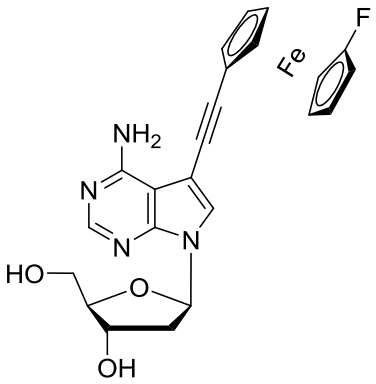


Compound **dC$^{Fc(am)}$** was prepared from **dC^{I}** according to general procedure (Method B). The product was isolated as a yellow solid (48 mg, 98%); ^{1}H NMR (500.0 MHz, CD_3OD): 2.17 (dt, 1H, J_{gem} = 13.6, $J_{2'b,1'}$ = $J_{2'b,3'}$ = 6.3, H-2′b); 2.41 (ddd, 1H, J_{gem} = 13.6, $J_{2'a,1'}$ = 6.3, $J_{2'a,3'}$ = 4.1, H-2′a); 3.01, 3.30 (2 × bs, 2 × 3H, (CH$_3$)$_2$N); 3.77 (dd, 1H, J_{gem} = 12.1, $J_{5'b,4'}$ = 3.5, H-5′b); 3.87 (dd, 1H, J_{gem} = 12.1, $J_{5'a,4'}$ = 3.0, H-5′a); 3.96 (ddd, 1H, $J_{4',3'}$ = 4.1, $J_{4',5'}$ = 3.5, 3.0, H-4′); 4.37 – 4.39 (m, 2H, H-3,4-cp); 4.40 (dt, 1H, $J_{3',2'}$ = 6.3, 4.1, $J_{3',4'}$ = 4.1, H-3′); 4.48 – 4.50 (m, 2H, H-2,5-cpCONMe$_2$); 4.56 – 4.58 (m, 2H, H-2,5-cp); 4.74 – 4.76 (m, 2H, H-3,4-cpCONMe$_2$); 6.24 (t, 1H, $J_{1',2'}$ = 6.3, H-1′); 8.42 (s, 1H, H-6).

^{13}C NMR (125.7 MHz, CD_3OD): 37.22, 39.80 ((CH$_3$)$_2$N); 42.47 (CH$_2$-2′); 62.42 (CH$_2$-5′); 67.78 (C-1-cp); 71.55, 71.56 (CH-3,4-cp); 71.67 (CH-3′); 72.57, 72.58 (CH-2,5-cpCONMe$_2$); 73.93 (CH-3,4-cpCONMe$_2$); 74.24, 74.25 (CH-2,5-cp); 77.99 (C5-**C**≡C-cp); 80.16 (C-1-cpCONMe$_2$); 87.91 (CH-1′); 89.07 (CH-4′); 93.34 (C-5); 93.58 (C5-C≡**C**-cp); 145.45 (CH-6); 156.71 (C-2); 166.151 (C-4); 172.14 (CONH$_2$).

MS (ESI-): *m/z* (%):507.1 (36) [M]; 529.1 (100) [M+Na]; HRMS (ESI-): calcd. 507.1325 for $C_{24}H_{27}O_5N_4Fe$, found 507.1323

7-(1'fluoro-ferrocene-1-yl-ethynyl)-7-deaza-2'-deoxyadenosine (dA^{FcF})



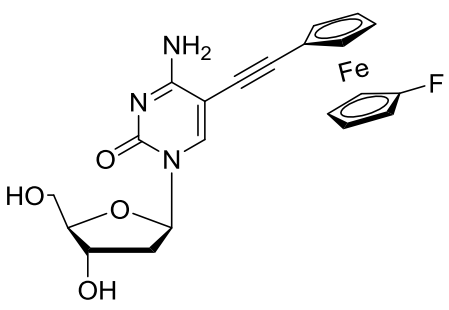
Compound **dA^{FcF}** was prepared from **dA^{I}** according to general procedure (Method B). The product was isolated as a yellow solid (51 mg, 98%); ^{1}H NMR (500.0 MHz, CD_3OD): 2.34 (ddd, 1H, J_{gem} = 13.7, $J_{2'b,1'}$ = 6.0, $J_{2'b,3'}$ = 2.7, H-2′b); 2.65 (ddd, 1H, J_{gem} = 13.7, $J_{2'a,1'}$ = 8.1, $J_{2'a,3'}$ = 5.9, H-2′a); 3.73 (dd, 1H, J_{gem} = 12.1, $J_{5'b,4'}$ = 3.6, H-5′b); 3.81 (dd, 1H, J_{gem} = 12.1, $J_{5'a,4'}$ = 3.3, H-5′a); 3.94 – 3.96 (m, 2H, H-2,5-cpF); 4.02 (ddd, 1H, $J_{4',5'}$ = 3.6, 3.3 $J_{4',3'}$ = 2.7, H-4′); 4.40 – 4.42 (m, 2H, H-3,4-cp); 4.43 – 4.45 (m, 2H, H-3,4-cpF); 4.53 (dt, 1H, $J_{3',2'}$ = 5.9, 2.7, $J_{3',4'}$ = 2.7, H-3′); 4.63 – 4.65 (m, 2H, H-2,5); 6.52 (dd, 1H, $J_{1',2'}$ = 8.1, 6.0, H-1′); 7.64 (s, 1H, H-6); 8.12 (bs, 1H, H-2).

^{13}C NMR (125.7 MHz, CD_3OD): 41.59 (CH_2-2′); 58.68 (d, $J_{C,F}$ = 15.3, CH-3,4-cpF); 63.64 (CH_2-5′); 64.25 (d, $J_{C,F}$ = 3.9, CH-2,5-cpF); 67.76 (C-1-cp); 71.37 (CH-3,4-cp); 73.02 (CH-3′); 73.56 (CH-2,5-cp); 79.57 (C5-**C**≡C-cp); 86.66 (CH-1′); 89.22 (CH-4′); 90.79 (C5-C≡**C**-cp); 97.87 (C-5); 104.64 (C-4a); 127.61 (CH-6); 136.96 (d, $J_{C,F}$ = 268.3, C-1-cpF); 149.89 (C-7a); 153.25 (CH-2); 159.34 (C-4).

^{19}F NMR (470.4 MHz, CD_3OD): -186.85.

MS (ESI-): *m/z* (%):477.1 (100) [M]; 499.1 (50) [M+Na]; HRMS (ESI-): calcd. 477.1020 for $C_{23}H_{22}O_3N_4FFe$, found 477.1018, calcd. 499.0839 for $C_{23}H_{21}O_3N_4FFeNa$, found 499.0836

5-(1'fluoro-ferrocene-1-yl-ethynyl)-2'-deoxycytidine (dC^{FcF})



Compound **dC^{FcF}** was prepared from **dC^{I}** according to general procedure (Method B). The product was isolated as a yellow solid (30 mg, 92%); ^{1}H NMR (500.0 MHz, CD_3OD): 2.17 (dt, 1H, J_{gem} = 13.6, $J_{2'b,1'}$ = $J_{2'b,3'}$ = 6.3, H-2′b); 2.40 (ddd, 1H, J_{gem} = 13.6, $J_{2'a,1'}$ = 6.3, $J_{2'a,3'}$ = 4.1, H-2′a); 3.76 (dd, 1H, J_{gem} = 12.1, $J_{5'b,4'}$ = 3.6, H-5′b); 3.85 (dd, 1H, J_{gem} = 12.1, $J_{5'a,4'}$ = 3.1, H-5′a); 3.94 (td, 2H, $J_{2\&5,3\&4}$ = 2.1, $J_{H,F}$ = 1.3, H-2,5-cpF); 3.95 (ddd, 1H, $J_{4',3'}$ = 4.1, $J_{4',5'}$ = 3.6, 3.1, H-4′); 4.39 (dt, 1H, $J_{3',2'}$ = 6.3, 4.1, $J_{3',4'}$ = 4.1, H-3′); 4.40 (t, 2H, $J_{3\&4,2\&5}$ = 1.9, H-3,4-cp); 4.44 (dt, 2H, $J_{H,F}$ = 2.7, $J_{3\&4,2\&5}$ = 2.1, H-3,4-cpF); 4.65 (t, 2H, $J_{2\&5,3\&4}$ = 1.9, H-2,5); 6.24 (t, 1H, $J_{1',2'}$ = 6.3, H-1′); 8.38 (s, 1H, H-6).

^{13}C NMR (125.7 MHz, CD_3OD): 42.42 (CH_2-2′); 58.69 (d, $J_{C,F}$ = 17.5, CH-3,4-cpF); 62.40 (CH_2-5′); 64.44 (d, $J_{C,F}$ = 4.0, CH-2,5-cpF); 67.26 (C-1-cp); 71.42 (CH-3,4-cp); 71.65 (CH-3′); 73.79, 73.81 (CH-2,5-cp); 77.32 (C5-**C**≡C-cp); 87.92 (CH-1′); 89.05 (CH-4′); 93.42 (C-5); 94.22 (C5-C≡**C**-cp); 136.92 (d, $J_{C,F}$ = 268.3, C-1-cpF); 145.25 (CH-6); 156.71 (C-2); 166.15 (C-4).

^{19}F NMR (470.4 MHz, CD_3OD): -187.33.

MS (ESI-): *m/z* (%):454.1 (10) [M]; 476.1 (100) [M+Na]; HRMS (ESI-): calcd. 454.0860 for $C_{21}H_{21}O_4N_3FFe$, found 454.0858; calcd. 476.0680 for $C_{21}H_{21}O_4N_3FFeNa$, found 476.0678

6.3.3 Synthesis of ferrocene-labeled dNTPs

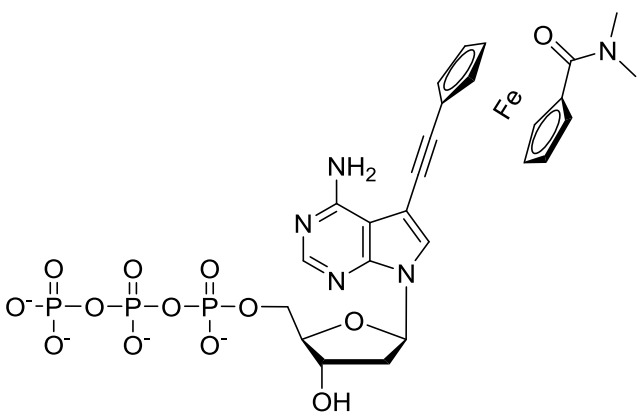
Synthesis of modified nucleoside triphosphates - Sonogashira cross-coupling:

Method A: A 1:1 mixture of H_2O-CH_3CN (2 mL) was added through a septum to an argon-purged flask containing a halogenated nucleotide **dN^{I}TP** (1 equiv.),**FcX** (1.5 equiv.), CuI (10 mol-%), Et_3N (10 equiv.), PPh_3 (2 mol-%) and [$Pd(PPh_3)_2Cl_2$] (5 mol-%). The flask was evacuated and purged with argon, and then the reaction mixture was stirred at 60ºC for 1 h until complete consumption of the starting material and then evaporated in vacuo. Product was isolated from the crude reaction mixture by HPLC on a C18 column with the use of a linear gradient of 0.1 M TEAB (triethylammonium bicarbonate) in H_2O to 0.1 M TEAB in H_2O–MeOH (1 : 1) as eluent. Several co-distillations with water followed by freeze-drying from water gave a solid product.

Synthesis of modified nucleoside triphosphates - Triphosphorylation:

Method B: $POCl_3$(2.5 equiv) in $PO(OMe)_3$ (1 ml) was added through a septum to an argon-purged flask containing modified nucleosides **dN^{EPT}**(1 equiv.), Reaction mixture was then stirred at 0ºC for 12 h until complete consumption of the starting material. Then an ice-cooled solution of $(NHBu_3)_2H_2P_2O_7$ (5 equiv) and Bu_3N (4.2 equiv) in dry DMF (2 ml) was added and the mixture was stirred at 0°C for another 1.5 h. The reaction was quenched by addition of 2 M aqueous TEAB (2 ml) and the solvents were evaporated in vacuo and the residue was co-distilled with water three times. The product was isolated by HPLC on a C18 column with the use of a linear gradient of 0.1 M TEAB (triethylammonium bicarbonate) in H_2O to 0.1 M TEAB in H_2O–MeOH (1 : 1) as eluent. Several co-distillations with water followed by freeze-drying from water gave solid product.

7-[1'-(N,N-dimethylaminocarbonyl)-ferrocene-1-yl-ethynyl]-7-deaza-2'-deoxyadenosine 5'-*O*-triphosphate (dA$^{Fc(am)}$TP)



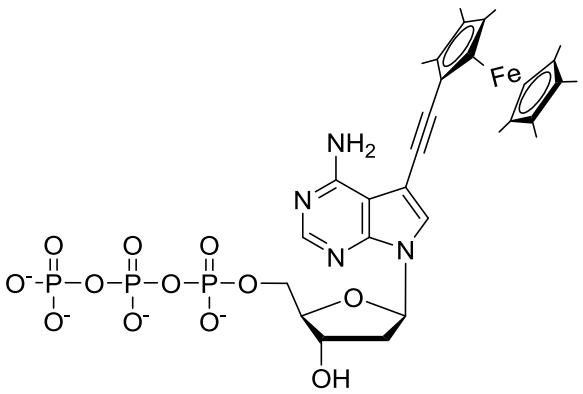
Compound **dA$^{Fc(am)}$TP** was prepared from **dA$^{Fc(am)}$** according to general triphosphorylation procedure (Method B). The product was isolated as a yellow solid (8 mg, 11%); ^{1}H NMR (500.0 MHz, D_2O, ref(dioxane) = 3.75 ppm): 1.27 (bm, 36H, C**H$_3$**CH$_2$N); 2.49 (bm, 1H, H-2′b); 2.72 (dt, 1H, J_{gem} = 14.0, $J_{2'a,1'}$ = $J_{2'a,3'}$ = 7.2, H-2′a); 2.78 (bs, 3H, $(CH_3)_2$N); 3.18 (bm, 24H, CH_3C**H$_2$**N); 3.30 (bs, 3H, $(CH_3)_2$N); 4.14, 4.20 (2 × dt, 2 × 1H, J_{gem} = 11.8, $J_{5',4'}$ = $J_{H,P}$ = 4.8, H-5′b); 4.25 (bm, 1H, H-4′); 4.39 – 4.46 (m, 2H, H-3,4-cp); 4.54 – 4.58 (m, 2H, H-2,5-cpCONMe$_2$); 4.60 – 4.65 (m, 2H, H-2,5-cp); 4.76 – 4.87 (m, 3H, H-3′, H-3,4-cpCONMe$_2$); 6.57 (t, 1H, $J_{1',2'}$ = 7.2, H-1′); 7.71 (s, 1H, H-6); 8.13 (bs, 1H, H-2).

^{13}C NMR (125.7 MHz, D_2O, ref(dioxane) = 69.3 ppm): 11.02 (**C**H_3CH_2N); 39.74 ($(CH_3)_2N$); 41.12 (CH_2-2′); 42.13 ($(CH_3)_2N$); 49.36 (CH_3**C**H_2N); 68.22 (d, $J_{C,P}$ = 5.3, CH_2-5′); 68.70 (C-1-cp); 73.50, 73.55 (CH-3,4-cp); 73.68 (CH-3′); 74.88, 74.96 (CH-2,5-cpCONMe$_2$); 75.21, 75.25 (CH-3,4-cpCONMe$_2$); 75.34, 75.36 (CH-2,5-cp); 80.22, 81.63 (C5-**C**≡C-cp, C-1-cpCONMe$_2$); 85.58 (CH-1′); 88.15 (d, $J_{C,P}$ = 8.7, CH-4′); 92.55 (C5-C≡**C**-cp); 99.93 (C-5); 105.59 (C-4a); 128.12 (CH-6); 151.49 (C-7a); 155.19 (CH-2); 160.33 (C-4); 174.54 ($CONH_2$).

^{31}P{^{1}H} NMR (202.3 MHz, D_2O): -21.99 (bdd, J = 17.6, 15.6, P_b); -10.57 (bd, J = 17.6, P_a); -5.85 (bd, J = 15.6, P_γ).

m/z (%): 768,0 [M-H]; HRMS (ESI-): calcd 768.0330 for $C_{26}H_{29}O_{13}N_5FeP_3$, found 768.0340.

7-(1',2,2',3,3',4,4',5-octamethylferrocene-1-yl-ethynyl)-7-deaza-2'-deoxyadenosine 5'-*O*-triphosphate (dA^{FcM}TP)

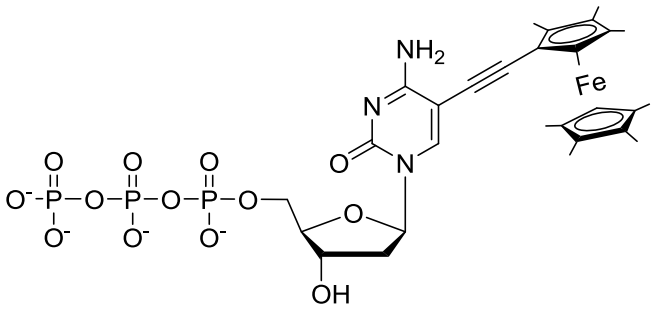


Compound **dA^{FcM}TP** was prepared from dA^{I}TP by aqueous Sonogashira cross-coupling (Method A, 20mg, 38%) and from **dA^{FcM}** according to general triphosphorylation procedure (Method B, 8mg, 15%). The product was isolated as a yellow solid. ^{1}H and ^{13}C NMR spectra were not possible to analyze because of partial oxidation of ferrocene moiety.

^{31}P{^{1}H} NMR (162 MHz, D_2O): -20.41 (t, J = 19,6 P_b); -10.29 (d, J = 19.6, P_a); -4.10 (d, J = 20.2, P_γ).

m/z (%): 729.2 (100) [M-H_2PO_3]; 649.2 (60) [M-$H_3P_2O_6$]; HRMS (ESI-): calcd 808.1132 for $C_{31}H_{39}O_{12}N_4FeP_3$, found 808.1119.

5-(1',2,2',3,3',4,4',5-octamethylferrocene-1-yl-ethynyl)-2'-deoxycytidine 5'-*O*-triphosphate (dC^{FcM}TP)

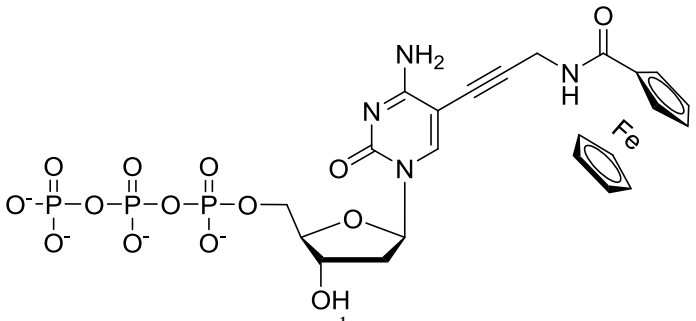


Compound **dC^{FcM}TP** was prepared from dC^{I}TP by aqueous Sonogashira cross-coupling (Method A, 18mg, 30%) and from **dC^{FcM}** according to general triphosphorylation procedure (Method B, 7mg, 20%). The product was isolated as a yellow solid. The product was isolated as a yellow solid. ^{1}H and ^{13}C NMR spectra were not possible to analyze because of partial oxidation of ferrocene moiety.

^{31}P{^{1}H} NMR (162 MHz, D_2O): -19.62 (t, J = 20.5, P_b); -9.40 (d, J = 20.1, P_a); -3.48 (d, J = 20.5, P_γ).

m/z (%):706.1 (100) [M-H_2PO_3]; 626.2 (50) [M-$H_3P_2O_6$]; HRMS (ESI-): calcd. 785.0972 for $C_{29}H_{38}O_{13}N_3FeP_3$, found 785.0964.

5-[3-(ferrocene-1-carboxamido)prop-1-yn-1-yl]-2'-deoxycytidine 5'-*O*-triphosphate (dC^{FcPa}TP)



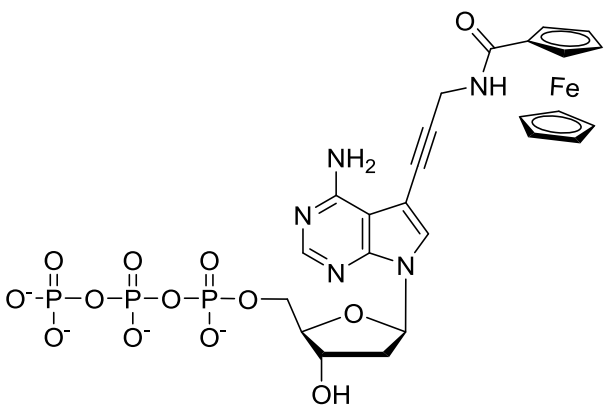
Compound **dC^{FcPa}TP** was prepared from dC^{I}TP by aqueous Sonogashira cross-coupling (Method A, 10mg, 16%) and from **dC^{FcPa}** according to general triphosphorylation procedure (Method B, 20mg, 18%). The product was isolated as a yellow solid. ^{1}H NMR (500.0 MHz, D_2O, ref(dioxane) = 3.75 ppm): 1.26 (bm, 36H, C**H$_3$**CH$_2$N); 2.26 (dt, 1H, J_{gem} = 13.7, $J_{2'b,1'}$ = 6.8, $J_{2'b,3'}$ = 6.4, H-2′b); 2.40 (ddd, 1H, J_{gem} = 13.7, $J_{2'a,1'}$ = 6.1, $J_{2'a,3'}$ = 4.7, H-2′a); 3.17 (bm, 24H, CH$_3$C**H$_2$**N); 4.17 (m, 1H, H-4′); 4.18 – 4.26 (m, 2H, H-5′); 4.26 – 4.33 (m, 7H, CH$_2$N, H-cp); 4.54 (m, 2H, H-2″,5″); 4.58 (dt, H, $J_{3',2'}$ = 6.4, 4.7, $J_{3',4'}$ = 4.7, H-3′); 4.85 (m, 2H, H-3″,4″); 6.22 (dd, 1H, $J_{1',2'}$ = 6.8, 6.1, H-1′); 8.18 (s, 1H, H-6).

^{13}C NMR (125.7 MHz, D_2O, ref(dioxane) = 69.3 ppm): 10.98 (**C**H$_3$CH$_2$N); 32.52 (CH$_2$N); 42.02 (CH$_2$-2′); 49.33 (CH$_3$**C**H$_2$N); 67.75 (d, $J_{C,P}$ = 4.6, CH$_2$-5′); 71.30, 71.34 (CH-3″,4″); 72.69 (CH-3′); 72.84 (CH-cp); 74.64, 74.65 (CH-2″,5″); 75.73 (C1″, C5-**C**≡C-CH$_2$); 88.21 (d, $J_{C,P}$ = 8.6, CH-4′); 88.82 (CH-1′); 95.05 (C-5); 95.60 (C5-C≡**C**-CH$_2$); 147.67 (CH-6); 158.68 (C-2); 167.83 (C-4); 177.10 (CONH).

^{31}P NMR (202.3 MHz, D_2O): -22.42 (t, J = 20.0, P_b); -10.83 (d, J = 20.0, P_a); -8.33 (bd, J = 20.0, P_γ).

MS (ESI-): 731.0 [M-H]; 651.0 [M-H$_2$PO$_3$]; HRMS (ESI-): calcd. 731.0013 for $C_{23}H_{26}O_{14}N_4FeP_3$, found 731.0006.

7-[3-(ferrocene-1-carboxamido)prop-1-yn-1-yl]-7-deaza-2'-deoxyadenosine 5'-*O*-triphosphate (dA^{FcPa}TP)



Compound **dA^{FcPa}TP** was prepared from dA^{I}TP by aqueous Sonogashira cross-coupling (Method A, 8mg, 13%) and from **dA^{FcPa}** according to general triphosphorylation procedure (Method B, 25 mg, 22%). The product was isolated as a yellow solid.^{1}H NMR (500.0 MHz, D_2O, ref(dioxane) = 3.75 ppm): 1.20 (bm, 36H, C**H$_3$**CH$_2$N); 2.43 (ddd, 1H,

J_{gem} = 13.7, $J_{2'b,1'}$ = 6.0, $J_{2'b,3'}$ = 3.4 H-2′b); 2.61 (ddd, 1H, J_{gem} = 13.7, $J_{2'a,1'}$ = 7.8, $J_{2'a,3'}$ = 6.0, H-2′a); 3.11 (bm, 24H, $CH_3C\mathbf{H_2}N$); 4.04 – 4.13 (m, 2H, H-5′); 4.17 (q, 1H, $J_{4',3'} = J_{4',5'}$ = 3.4, H-4′); 4.22 (bs, 5H, H-cp); 4.26 (bs, 2H, CH_2N); 4.46 (m, 2H, H-2″,5″); 4.68 (dt, H, $J_{3',2'}$ = 6.0, 3.4, $J_{3',4'}$ = 3.4, H-3′); 4.79 (bs, 2H, H-3″,4″); 6.46 (dd, 1H, $J_{1',2'}$ = 7.8, 6.0, H-1′); 7.60 (s, 1H, H-6); 8.03 (bs, 1H, H-2).

^{13}C NMR (125.7 MHz, D_2O, ref(dioxane) = 69.3 ppm): 10.94 ($\mathbf{C}H_3CH_2N$); 32.68 (CH_2N); 41.15 (CH_2-2′); 42.97 ($CH_3\mathbf{C}H_2N$); 68.28 (d, $J_{C,P}$ = 4.9, CH_2-5′); 71.19, 71.21 (CH-3″,4″); 72.74 (CH-cp); 73.69 (CH-3′); 74.50 (CH-2″,5″); 75.88 (C-1″); 7.47 (C5-**C**≡C-CH_2); 85.50 (CH-1′); 87.96 (d, $J_{C,P}$ = 8.7, CH-4′); 92.15 (C5-C≡**C**-CH_2); 99.01 (C-5); 105.56 (C-4a); 128.80 (CH-6); 151.18 (C-7a); 154.90 (CH-2); 159.84 (C-4); 176.97 (CONH).

^{31}P NMR (202.3 MHz, D_2O): -22.36 (bt, J = 19.4, P_b); -10.75 (d, J = 19.4, P_a); - 7.87 (bd, J = 19.4, P_γ).

MS (ESI-): 754.0 [M-H]; 674.0 [M-H_2PO_3]; HRMS (ESI-): calcd. 754.0173 for $C_{25}H_{27}O_{13}N_5FeP_3$, found 54.0169.

6.3.4 Analysis and isolation of PEX products

Primer extension experiment:

Single incorporation:

Method A: reaction mixture (20 µL) contained temp^A (3 µM, 1 µl), 5′-(FAM)-labeled primer^{rnd} (3 µM, 1.5 µl), dGTP (4 mM, 0.1µl), either dATP or dA^{XFc}TP (4 mM, 1 µl) KOD XL DNA polymerase (0.125 U) and reaction buffer (2 µl) supplied by manufacturer. The reaction mixture was incubated for 40 minutes at 60 °C. The PEX reaction was stopped by the addition of PAGE stop solution [20 µL, formamide (80%, v/v), EDTA (20 mM), bromophenol blue (0.025%, w/v), xylene cyanol (0.025%,w/v) and Milli-Q water] and heated for 2 minutes at 95 °C. Samples were analyzed by use of a 12.5 % denaturizing polyacrylamide gel electrophoresis (1 h, 50 °C) and visualized by fluorescence imaging.

Method B: PEX reactions with temp^C were performed in the same way as described for temp^A exept either dCTP or **dC^{XFc}TP** (4 mM, 1µl) were used.

Multiple incorporation:

The reaction mixture (20 µL) contained template^{rnd16} (3 µM, 1µl), 5′-(FAM)-labeled primer^{rnd} (3 µM, 1.5 µl), dNTP (either natural or modified, 4 mM, 1µl), KOD XL DNA polymerase (0.25 U) and reaction buffer (2 µl) supplied by manufacturer. The reaction mixture was incubated for 40

minutes at 60 °C. The PEX reaction was stopped by the addition of PAGE stop solution [20 µL, formamide (80%, v/v), EDTA (20 mM), bromophenol blue (0.025%, w/v), xylene cyanol (0.025%,w/v) and Milli-Q water] and heated for 2 minutes at 95 °C. Samples were analyzed by use of a 12.5 % denaturizing polyacrylamide gel electrophoresis (1h, 50 °C) and visualized by a fluorescence imaging.

Kinetics of incorporation of modified dNTPs:

Rate of incorporation was compared by preparation of samples with natural and modified dNTPs with various time periods. PEX reaction mixture (20 µl) was performed with 5′-(FAM)-labeled $prim^{rnd}$ (3 µM, 1 µl), $temp^{termA}$ (3 µM, 1.5µl) or $temp^{termC}$ (3 µM, 1.5 µl), dNTPs (4 mM, 1 µl) with KOD XL polymerase (0.125 U) in enzyme reaction buffer (1 µL) supplied by the manufacturer by followed stopping of reaction by using of PAGE stop solution [20µL, formamide (80%, v/v), EDTA (20 mM), bromophenol blue (0.025%, w/v), xylene cyanol (0.025%,w/v) and Milli-Q water] and immidiate heating for 2 minutes at 95 °C. Samples were analyzed by use of a 12.5 % denaturizing polyacrylamide gel electrophoresis (1h, 50 °C) and visualized by a fluorescence imaging.

6.3.5 Electrochemical analysis .

Nucleosides were analyzed by conventional in situ square-wave voltammetry (SWV). SWV settings: initial potential -0.5 V, end potential +1.5 V, frequency 200 Hz, amplitude 50 mV. Background electrolyte: 0.2m sodium acetate pH 5.0. All measurements were performed at room temperature using an Autolab analyzer (Eco Chemie, The Netherlands) in connection with VA-stand 663 apparatus (Metrohm, Herisau, Switzerland). The three-electrode system was used with an Ag/AgCl/3m KCl electrode as a reference and platinum wire as an auxiliary electrode.

7 Appendices

Appendix 1. Full gels of TdT elongation

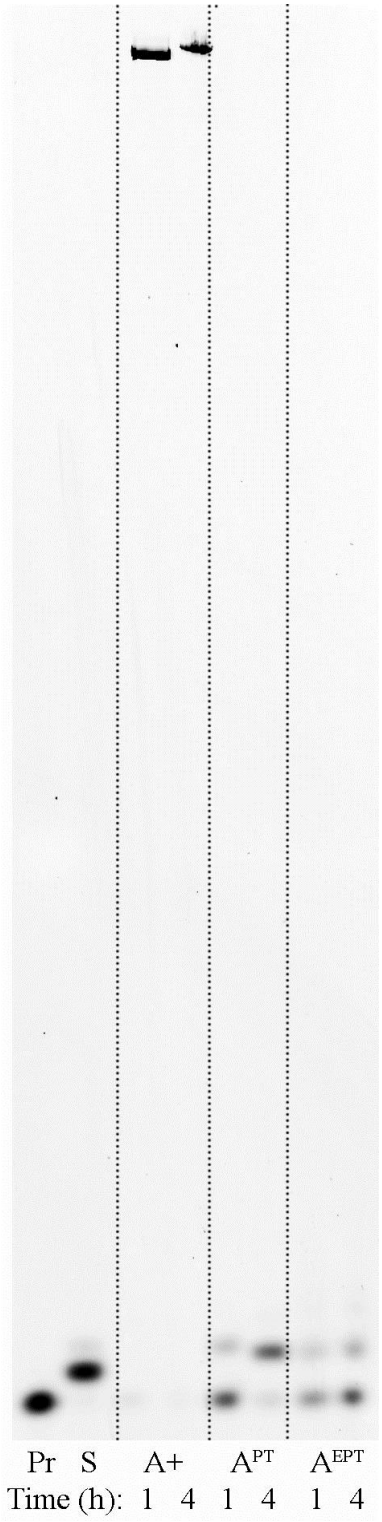


Figure 57. TdT-catalyzed DNA chain elongation. Pr: primer; S-standard (PEX product of temp^{termA} with dATP); A+, A^{PT} and A^{EPT}: products of primer^{rnd} elongation using terminal transferase and either dATP, **dA^{PT}TP** or **dA^{EPT}TP** respectively. Time intervals are given in hours.

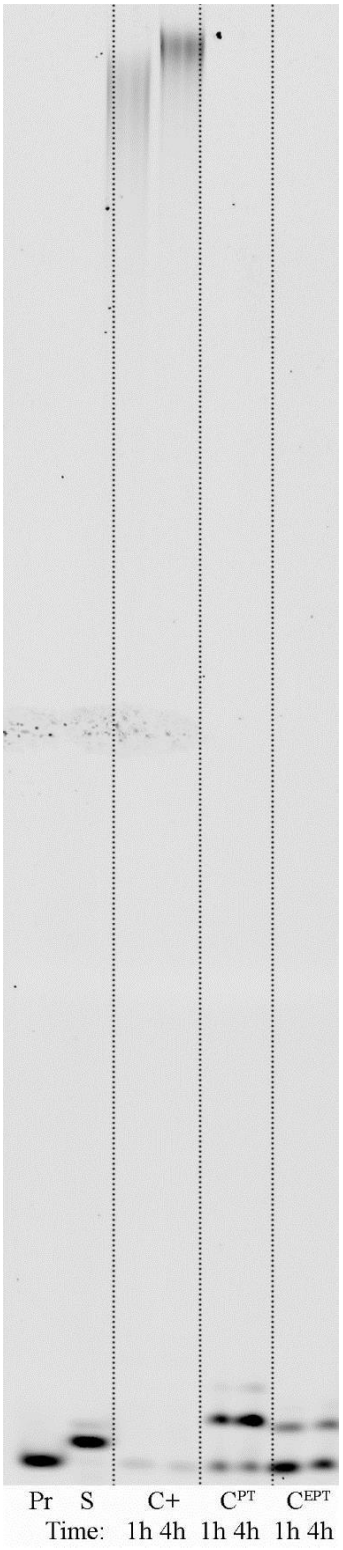


Figure 58. TdT-catalyzed DNA chain elongation. Pr: primer; S-standard (PEX product of temp^{termC} with dCTP); C+, C^{PT} and C^{EPT}: products of primer^{rnd} elongation using terminal transferase and either dCTP, **$dC^{PT}TP$** or **$dC^{EPT}TP$** respectively. Time intervals are given in hours.

8 References

1. R. Dahm, *Human Genetics*, 2008, **122**, 565–581.
2. J. D. Watson, and F. H. C. Crick, *Nature, 1953,* **171**, 737-738
3. G. M. Blackburn, M. J. Gait, D. Loakes and D. M. Williams, *Nucleic acids in chemistry and biology*, RSC Pub, Cambridge, UK, 3rd ed., 2006.
4. A.-C. Syvänen, *Nature Reviews Genetics*, 2001, **2**, 930–942.
5. J. M. Heather and B. Chain, *Genomics*, 2016, **107**, 1–8.
6. M. Fakruddin, K. B. Mannan, M. Hossain, S. Islam, R. Mazumdar, A. Chowdhury and M. Chowdhury, *Journal of Pharmacy and Bioallied Sciences*, 2013, **5**, 245.
7. H. G. Khorana, K. L. Agarwal, H. Buchi, M. H. Caruthers, N. K. Gupta, K. Kleppe, A. Kumar, E. Ohtsuka, U. L. RajBhandary, J. H. Van de Sande, V. Suramella, T. Terao, H. Weber and T. Yamada, *Journal of Molecular Biology*, 1972, **72**, 209-217
8. D. A. Malyshev, K. Dhami, T. Lavergne, T. Chen, N. Dai, J. M. Foster, I. R. Corrêa and F. E. Romesberg, *Nature*, 2014, **509**, 385–388.
9. A. M. Michelson and Sir A. R. Tod, *Journal of the Chemical Society*, 1955, 2632-2638.
10. R. H. Hall, Sir A. Todd and R. F. Webb, *Journal of the Chemical Society*, 1957, 3291-3296
11. P. T. Gilham and H. G. Khorana, *Journal of the American Chemical Society*, 1958, **80**, 6212–6222.
12. R. L. Letsinger and K. K. Ogilvie, *Journal of the American Chemical Society*, 1969, **91**, 3350–3355.
13. C. B. Reese, Tetrahedron, 1978, **34**, 3143-3179.
14. R. L. Letsinger, J. L. Finnan, G. A. Heavner and W. B. Lunsford, *Journal of the American Chemical Society*, 1975, **97**, 3278–3279.
15. S. L. Beaucage and M. H. Caruthers, *Tetrahedron Letters*, 1981, **22**, 1859–1862.
16. M. D. Matteucci and M. H. Caruthers, *Journal of the American Chemical Society*, 1981, **103**, 3185–3191.
17. M. H. Caruthers, *Biochemical Society Transactions*, 2011, **39**, 575–580.
18. M. H. Caruthers, *Journal of Biological Chemistry*, 2013, **288**, 1420–1427.
19. E. M. LeProust, B. J. Peck, K. Spirin, H. B. McCuen, B. Moore, E. Namsaraev and M. H. Caruthers, *Nucleic Acids Research*, 2010, **38**, 2522–2540.
20. H. K. Schachman, J. Adler, C. M. Radding, I. R. Lehman and A. Kornberg, *Journal of Bioloqical Chemistry*, 1960, **235**, 3242-3249

21. K. Kleppe, E. Ohtsuka, R. Kleppe, I. Molweux, and H.G. Khorana, *Journal of Molecular Biology*, 1971, **56**, 341-361
22. G. Heilek, in *Nucleic Acids - From Basic Aspects to Laboratory Tools*, eds. M. L. Larramendy and S. Soloneski, InTech, 2016.
23. K. Reddington, N. Tuite, E. Minogue and T. Barry, *Biomolecular Detection and Quantification*, 2014, **1**, 3–7.
24. A. Keller and E. Meese, Eds., in *Nucleic Acids as Molecular Diagnostics*, Wiley-VCH, Weinheim, Germany, 2014.
25. E. A. Motea and A. J. Berdis, *Biochimica et Biophysica Acta*, 2010, **1804**, 1151–1166.
26. P. Horáková, H. Macíčková-Cahová, H. Pivoňková, J. Špaček, L. Havran, M. Hocek and M. Fojta, *Organic & Biomolecular Chemistry*, 2011, **9**, 1366.
27. K. B. Mullis and F. A. Faloona, Methods in Enzymology, 1987, **155**, 335-350.
28. J. M. S. Bartlett and D. Stirling, *PCR Protocols*, Second Edition.
29. M. Louie, L. Louie and A. E. Simor, Canadian Medical Association or its licensors, 2000, **163**, 301-309
30. P. Gill and A. Ghaemi, *Nucleosides, Nucleotides and Nucleic Acids*, 2008, **27**, 224–243.
31. Y. Zhao, F. Chen, Q. Li, L. Wang and C. Fan, *Chemical Reviews*, 2015, **115**, 12491–12545.
32. J. Van Ness, L. K. Van Ness and D. J. Galas, *Proceedings of the National Academy of Sciences*, 2003, **100**, 4504–4509.
33. P. Ménová and M. Hocek, *Chemical Communications*, 2012, **48**, 6921.
34. P. Ménová, D. Dziuba, P. Güixens-Gallardo, P. Jurkiewicz, M. Hof and M. Hocek, *Bioconjugate Chemistry*, 2015, **26**, 361–366.
35. V. Troger. K. Niemann, C. Gartig and D. Kuhlmeier, *Journal of Nanomedicine & Nanotechnology*, 2015, 6, 1-19.
36. F. Pertusat, M. Serpi and C. McGuigan, *Antiviral Chemistry and Chemotherapy*, 2012, **22**, 181–203.
37. Y. Zhang, Y. Gao, X. Wen and H. Ma, *Asian Journal of Pharmaceutical Sciences*, 2014, **9**, 65–74.
38. M. Hennig, L. G. Scott, E. Sperling, W. Bermel and J. R. Williamson, *Journal of the American Chemical Society*, 2007, **129**, 14911–14921.
39. J. Riedl, R. Pohl, L. Rulíšek and M. Hocek, *The Journal of Organic Chemistry*, 2012, **77**, 1026–1044.

40. I. Lee and A. J. Berdis, *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, 2010, **1804**, 1064–1080.
41. M. Hocek, *Chemical Reviews*, 2009, **109**, 6729–6764.
42. Y. Motorin, S. Seidu-Larry and M. Helm, in *DNA Methyltransferases - Role and Function*, , Springer International Publishing, 2016, **945**, 19–33.
43. F. Seela and A. Roling, *Nucleic Acids Research*, 1991, **20**, 55-61.
44. P. Kielkowski, J. Fanfrlík and M. Hocek, *Angewandte Chemie*, 2014, **126**, 7682–7685.
45. H. Cahová, A. Panattoni, P. Kielkowski, J. Fanfrlík and M. Hocek, *ACS Chemical Biology*, 2016, **11**, 3165–3171.
46. J. Matyašovský, P. Perlíková, V. Malnuit, R. Pohl and M. Hocek, *Angewandte Chemie International Edition*, 2016, **55**, 15856–15859.
47. M. Yoshikawa, T. Kato and T. Takenishi, Tetrahedron Letters, 1967, **50,** 5065-5068.
48. M. Yoshikawa, T. Kato and T. Takenishi, **42**, Bulletin of the Chemical Soceity of Japan, 1969, 42, 3505-3508.
49. J. Ludwig, *Acta Biochimica et Biophysica Academiae Scientiarum Hungaricae*, 1981, **16**, 131-133.
50. K. Burgess and D. Cook, *Chemical Reviews*, 2000, **100**, 2047–2060.
51. G. S. Cremosnik, A. Hofer and H. J. Jessen, *Angewandte Chemie International Edition*, 2014, **53**, 286–289.
52. S. Mohamady and S. D. Taylor, *Organic Letters*, 2016, **18**, 580–583.
53. J. G. Moffatt, *Canadian Journal of Chemistry*, 1964, **42**, 599–604.
54. J. H. van Boom, R. Crea, W. C. Luyten and A. B. Vink, *Tetrahedron Letters*, 1975, **32**, 2779-2782.
55. A. Simoncsits and J. Tomasz, *Nucleic Acids Research*, 1975, **2**, 1223–1234.
56. J. Ludwig and F. Eckstein, *The Journal of Organic Chemistry*, 1989, **54**, 631–635.
57. M. Saady, L. Lebeau and C. Mioskowski, *Tetrahedron Letters*, 1995, **36**, 5183-5186.
58. Y. Ahmadibeni and K. Parang, *Organic Letters*, 2005, **7**, 5589–5592.
59. K. Sonogashira, Y. Tohda and N. Hagihara, *Tetrahedron Letters*, 1975, **50**, 4467-4470.
60. M. J. Corr, S. V. Sharma, C. Pubill-Ulldemolins, R. T. Bown, P. Poirot, D. R. M. Smith, C. Cartmell, A. Abou Fayad and R. J. M. Goss, *Chemical Science*, 2017, **8**, 2039–2046.
61. D. Wang and S. Gao, *Org. Chem. Front.*, 2014, **1**, 556–566.
62. R. Chinchilla and C. Nájera, *Chemical Reviews*, 2007, **107**, 874–922.
63. A. Soheili, J. Albaneze-Walker, J. A. Murry, P. G. Dormer and D. L. Hughes, *Organic Letters*, 2003, **5**, 4191–4194.

64. N. Miyaura, K. Yamada and A. Suzuki, *Tetrahedron Letters*, 1979, **36**, 3437-3440
65. N. Miyaura and A. Suzuki, *Chemical Reviews*, 1995, **95**, 2457–2483.
66. S. S. Gujral, S. Khatri and P. Riyal, *Indo Global Journal of Pharmaceutical Sciences*, 2012, **2**, 351-367
67. A. L. Casalnuovo and J. C. Calabrese, *Journal of the American Chemical Society*, 1990, **112**, 4324–4330.
68. L. H. Thoresen, G.-S. Jiao, W. C. Haaland, M. L. Metzker and K. Burgess, *Chemistry - A European Journal*, 2003, **9**, 4603–4610.
69. N. K. Garg, C. C. Woodroofe, C. J. Lacenere, S. R. Quake and B. M. Stoltz, *Chemical Communications*, 2005, 4551.
70. N. Amann, E. Pandurski, T. Fiebig and H.-A. Wagenknecht, *Angewandte Chemie International Edition*, 2002, **41**, 2978.
71. E. C. Western, J. R. Daft, E. M. Johnson, P. M. Gannett and K. H. Shaughnessy, *The Journal of Organic Chemistry*, 2003, **68**, 6767–6774.
72. L. Lercher, J. F. McGouran, B. M. Kessler, C. J. Schofield and B. G. Davis, *Angewandte Chemie International Edition*, 2013, **52**, 10553–10558.
73. J. M. Chalker, C. S. C. Wood and B. G. Davis, *Journal of the American Chemical Society*, 2009, **131**, 16346–16347.
74. T. Kottysch, C. Ahlborn, F. Brotzel and C. Richert, *Chemistry - A European Journal*, 2004, **10**, 4017–4028.
75. S. Hauke, M. Best, T. T. Schmidt, M. Baalmann, A. Krause and R. Wombacher, *Bioconjugate Chemistry*, 2014, **25**, 1632–1637.
76. J. Riedl, P. Ménová, R. Pohl, P. Orság, M. Fojta and M. Hocek, *The Journal of Organic Chemistry*, 2012, **77**, 8287–8293.
77. D. Dziuba, P. Jurkiewicz, M. Cebecauer, M. Hof and M. Hocek, *Angewandte Chemie International Edition*, 2016, **55**, 174–178.
78. P. Kielkowski, H. Macíčková-Cahová, R. Pohl and M. Hocek, *Angewandte Chemie International Edition*, 2011, **50**, 8727–8730.
79. Z. Vaníková and M. Hocek, *Angewandte Chemie International Edition*, 2014, **53**, 6734–6737.
80. J. Dadová, P. Orság, R. Pohl, M. Brázdová, M. Fojta and M. Hocek, *Angewandte Chemie International Edition*, 2013, **52**, 10515–10518.
81. A. Olszewska, R. Pohl, M. Brázdová, M. Fojta and M. Hocek, *Bioconjugate Chemistry*, 2016, **27**, 2089–2094.

82. E. Palacek, Nature, 1960, 188, 656-657.
83. D. L. Smith and P. J. Elving, *Journal of the American Chemical Society*, 1962, **84**, 2741–2747.
84. D. L. Smith and P. J. Elving, *Journal of the American Chemical Society*, 1962, **84**, 1412–1420.
85. E. Paleček and M. Bartošík, *Chemical Reviews*, 2012, **112**, 3427–3481.
86. B. Janik and P. J. Elving, *Chemical Reviews*, 1968, **68**, 295–319.
87. T. Cummings, *Journal of Electroanalytical Chemistry*, 1978, **94**, 123–145.
88. T. E. Cummings and P. J. Elving, *Journal of Electroanalytical Chemistry*, 1979, **102**, 237-248.
89. M. V. B. Zanoni, E. I. Rogers, C. Hardacre and R. G. Compton, *Analytica Chimica Acta*, 2010, **659**, 115–121.
90. L. Trnková, *Talanta*, 2002, **56**, 887–894.
91. P. Singhal and W. G. Kuhr, *Analytical Chemistry*, 1997, **69**, 3552–3557.
92. E. E. Ferapontova and E. Domínguez, *Electroanalysis*, 2003, **15**, 629–634.
93. F. Jelen, M. Tomschik and E. Paleček, *Journal of Electroanalytical Chemistry*, 1997, **423**, 141–148.
94. A. Brotons, F. J. Vidal-Iglesias, J. Solla-Gullón and J. Iniesta, *Analytical Methods*, 2016, **8**, 702–715.
95. L. M. Gonçalves, C. Batchelor-McAuley, A. A. Barros and R. G. Compton, *The Journal of Physical Chemistry C*, 2010, **114**, 14213–14219.
96. A. M. Oliveira Brett and F.-M. Matysik, *Journal of Electroanalytical Chemistry*, 1997, **429**, 95–99.
97. C.-X. Ruan, J. Lou, Y.-Y. Duan and W. Sun, *Journal of the Chinese Chemical Society*, 2010, **57**, 1056–1060.
98. M. Arvand, R. Motaghed Mazhabi and A. Niazi, *Electrochimica Acta*, 2013, **89**, 669–679.
99. W. Sun, M. Xi, L. Zhang, T. Zhan, H. Gao and K. Jiao, *Electrochimica Acta*, 2010, **56**, 222–226.
100. T. A. Joshi, *International Journal of Research in Pharmacy and Chemistry*, 2011, **13**, 1015-1027
101. C. N. Jones, C. I. Jones, W. D. Graham, P. F. Agris and L. L. Spremulli, *Journal of Biological Chemistry*, 2008, **283**, 34445–34456.
102. E. Freese, *Journal of Molecular Biology*, 1959, **1**, 87–105.

103. B. P. O'Sullivan, S. D. Freedman, *Lancet*, 2009, **373**, 1891–1904.
104. J. H. J. Hoeijmakers, *Nature*, 2001, **411**, 366-374.
105. E. Serra, E. Ars, A. Ravella, A. Sánchez, S. Puig, T. Rosenbaum, X. Estivill and C. Lázaro, *Human Genetics*, 2001, **108**, 416–429.
106. C. F. Taylor and G. R. Taylor, in *Molecular Diagnosis of Genetic Diseases*, Humana Press, New Jersey, 2003, vol. 92, pp. 9–44.
107. H. Cahová, L. Havran, P. Brázdilová, H. Pivoňková, R. Pohl, M. Fojta and M. Hocek, *Angewandte Chemie International Edition*, 2008, **47**, 2059–2062.
108. M. Vrábel, P. Horáková, H. Pivoňková, L. Kalachova, H. Černocká, H. Cahová, R. Pohl, P. Šebest, L. Havran, M. Hocek and M. Fojta, *Chemistry - A European Journal*, 2009, **15**, 1144–1154.
109. J. Riedl, P. Horáková, P. Šebest, R. Pohl, L. Havran, M. Fojta and M. Hocek, *European Journal of Organic Chemistry*, 2009, **2009**, 3519–3525.
110. V. Raindlová, R. Pohl, B. Klepetářová, L. Havran, E. Šimková, P. Horáková, H. Pivoňková, M. Fojta and M. Hocek, *ChemPlusChem*, 2012, **77**, 652–662.
111. J. Dadová, P. Vidláková, R. Pohl, L. Havran, M. Fojta and M. Hocek, *The Journal of Organic Chemistry*, 2013, **78**, 9627–9637.
112. J. Balintová, R. Pohl, P. Horáková, P. Vidláková, L. Havran, M. Fojta and M. Hocek, *Chemistry - A European Journal*, 2011, **17**, 14063–14073.
113. J. Balintová, J. Špaček, R. Pohl, M. Brázdová, L. Havran, M. Fojta and M. Hocek, *Chemical Science*, 2015, **6**, 575–587.
114. M. Hocek and M. Fojta, *Chemical Society Reviews*, 2011, **40**, 5802.
115. E. Paleček, *Electrochemistry of nucleic acids and proteins: towards electrochemical sensors for genomics and proteomics*, Elsevier, Amsterdam, 2005.
116. J. Gajdar, E. Horakova, J. Barek, J. Fischer and V. Vyskocil, *Electroanalysis*, 2016, **28**, 2659–2671.
117. K. Cizek, J. Barek, J. Fischer, K. Peckova and J. Zima, *Electroanalysis*, 2007, **19**, 1295–1299.
118. V. Vyskocil and J. Barek, *Current Organic Chemistry*, 2011, **15**, 3059–3076.
119. P. Zuman, *Collection of Czechoslovak Chemical Communications*, 1993, **58**, 41–46.
120. K. Pecková, J. Barek, T. Navrátil, B. Yosypchuk and J. Zima, *Analytical Letters*, 2009, **42**, 2339–2363.
121. S. Akoudad, P. Frère, N. Mercier and J. Roncali, *The Journal of Organic Chemistry*, 1999, **64**, 4267–4272.

122. J. Balintová, M. Plucnara, P. Vidláková, R. Pohl, L. Havran, M. Fojta and M. Hocek, *Chemistry - A European Journal*, 2013, **19**, 12720–12731.
123. D. R. van Staveren and N. Metzler-Nolte, *Chemical Reviews*, 2004, **104**, 5931–5986.
124. B. Wu, M.-W. Chen, Z.-S. Ye, C.-B. Yu and Y.-G. Zhou, *Advanced Synthesis & Catalysis*, 2014, **356**, 383–387.
125. H. M. Peng and R. D. Webster, *The Journal of Organic Chemistry*, 2008, **73**, 2169–2175.
126. Y. Samet, R. Abdelhedi and A. Savall, *Physical and Chemical News*, 2002, **8**, 89-99.
127. H. Pivoňková, P. Horáková, M. Fojtová and M. Fojta, *Analytical Chemistry*, 2010, **82**, 6807–6813.
128. L. Qi and Y. Ding, *Science China Life Sciences*, 2013, **56**, 1020–1027.
129. M. J. Ohlow and B. Moosmann, *Drug Discovery Today*, 2011, **16**, 119–131.
130. S. C. Mitchell, *Current Drug Targets*, 2006, **7**, 1181-1189.
131. A. A. Golriz, T. Kaule, M. B. Untch, K. Kolman, R. Berger and J. S. Gutmann, *ACS Applied Materials & Interfaces*, 2013, **5**, 2485–2494.
132. N. Nakadan, S. Imabayashi and M. Watanabe, *Journal of Electroanalytical Chemistry*, 2009, **632**, 59–63.
133. N. Nakadan, S. Imabayashi and M. Watanabe, *Langmuir*, 2004, **20**, 8786–8791.
134. K. Mielech-Łukasiewicz, H. Puzanowska-Tarasiewicz and A. Panuszko, *Analytical Letters*, 2008, **41**, 789–805.
135. G. Viola, L. Latterini, D. Vedaldi, G. G. Aloisi, F. Dall'Acqua, N. Gabellini, F. Elisei and A. Barbafina, *Chemical Research in Toxicology*, 2003, **16**, 644–651.
136. M. T. Tierney and M. W. Grinstaff, *Organic Letters*, 2000, **2**, 3413–3416.
137. M. T. Tierney and M. W. Grinstaff, *The Journal of Organic Chemistry*, 2000, **65**, 5355–5359.
138. X. Hu, M. T. Tierney and M. W. Grinstaff, *Bioconjugate Chemistry*, 2002, **13**, 83–89.
139. S. A. N. Hashmi, X. Hu, C. E. Immoos, S. J. Lee and M. W. Grinstaff, *Organic Letters*, 2002, **4**, 4571–4574.
140. C. Wagner and H.-A. Wagenknecht, *Chemistry - A European Journal*, 2005, **11**, 1871–1876.
141. C. S. Krämer and T. J. J. Müller, *European Journal of Organic Chemistry*, 2003, **2003**, 3534–3548.
142. A. M. Debela, S. Thorimbert, B. Hasenknopf, C. K. O'Sullivan and M. Ortiz, *Chemical Communications*, 2016, **52**, 757–759.

143. A. M. Debela, M. R. Ortiz, V. Beni, D. Lesage, R. Cole, C. O'Sullivan, S. Thorimbert and B. Hasenknopf, *ECS Transactions*, 2017, **77**, 1873–1883.

144. K. Nesměrák, V. Červený, J. Hraníček and P. Rychlovský, *Microchemical Journal*, 2013, **106**, 226–232.

145. A. M. M. Rawashdeh, *Basic science and Engineering*, 2005, **14**, 195-208.

146. L. A. Tinker and A. J. Bard, *Journal of the American Chemical Society*, 1979, **101**, 2316–2319.

147. B. Paduszek and M. K. Kalinowski, *Electrochimica Acta*, 1983, **28**, 639-642.

148. P. Liu, M. Lu, Q. Zheng, Y. Zhang, H. D. Dewald and H. Chen, *The Analyst*, 2013, **138**, 5519.

149. H. Puzanowska-Tarasiewicz, L. Kuzmicka, J. Karpinska and K. Mielech-Lukasiewicz, *Analytical Sciences*, 2005, **21**, 1149–1153.

150. T. J. Kealy, P. L. Pauson, Nature, 1951, 1039-1040.

151. G. Wilkinson, M. Rosenblum, M. C. Whiting and R. B. Woodward, *Journal of the American Chemical Society*, 1952, **74**, 2125–2126.

152. E. O. Fischer and W. Pfab, *Zeitschrift für Naturforschung,* 1952, **7**, 377–379.

153. D. Astruc, *European Journal of Inorganic Chemistry*, 2017, **2017**, 6–29.

154. M. Gallei and C. Rüttiger, *Chemistry - A European Journal*, 2018, **24**, 10006–10021.

155. D. Kong, F. Liao, Y. Lin, L. Cheng, H. Peng, J. Zhang, H. Cui, N. Hong, C. Chen, G. Wei and H. Fan, *Sensors and Actuators B: Chemical*, 2018, **266**, 288–293.

156. H. Song, P. M. Diakowski, R. H. E. Hudson and H.-B. Kraatz, *Journal of Inorganic and Organometallic Polymers and Materials*, 2012, **22**, 178–182.

157. R. Ikeda, S. Kitagawa, J. Chiba and M. Inouye, *Chemistry - A European Journal*, 2009, **15**, 7048–7051.

158. P. Meunier, I. Ouattara, B. Gautheron, J. Tirouflet, D. Camboli, J. Besancon, *European Journal of Medicinal Chemistry*, 1991, **26**,351-362.

159. H. Song, X. Li, Y. Long, G. Schatte and H.-B. Kraatz, *Dalton Transactions*, 2006, 4696.

160. P. Brázdilová, M. Vrábel, R. Pohl, H. Pivoňková, L. Havran, M. Hocek and M. Fojta, *Chemistry - A European Journal*, 2007, **13**, 9527–9533.

161. B. Dou, J. Li, B. Jiang, R. Yuan and Y. Xiang, *Analytica Chimica Acta*, 2018, 1-7.

162. J.-B. Raoof and M. Kolbadinezhad, *Electroanalysis*, 2005, **17**, 2043–2051.

163. I. Noviandri, K. N. Brown, D. S. Fleming, P. T. Gulyas, P. A. Lay, A. F. Masters and L. Phillips, *Journal of Physical Chemistry B,* 1999**,** ***103,*** 6713-6722.

164. M. Hocek, P. Štěpnička, J. Ludvík, I. Císařová, I. Votruba, D. Řeha and P. Hobza, *Chemistry - A European Journal*, 2004, **10**, 2058–2066.

165. G. Ilyashenko, R. Al-Safadi, R. Donnan, R. Dubrovka, J. Pancholi, M. Watkinson and A. Whiting, *RSC Advances*, 2013, **3**, 17081.

166. T.-Y. Dong and L.-L. Lai, *Journal of Organometallic Chemistry*, 1996, **509**, 131–134.

167. M. S. Inkpen, S. Du, M. Driver, T. Albrecht and N. J. Long, *Dalton Trans.*, 2013, **42**, 2813–2816.

168. S. K. Ghag, M. L. Tarlton, E. A. Henle, E. M. Ochoa, A. W. Watson, L. N. Zakharov and E. J. Watson, *Organometallics*, 2013, **32**, 1851–1857.

169. C. Zou and M. S. Wrighton*, Journal of the American Chemical Society,* 1990, **112,** 7578-7584.

170. P. Jutzi and B. Kleinebekel, *Journal of Organometallic Chemistry*, 1997, **545**–**546**, 573–576.

171. A. E. Beilstein and M. W. Grinstaff, *Chemical Communications*, 2000, 509–510.

172. C. J. Yu, H. Yowanto, Y. Wan, T. J. Meade, Y. Chong, M. Strong, L. H. Donilon, J. F. Kayyem, M. Gozin and G. F. Blackburn, *Journal of the American Chemical Society*, 2000, **122**, 6767–6768.

173. M. J. Robins and P. J. Barr, *The Journal of Organic Chemistry*, 1983, **48**, 1854–1862.

174. P. Kielkowski, R. Pohl and M. Hocek, *The Journal of Organic Chemistry*, 2011, **76**, 3457–3462.

175. A. R. Pike, L. C. Ryder, B. R. Horrocks, W. Clegg, B. A. Connolly and A. Houlton, *Chemistry - A European Journal*, 2005, **11**, 344–353.

176. P. Čapek, H. Cahová, R. Pohl, M. Hocek, C. Gloeckner and A. Marx, *Chemistry - A European Journal*, 2007, **13**, 6196–6203.

177. W. H. Melhuish, *The Journal of Physical Chemistry*, 1961, **65**, 229–235.

178. D. Bulfield, M. Maschke, M. Lieb and N. Metzler-Nolte, *Journal of Organometallic Chemistry*, 2015, **797**, 125–130.