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Jevgenija
JAKUBOVSKA

Modified pyrimidine nucleotides for the synthesis of oligonucleotides

DOCTORAL DISSERTATION

Natural sciences,
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VILNIAUS UNIVERSITETAS

Jevgenija
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Modifikuotų pirimidino nukleotidų naudojimas oligonukleotidų sintezei

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Gamtos mokslai,
biochemija N004

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications (4 papers). The arrangement of publications is in agreement with Results and Disccusion part.

Paper I. Jakubovska, J., Tauraitė, D., Birštonas, L., Meškys, R. (2018) N^4-acyl-2′-deoxycytidine-5′-triphosphates for the enzymatic synthesis of modified DNA. *Nucleic Acids Res.*, **46**, 5911–5923.

Paper II. Tauraitė, D., Jakubovska, J., Dabužinskaitė, J., Bratchikov, M., Meškys, R. (2017) Modified nucleotides as substrates of terminal deoxynucleotidyl transferase. *Molecules*, **22**, 672.

Paper III. Jakubovska, J., Tauraitė, D., Meškys, R. (2019) Transient N^4-acyl-DNA protection against the cleavage by restriction endonucleases. *ChemBioChem*, **20**, doi:10.1002/cbic.201900280.

Paper IV. Jakubovska, J., Tauraitė, D., Meškys, R. (2018) A versatile method for the UVA-induced cross-linking of acetophenone-or benzophenone-functionalized DNA. *Sci. Rep.*, **8**, 16484.

LIST OF ABBREVIATIONS

(exo–)	exonuclease-deficient
AcBz	acetylbenzoyl
AP	acetophenone
BP	benzophenone
BS	borosilicate
BzBz	benzoylbenzoyl
$dC^{Acyl}TP$	N^4-acyl-2′-deoxycytidine triphosphate
dNTP	2′-deoxyribonucleoside 5′-triphosphate
EA	ethanolamine
KF (exo–)	Klenow fragment (exo–)
ON	oligonucleotide
PAGE	polyacrylamide gel electrophoresis
PDMS	polydimethylsiloxane
PEX	primer extension
PLA	polylactate
PP	polypropylene
PS	polystyrene
RE	restriction endonuclease
SSB	single-stranded DNA binding protein
TdT	terminal deoxynucleotidyl transferase

INTRODUCTION

Nucleotides bearing nucleobase modifications are widely used for the specific labeling of nucleic acids, bioconjugation, therapeutics and diagnostics, development of aptamers, DNAzymes, and biosensors, surface functionalization, as well as the construction of DNA nanomaterials and DNA-based nanodevices (1–24). Introduction of the functional groups into the oligonucleotides (ONs) can be achieved by the solid-phase synthesis, enzymatic synthesis or post-synthetic modification (25–27). Due to robustness, efficiency and simplicity modified ONs are mostly synthesized chemically by a standard phosphoramidite method on a solid support. However, chemical incompatibility with synthesis conditions, the limited selection of available phosphoramidites, complicated synthesis of long ONs (>100 nt), and the poor yield caused by difficult multistep synthesis broaden the development and utilization of other approaches. As such, ONs with a small functional group (amine, thiol, ethynyl, azide, etc.) introduced during the solid-phase synthesis can be further conjugated with a great variety of labels by a chemoselective reaction, a process called the post-synthetic modification. Indeed, the post-synthetic approaches can overcome some of the aforementioned limitations, yet it suffers from low chemoselectivity, high cross reactivity and troublesome attachment of more complex labels.

An alternative method for the synthesis of modified nucleic acids brings enzymes into play. Different DNA/RNA polymerases use modified (2′-deoxy)ribonucleoside 5′-triphosphates (dNTPs/NTPs) as substrates providing a variety of amplification techniqes such as PCR, primer extension (PEX), site-specific modification, terminal deoxynucleotidyl transferase (TdT)-catalyzed 3′-end tailing or nicking enzyme amplification reaction that can be employed (28–35). Even slightly unusual yet impressive methods for the nucleic acids labeling such as a polymerase ribozyme-catalyzed 3′-end tailing or programmable *de novo* DNA synthesis based on TdT have been proposed (36, 37). Such a diversity of enzymatic nucleic acid modification approaches ensures a strict programmability regarding the arrangement of the functional groups. In fact, one can (i) generate a modified nucleic acid molecule bearing either internal or external modifications, (ii) predetermine the exact quantity and location of functional groups, and (iii) choose if DNA should be labeled on one or both strands. Remarkably, polymerases are able to tolerate different types of modifications, including strong nucleophiles or electrophiles as well as massive cargos that sometimes might exceed the size of the polymerase itself, making it superior than the other methods.

Nevertheless, not all nucleobase modifications are acceptable and tolerable by polymerases. A number of factors determines the selection of a right nucleotide including steric effects, tighthness of the nucleotide binding pocket, hydrogen bonding, minor groove scanning and nucleobase stacking (38, 39). In general, the most favoured nucleobase positions for modifications are position 7 of 7-deazapurines as well as position 5 of pyrimidines, because they do not alter correct base-pairing (26, 40, 41). Since C5/C7 positions orient the modified residue toward the major groove of the double helix, this arrangement is commonly considered to minimize the interference with enzymatic activity. Therefore, such nucleotide analogues serve as good to excellent substrates for a variety of commercially available as well as engineered DNA and RNA polymerases (29, 42–46).

Among a large spectrum of nucleotide analogues that are known to be substrates for DNA polymerases, modifications are very diverse, varying from small substituents to considerably large modifications. Sterically demanding groups, such as bulky fluorescent labels or affinity tags are often anchored to the C5 position of pyrimidines through a linker. It can be either rigid and short (e.g. acetylen or phenylen) or long and flexible, which properly positions the modification for further needs (28). Remarkably, the optimization of both the composition and the length of a flexible tether has led to the successful enzymatic synthesis of artificial DNA bearing extremely bulky C5-substituents, such as grafted organic-polymers, 40 nt long ONs, G-quadruplex derived DNAzymes, antibodies or even a 40 kDa horseradish peroxidase (47–51).

A great number of novel nucleotide analogues are reported to carry redox, fluorescent, protective, photo-sensitive or reactive groups that can provide a wide variety of new and unique properties, e.g. electrical, magnetic or light transporting properties, enhance hydrophobicity, lipophilicity, affinity to different substrates, resistance to nucleases or complementarity, or even originate a catalytic activity. Introduction of a functional group by DNA polymerase mainly depends on the application of a modified ON. A fluorescent label bearing canonical nucleobases or artificial fluorescent nucleobases are a powerfull class of molecular reporters to analyze the location, conformation and interaction of nucleic acids and to monitor the intracellular processes (2, 34, 52, 53). A wide spectrum of modified nucleosides bearing redox indicators and markers based on the amino, nitro/amino-phenyl, alkylsulphonylphenyl, tetrathiafulvalene, ferrocene or Os^{2+}/Ru^{2+} complexes are used for the electrochemical detection and further bioanalytical purposes (1, 54–57). Reactive aldehyde, acrylamide,

vinylsulphonamide, chloroacetamide or trifluoroacetophenone groups are the perfect choice for the DNA bioconjugation studies (3, 4, 58, 59). Due to direct and unique contacts with proteins uridine analogues with protein-like side chain motifs such as histamine, arginine, phenylalanine, benzyl, naphtyl, indolyl, phenol, hydroxyl or carboxyl are irreplaceable building blocks for aptamers or DNAzymes (13, 28, 60–62). Furthermore, considering its remarkable labeling efficiency, ethynyl or azide modified nucleotides suitable for click chemistry are used to further link the reporter group, to cyclise DNA, to generate DNA catenanes or otherwise alter the structure of DNA (63, 64). A variety of photocleavable groups are used to generate the photocaged DNA that can be temporary inactive and protected against the cleavage by nucleases, whereas bulky modifications can be used to tune the resistance of DNA to degradation by exonucleases (65–67). In addition, there are nucleotides with spin labels for probing DNA dynamics, amphiphilic groups for extended bioavailability, hydrophobic moieties for the investigation of hydrophobic DNA interactions, steroids, fluoroalkyls or sugars for DNA-functionalization for other applications (68–74). The aforementioned examples prove the extraordinary ability of DNA polymerases to tolerate a toolbox of modifications.

While a broad spectrum of modified nucleotides has been already designed, little attention has been paid to altering other positions. Although C6- or C2/C8-modified pyrimidines and purines, respectively, have been subjected to modifications, such nucleotide analogues are less prone to be incorporated by polymerases, and this leads to poorer manipulation (75–78). Other positions, such as O^4 of purines/pyrimidines or N^4 of pyrimidines, have been exploited even less. To increase the portfolio of accessible modified nucleotides not only the functional group per se should be the variable, but rather the combination of the latter with the proper nucleobase position. In fact, it can be the rationaly chosen nucleobase position with the already known and well-studied modification that will give the major impact. Moreover, artificial nucleobases as well as nucleobase imitators bearing nucleotides have a great potential to positively influence the characteristics of the nucleic acid, thus should be explored more.

To expand the diversity of the base-functionalized DNA and to deepen the current knowledge about the N^4-modified nucleotide analogues and its possible uses, **the aim of this study** was to investigate the use of novel modified pyrimidine nucleotides. The following tasks have been formulated to attain this aim:

- To investigate the potential of modified pyrimidine nucleotides for the enzymatic synthesis of modified DNA.
- To identify DNA polymerases that utilize these nucleotides as substrates.
- To determine the base-pairing properties of N^4-acyl cytosine.
- To evaluate the effect of the N^4-acyl modification present in a nucleic acid molecule to the activity of restriction endonucleases.
- To study the demodification process of N^4-acyl-modified oligonucleotides.
- To examine the application of the photoactive modified nucleotides bearing oligonucleotides for the UVA-induced covalent cross-linking.

Scientific novelty and practical value

N^4-acetylcytidine is a naturally occurring minor nucleoside found in RNA whereas N^4-hydroxycytidine as well as N^4-methoxycytidine are the products generated by some mutagenic agents that cause misincorporations (79, 80). Yet the information regarding the N^4-substituted nucleotides and the corresponding DNA is obscure. Chemical synthesis, thermal stabilities and hybridization capabilities of ONs containing N^4-acyl, N^4-alkoxycarbonyl or N^4-carbamoyl-cytidine nucleotides have been previously described (81). Moreover, it is known that N^4-acetyl-CTP is efficiently used as a substrate in a T7 RNA polymerase-catalyzed *in vitro* transcription, while N^4-alkyl-deoxycytidines have been tested for PCR amplification of GC-rich DNA regions (82, 83). Here, for the first time a set of novel N^4-acyl-2′-deoxycytidine triphosphates ($dC^{Acyl}TPs$) was investigated showing that these nucleotides are promising building blocks for the enzymatic synthesis of N^4-modified DNA. It was demonstrated that a variety of DNA polymerases uses $dC^{Acyl}TPs$ as substrates and these nucleotides can be successfully recruited for the template dependent as well as template independent synthesis of modified DNA. Importantly, base-pairing properties of *N*-acylated cytidine nucleotides using different DNA polymerases were examined. DNA containing specific *N*-acyl functional groups was found to be resistant to the cleavage by several restriction endonucleases. These findings also indicate that N^4-acyl functionalization is an easily removable DNA modification, making it superior to the permanent one. Furthermore, it was demonstrated that N^4-cytidine modified ONs containing reactive acetylbenzoyl or

benzoylbenzoyl groups are suitable for the UVA-induced cross-linking. A straightforward cross-linking approach can be used for the formation of covalent complexes between DNA and interacting proteins. In addition, 3′-tailed DNA bearing the same photosensitive moieties is easily photoimmobilized onto a variety of untreated surfaces. As such, it was shown to be functional for the detection of complementary DNA targets. N^4-acyl-cytidine nucleotides have been proposed as substrates for the enzymatic synthesis of functionalized DNA (Lithuanian Patent No. 6615 and International application No. PCT//B2018/056961).

Synthetic pyridone-based nucleotides were shown to be good substrates for the TdT-catalyzed 3′-elongation, where halogenated derivatives could be used as radiosensitizing agents. It was also demonstrated that 4-thio-2′-deoxyuridine containing DNA can be utilized for the UV-mediated cross-linking with interacting proteins. Similarly, due to its reactive hydrazine functionality, N^4-amino-2′-deoxycytidine bearing DNA could form stable adducts with the aldehyde-containing substrates. These findings indicate that template-independent labeling is a suitable and convenient approach for DNA 3′-end modification with reactive groups.

MATERIALS AND METHODS

To accomplish the aim of this thesis a variety of common as well as adapted methods of molecular biology and biochemistry was utilized. All materials and methods are described in detail in Paper I–IV. The thesis mainly involved various techniques of manipulation and analysis of DNA, including PEX (Paper I–III), competitive PEX (Paper III), TdT-catalyzed 3′-elongation (Paper I, II), isothermal amplification (Paper III), agarose gel electrophoresis (Paper III, IV), polyacrylamide gel electrophoresis (PAGE) (Paper I–IV), restriction endonuclease (RE) treatment (Paper III), photo-cross-linking of nucleic acids and proteins (Paper II, IV), and adapted DNA microarray procedures (immobilization, hybridization, denaturation) (Paper IV).

RESULTS AND DISCUSSION

The results of this thesis include several major parts: the incorporation of modified pyrimidine nucleotides by enzymatic means (Paper I, II), the base-pairing properties of N^4-acyl-cytosine (Paper I), the influence of the N^4-acyl-modification on the activity of restriction enzymes (Paper III), the removal of N^4-acyl-residues from the N^4-acyl-ONs (Paper III), and the covalent cross-linking of modified DNA to proteins and other supports (Paper II, IV). The synthesis of the nucleotides presented here was carried out by Dr. D. Tauraitė, and was described in detail earlier (84, Paper I, II).

1. Functionalization of DNA using modified pyrimidine nucleotides

To utilize novel modified pyrimidine nucleotides (Figure 1) for the functionalization of DNA, several enzymatic approaches such as PEX, TdT-catalyzed 3′-elongation and isothermal amplification were applied. A variety of family A and B template dependent DNA polymerases was studied including Klenow Fragment (exo–) (KF (exo–)) of *E. coli* (family A), thermostable *Thermus aquaticus Taq* DNA polymerase (family A) and *Thermococcus kodakaraenis* KOD XL (family B). In addition, two DNA polymerases – Bsm (family A) and phi29 (family B) – featuring strong strand displacement activity were tested. Contrary to the others, phi29 DNA polymerase possesses a 3'→5' exonuclease (proofreading) activity, and therefore performs a very accurate DNA synthesis (85).

To further expand the utilization of novel nucleotide derivatives, a template-independent 3'-tailing based on TdT was applied. TdT is an X family DNA polymerase catalyzing a step-wise addition of random nucleotides to the 3'-OH terminus of DNA (86, 87). Unlike most DNA polymerases, TdT displays an uncommonly low discrimination between natural and artificial nucleotides.

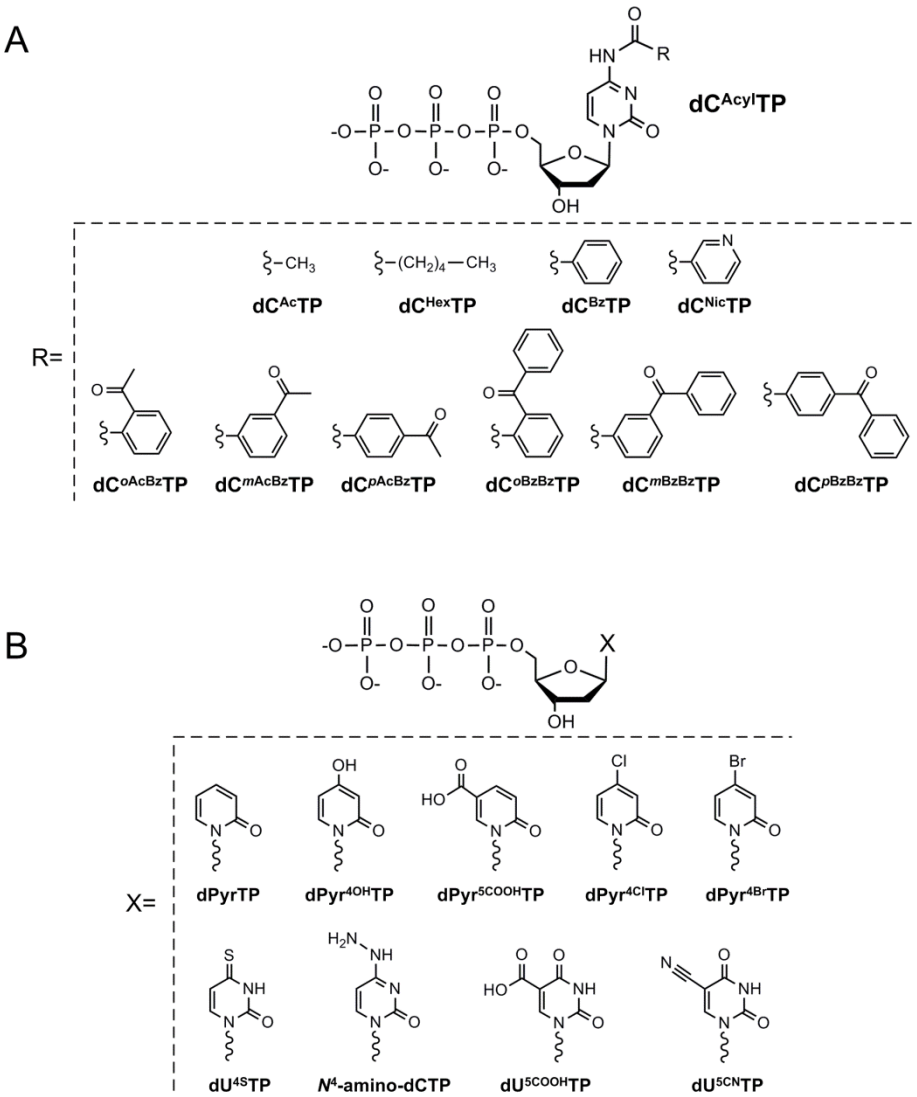


Figure 1. Chemical structures of modified pyrimidine nucleotides. (**A**) dC^{Acyl}TP: N^4-acyl-2'-deoxycytidine-5'-triphosphate (N^4-acyl-dCTP), dC^{Ac}TP: N^4-acetyl-dCTP, dC^{Hex}TP: N^4-hexanoyl-dCTP, dC^{Bz}TP: N^4-benzoyl-dCTP, dC^{Nic}TP: N^4-nicotinoyl-dCTP, dC^{oAcBz}TP: N^4-(2-acetyl-benzoyl)-dCTP, dC^{mAcBz}TP: N^4-(3-acetyl-benzoyl)-dCTP, dC^{pAcBz}TP: N^4-(4-acetyl-benzoyl)-dCTP; dC^{oBzBz}TP: N^4-(2-benzoyl-benzoyl)-dCTP, dC^{mBzBz}TP: N^4-(3-benzoyl-benzoyl)-dCTP, dC^{pBzBz}TP: N^4-(4-benzoyl-benzoyl)-dCTP. (**B**) dPyrTP: 2-pyridone-2′-deoxynucleoside-5'-triphosphate (2-pyridone-dNTP), dPyr^{4ON}TP: 4-hydroxy(2-pyridone)-dNTP, dPyr^{5COOH}TP: 5-carboxy(2-pyridone)-dNTP, dPyr^{4Cl}TP: 4-chloro(2-pyridone)-dNTP, dPyr^{4Br}TP: 4-bromo(2-pyridone)-dNTP, dU^{4S}TP: 4-thio-dUTP, dU^{5COOH}TP: 5-carboxy-2′-deoxyuridine-5'-triphosphate, dU^{5CN}TP: 5-cyano-2′-deoxyuridine-5'-triphosphate.

In fact, TdT is well-known to use a wide spectrum of base-modified nucleotides including the ones with artificial or pyridone/imidazole-based

bases, steric aromatic pyrene residues, 2′,4′-bridged structures, click-reaction suitable residues and other functional groups (88–95). As a consequence, TdT-mediated 3′-elongation with nucleotide derivatives is used to generate polymers of unnatural DNA that find plenty of applications such as functional labeling, immobilization, nuclease resistance or various conjugation approaches (96).

1.1 Incorporation of N^4-acyl-dC nucleotides by exonuclease-deficient polymerases

First, dC^{Acyl}TPs were tested as substitutes for dCTP using exonuclease-deficient (exo–) DNA polymerases (i.e., KF (exo–), *Taq*, KOD XL and Bsm). To determine whether these nucleotides can be incorporated at multiple positions as well as one after the other DNA templates containing guanosines at different positions were used (Table S1). It was demonstrated that all exo– DNA polymerases tested were able to incorporate dC^{Acyl}s in the adjacent as well as discrete positions (Paper I, Figure 2; Figure S1). As illustrated in Figure 2, modified ONs containing either three to four dC^{Acyl} in a row or up to six modifications throughout the entire ON could be successfully generated.

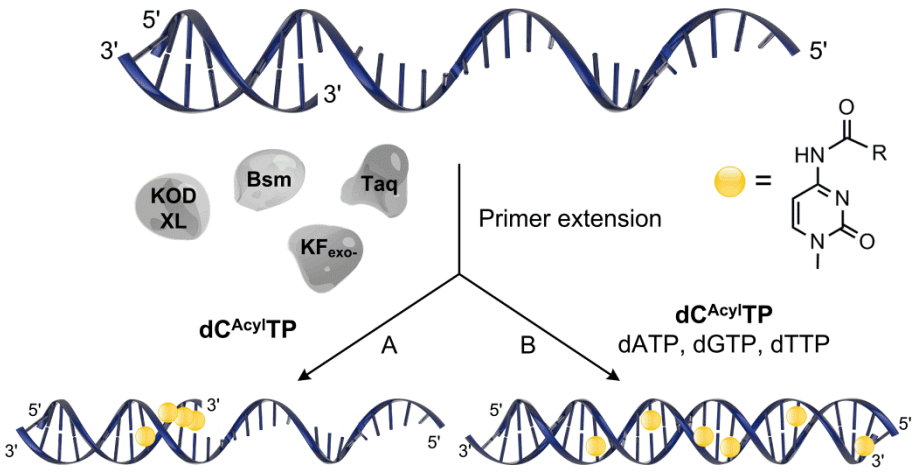


Figure 2. Two incorporation strategies of dC^{Acyl}TPs by exo– DNA polymerases: incorporation of up to four dC^{Acyl}TPs in a row (**A**) and incorporation of several dC^{Acyl}TPs at discrete positions (**B**). dC^{Acyl}TPs were used as substitutes for dCTP.

It was also revealed that exo– DNA polymerases tolerated smaller modifications bearing nucleotides (dC^{Ac}, dC^{Hex}, dC^{Bz}, dC^{Nic}) quite similarly whereas bulkier cytidine derivatives (dC^{AcBz} and dC^{BzBz}) were treated

differently (Paper I, Figure 2). In fact, *meta* and *para* isomers of dC^{AcBz} and dC^{BzBz} appeared to be better substrates for KF (exo–) and Bsm than for *Taq* and KOD XL DNA polymerases. Since the size of the N^4-modification had an obvious impact on the incorporation efficiency, it can be suggested that, in this case, the selection of the right nucleotide mostly depended on the nucleotide binding pocket of the polymerase used.

1.2. Incorporation of N^4-acyl-dC nucleotides by a proofreading DNA polymerase

Next, $dC^{Acyl}TP$s were tested in PEX reactions using a highly processive phi29 DNA polymerase. As shown in Figure 3, phi29 DNA polymerase was able to incorporate up to four modified nucleotides in a row and up to six cytidine analogues interchanged with natural ones (Paper I, Figure 5, 6). Contrary to exo– DNA polymerases, phi29 DNA polymerase strictly discriminated between *N*-acylated nucleotides selecting dC^{Acyl}s bearing acetyl, hexanoyl, nicotinoyl, *p*-acetylbenzoyl and *p*-benzoylbenzoyl groups (Figure 3).

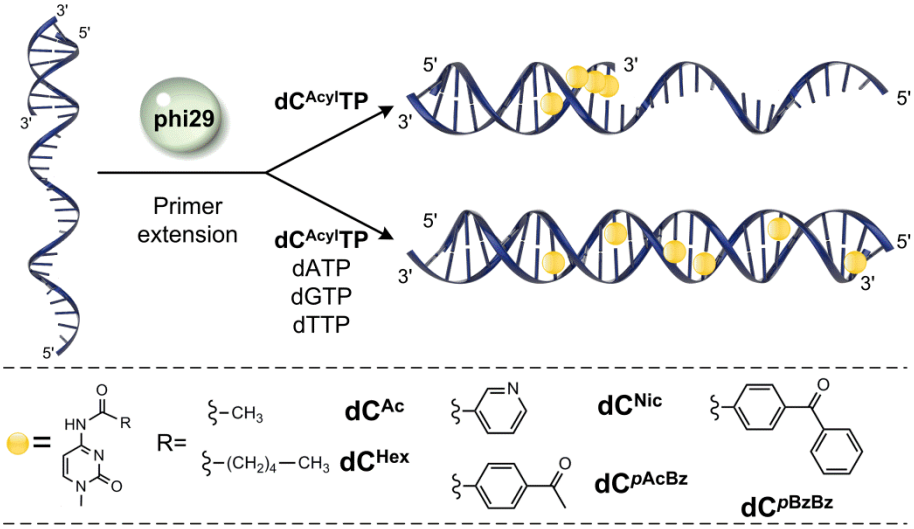


Figure 3. Incorporation of $dC^{Acyl}TP$s by phi29 DNA polymerase. Incorporation of up to four $dC^{Acyl}TP$s in a row and incorporation of several $dC^{Acyl}TP$s at discrete positions are shown at the top and the middle of the figure, respectively. The preferred $dC^{Acyl}TP$s are indicated at the bottom of the figure. $dC^{Acyl}TP$s were used as substitutes for dCTP.

Since phi29 DNA polymerase features not only a great processivity but also a strong strand displacement activity, a multiply-primed rolling circle isothermal amplification procedure was conducted using $dC^{Acyl}TP$ instead of

its natural counterpart (Figure 4). It was revealed that the only N^4-acylated cytidine nucleotide suitable for the isothermal amplification of plasmid DNA (~2.7 kb) was $dC^{Ac}TP$ (Paper III, Figure 4). The amplification efficiency using either $dC^{Ac}TP$ or dCTP was of a similar level, and phi29 DNA polymerase generated rather comparable amounts of smaller and large concatameric DNA molecules of natural as well as dC^{Ac}-modified DNA. Furthermore, it was confirmed that the newly generated modified DNA was indeed double-stranded (Paper III, Figure 4).

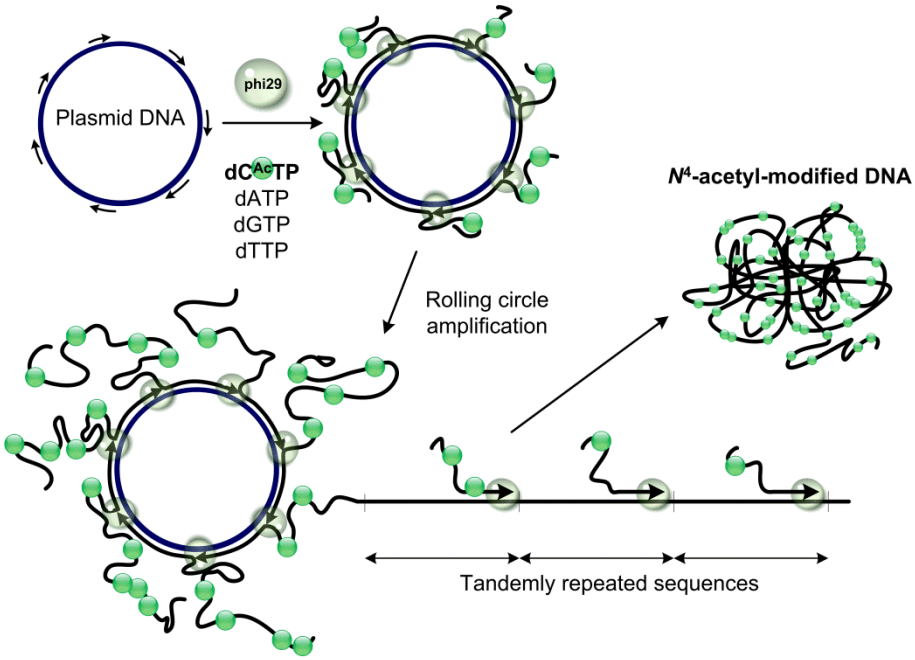


Figure 4. Multiply-primed rolling circle amplification performed by phi29 DNA polymerase using $dC^{Ac}TP$ instead of dCTP.

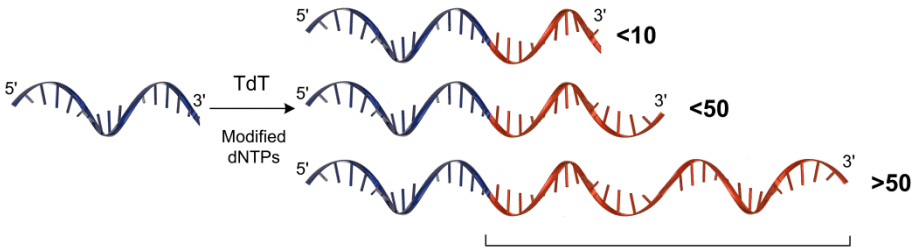
Not only phi29 DNA polymerase finds many applications in biotechnology, diagnostics and material sciences but it also offers a convenient alternative to the conventional amplification techniques requiring conditions of only minimal heating and cycling. The high interest in isothemal amplification that is often thought as more customer-friendly has been highlighted in several recent papers (97–99). Nonetheless, only few cases have been reported regarding the use of non-natural substrates by phi29 DNA polymerase (100–104). Therefore, the findings of this thesis can substantially contribute to the progress of isothermal amplification of the

nucleic acids since it offers a different approach to insert multiple dC^{Acyl}-functionalizations and presumably others into the DNA.

1.3. TdT-mediated 3′-end tailing using modified pyrimidine nucleotides

Next, modified nucleotides illustrated in Figure 1 were tested as substrates for TdT-catalyzed polymerization. Since TdT is unique for its broad utilization of different metal ions as cofactors (Mg^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+}), where nucleotide preference strongly depends on the metal ion used, two buffer systems based on Mg^{2+} and Co^{2+} were used. In general, a majority of modified nucleotides emerged as good to excellent substrates for TdT (Paper I, Figure 7; Paper II, Figure 1, 2; Figure S2). A substantial discrimination between various nucleotides using the Mg^{2+}-containing and Co^{2+}-containing buffers was observed, the latter being more favorable for the efficient incorporation. Data presented in Figure 5 indicate that utilization of a Co^{2+}-containing buffer induced incorporation of nearly every member of the library of nonnatural nucleotides. Notably, some exceptional examples can be distinguished such as TdT being able to append as many as several hundreds of consecutive monomers in the case of dC^{AcBz}TPs and dC^{BzBz}TPs (Paper I, Figure 7; Figure S2).

TdT was able to easily generate single-stranded DNA tailed with more than 40 halogenated pyridone-based nucleotides, which could be successfully used as radiosensitizing agents (Paper II, Figure 2). dPyrTP, dU^{4S}TP and N^4-amino-dCTP appeared as only moderate substrates for TdT, however, the ON bearing at least several reactive groups such as thio or hydrazine could be used for further modification, cross-linking or conjugation. Only a single incorporation of several analogues namely $dPyr^{4OH}$ and dU^{5CN} was observed suggesting that some sort of steric hindrance could provoke the dissociation of TdT and a subsequent termination of elongation (Paper II, Figure 2). Neither $dPyr^{5COOH}$TP nor dU^{5COOH}TP were used by TdT indicating that the negative charge of a carboxy group was detrimental to the activity of the enzyme (Paper II, Figure 1, 2).



	[a]		[a]
dPyrTP	+	$dC^{Ac}TP$	+++
$dPyr^{4OH}TP$	+	$dC^{Hex}TP$	++
$dPyr^{5COOH}TP$	–	$dC^{Bz}TP$	+++
$dPyr^{4Cl}TP$	++	$dC^{Nic}TP$	+++
$dPyr^{4Br}TP$	++	$dC^{oAcBz}TP$	++
$dU^{4S}TP$	+	$dC^{mAcBz}TP$	+++
N^4-amino-dCTP	+	$dC^{pAcBz}TP$	+++
$dU^{5COOH}TP$	–	$dC^{oBzBz}TP$	+
$dU^{5CN}TP$	+	$dC^{mBzBz}TP$	+++
		$dC^{pBzBz}TP$	+++

Figure 5. Relative incorporation efficiency of modified pyrimidine nucleotides by TdT. [a]Approximate number of nucleotides appended by TdT under optimal conditions: – = 0; + = <10; ++ = <50; +++ = >50.

As to the N^4-acylated cytidine nucleotides, the majority was found to be better substrates for TdT than the natural dCTP (Paper I, Figure 7). Interestingly, a possible 3′-tailing dependence on the bulkiness of a substituent at N^4 position of deoxycytidine was noticed. As such, with several exceptions, by increasing the size of a functional group (acetyl< benzoyl< nicotinoyl< acetylbenzoyl< benzoylbenzoyl) the elongation efficiency improved considerably. In addition, a straightforward tendency regarding dC^{AcBz} and dC^{BzBz} isomers was detected deducing that the *ortho* isomers were the least favorable while the *para* configuration enhanced the activity of TdT (Paper I, Figure 7; Figure S2). Remarkably, in the case of AcBz- or BzBz- modified elongation products, a considerable portion of such radioactive material was unable to enter the gel (Paper I, Figure 7; Figure S2). Identical accumulation of the bulky material near the gel wells was also observed performing conventional as well as pulse-field agarose gel electrophoresis (Figure S3, S4). Moreover, these aggregates were resistant to the hydrolysis by protease implying that they were not TdT-DNA complexes formed due to the exposure to UV present in a daylight. Altogether, it seemed likely that the presence of a highly hydrophobic tail could have caused the formation of specific steric structures, aggregates or even

nanoscaled architectures that were supported by the stacking of additional aromatic rings and prevented the modified DNA from migration (Figure 6).

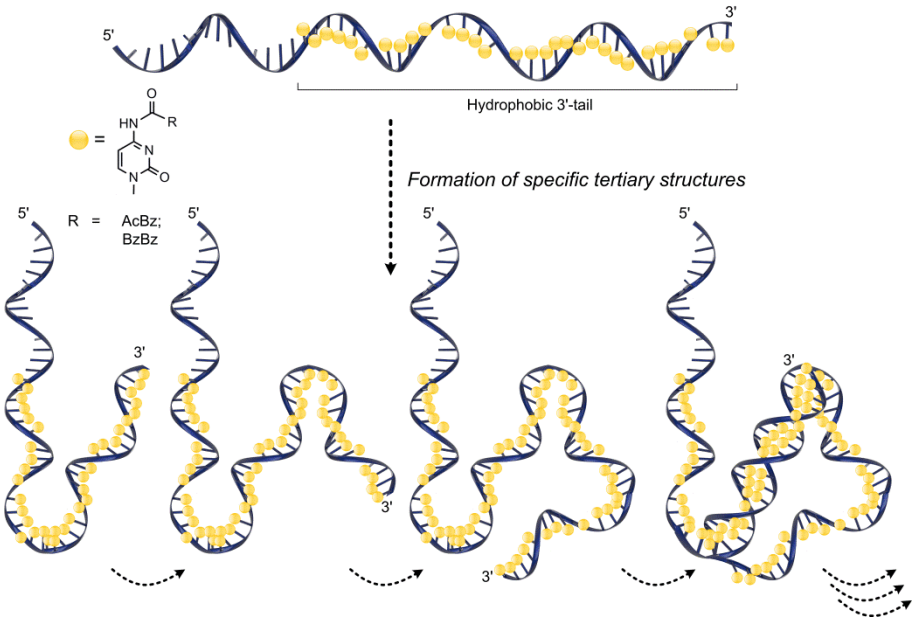


Figure 6. A possible formation of specific tertiary structures of a highly hydrophobic 3′-tail containing acetylbenzoyl or benzoylbenzoyl moieties.

According to the results, the more hydrophobic modifications were present on the ON, the more intense phenomenon of inability to migrate was observed (Paper IV, Figure 3). As illustrated in Figure 6, a larger amount of AcBz or BzBz groups could possibly stimulate the unpredictable formation of spatial structures. Indeed, such an assembly of modified DNA could prevent TdT from further translocation and force to dissociate explaining why only a certain amount of hydrophobic groups are appended (Paper I, Figure 8). Such a scenario could become beneficial for the construction of the three-dimensional DNA-ordered architectures or functionalized DNA-based nanomaterials.

2. Base pairing properties of N^4-acyl-cytosine

Since the nature of complementarity dictates the specific base-pair bonding structures and the general folding of the nucleic acids molecules, it was essential to evaluate whether C^{Acyl} follows a single or multiple complementarity pattern. During the studies on the chemical synthesis and hybridization properties of N^4-acyl-, N^4-alkoxycarbonyl- and N^4-(N-

arylcarbamoyl)-dC-containing ONs, it has been demonstrated that the particular N^4-groups present in an ON are oriented in a geometrically fixed manner (proximal or distal) (105). This, in turn, makes the formation of a conventional Watson-Crick type base pair with the guanine residue, owing to an intramolecular hydrogen bond between the carbonyl oxygen atom and the 5-vinyl proton of the cytosine ring. The results of this thesis showed that template-dependent DNA polymerases paired dC^{Acyl} nucleotides with G suggesting that the N^4-substituents might be fixed in a proximal geometry and form an additional hydrogen bond (Figure 7A, *I*). Clearly, this supports the general idea that N^4-acyl groups do not impair the correct hydrogen bond network with guanine. Nevertheless, as studies have shown, regarding the polarity of the solvent a conformational change between the proximal and the distal configurations is possible. Thus, a slightly different dC^{Acyl}•G base pair should be taken into consideration as well (Figure 7A, *II*).

To investigate if template-dependent DNA polymerases can incorporate modified cytidine analogues against other nucleobases, additional templates containing A, C or T were utilized (Table S1). In the case of pyrimidine-based templates, exo– DNA polymerases treated dC^{Acyl}TPs similarly as its natural counterpart, and only some combinations of nucleotides and polymerases exhibited a slightly enhanced incorporation (Figure S5, S6). As expected, phi29 DNA polymerase could not pair C^{Acyl} with neither cytosine nor thymine.

Surprisingly, PEX experiments using exo– DNA polymerases revealed a strong complementarity between C^{Acyl} and adenine base (Paper I, Figure 3). In fact, depending upon the exo– DNA polymerase and the N^4-acylated nucleotide, DNA products with up to four dC^{Acyl}s in a row could be obtained (Paper I, Figure 3). KF(exo–) and Bsm DNA polymerases were found to pair C^{Acyl} with A most effectively with high preference towards acetyl, hexanoyl and nicotinoyl groups. Furthermore, replacing dTTP with dC^{Acyl}TP and using the aforementioned combinations of DNA polymerases and nucleotides, synthesis of longer PEX products containing five modifications was feasible (Paper I, Figure 4). It was even more impressive that Bsm DNA polymerase was able to generate full-length products in the presence of only three nucleotides namely dATP, dGTP and dC^{Acyl}TP (dC^{Ac}TP, dC^{Hex}TP or dC^{Nic}TP) (Figure S7). These findings indicate a rather dual complementarity of C^{Acyl} nucleobase when using particular polymerases.

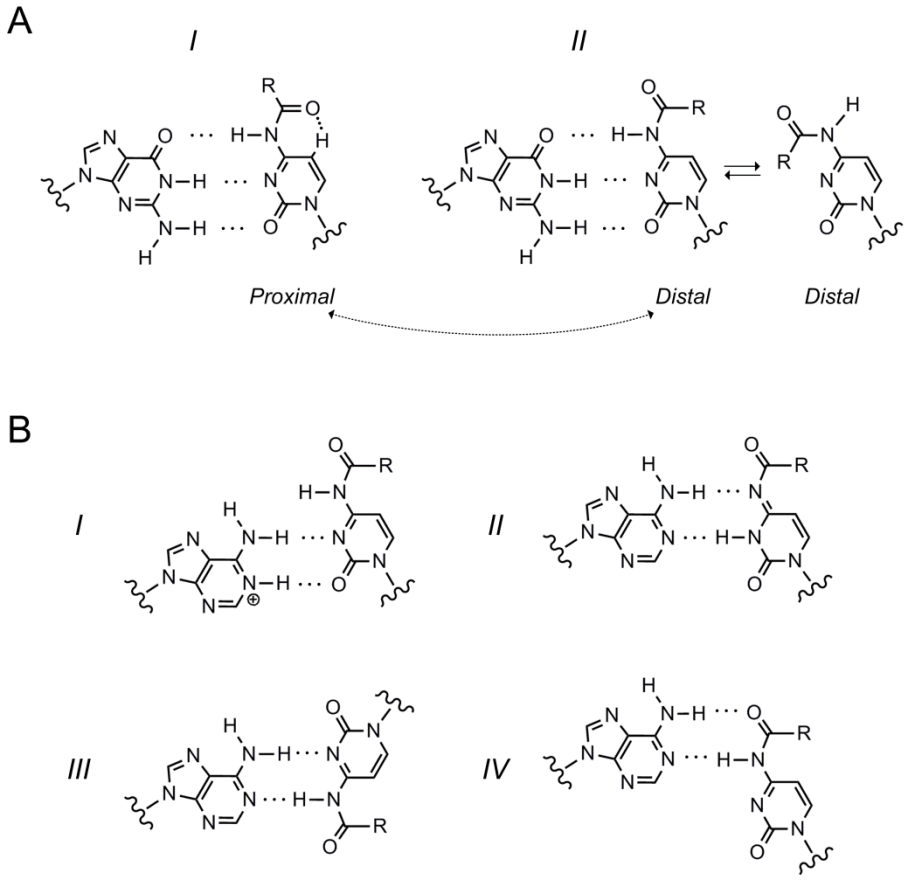


Figure 7. Suggested structures of C^{Acyl}•G (**A**) and C^{Acyl}•A (**B**) base pairs.

Contrary, phi29 DNA polymerase acted completely different than exo– DNA polymerases. It appeared that the adjacent nucleobase positions on the template strand as well as the combination of nucleotides had a slight impact on the behaviour of phi29 DNA polymerase. Predominantly, phi29 DNA polymerase was unable to initiate the elongation with N^4-acylated-nucleotides in the case of template that started with four adenines in a row (Figure S8). Nevertheless, the nucleotide shortage and the exact sequence of the template were the two determinants that caused phi29 DNA polymerase to misincorporate (Figure S9, S10). It seemed that upon an instant incorporation of the next correct nucleotide, phi29 DNA managed to pass through the C•A or C^{Acyl}•A mispair occasionally, and generated truncated DNA products to a certain extent. Regardless, an exclusively correct nucleotide incorporation by phi29 DNA polymerase can be easily achieved after the supplementation with all of the necessary dNTPs.

Very few studies have been reported on the natural A•C mispairing where experimental evidence and theoretical calculations suggest slightly different A•C structures. Based on the experimental data several possible A•C structures of wobble type have been proposed involving a single or double hydrogen bonds, major and minor tautomeric forms and protonation of adenine base at ^{1}N (106, 107). Meanwhile, strictly two N–H•••N hydrogen bonds were estimated to be more energetically favorable using theoretical methods predicting reverse wobble and reverse Hoogsteen A•C structures (108). As far as modified cytidine nucleobases are concerned, a strong complementarity between several cytosine analogues namely N^4-methoxy-C and N^4-alkoxycarbonyl-C and adenine has also been detected indicating a similar base-pair stability with both G and A (109, 110). It was also shown that the latter forms a base pair with A in a different geometry from the naturally occurring wobble A•C mismatch, involving two unique hydrogen bonds (C-^{4}NH•••A-^{1}N and C-Oe^{ster}•••A-^{6}NH) (110).

Based on the both previously published data and the results of this thesis several possible A•C^{Acyl} structures were proposed (Figure 7B). The *I*, *II* and *III* structures are equivalent to the proposed for the A•C base pair, where the *I* involves a protonated adenine, the *II* is based on the imino form of cytosine and the *III* is in accordance with theoretical modelling. The *IV* A•C^{Acyl} structure was proposed in compliance with the work by others (110). The exact conformation of the A•C^{Acyl} base pair may not only depend on the N^4-residue itself but also on the ON sequence, salvation as well as polymerase‘s characteristics. Further studies on the hybridization of modified ON and theoretical modelling are needed to resolve the actual geometry of various N^4-acyl groups.

3. The influence of N^4-acyl-modifications on the activity of restriction endonucleases

Nucleobase modifications exposed to the major groove may alter the structure of a nucleic acid molecule so that the interaction with the DNA-binding proteins are changed. Accordingly, to prepare any desired modified DNA (short ON, plasmid or genomic DNA) that is compatible for the downstream applications, such as cleavage by REs, amplification or ligation, the influence on the activity of DNA-binding proteins of interest must be examined. Despite the paramount role as well as use of REs in genetic engineering and synthetic biology, insufficient data on the influence of base-functionalizations to the activity of REases may hinder the application of

synthetic DNA. Only a small part of new DNA modifications is tested for its effects on REases (111–118). In addition, very few studies on the cleavage of DNA-bearing artificial base pairs by REs have been reported so far (119). Based on the recent findings, it is known that the specific modifications attached to the positions C5, C7 or C8 of the nucleobases can either block the cleavage or be tolerated by the appropriate restriction enzymes. Since N^4-modified nucleotides are investigated scarcely, the influence of such modifications remains unclear. A few methyltransferases specific to the position N^4 and the sensitivity of some REs towards N^4-methylation has been presented, however there is no data on the influence of other non-natural N^4-modifications on the cleavage performed by restriction enzymes (120–123). Hence, in this thesis the cleavage of dC^{Acyl}-containing DNA by the selected REs was investigated.

The template DNA (Paper III, Figure 2) was designed to contain one, two or three modified cytidine analogues either in the adjacent or discrete positions within the different palindromic recognition sequences. To study the influence of N^4-acyl-modifications incorporated next to the recognition regions of REs, the AT-containing recognition sequence surrounded with C^{Acyl} on the both sides was inserted. In addition, the cleavage of DNA containing either one or both modified strands was tested. Eight type II REs (KpnI, SmaI, Crf9I, HpaII, SacI, XbaI, PauI and Bsh1236I) were selected and tested for the ability to cleave the modified DNA that was prepared by PEX or isothermal amplification using phi29 DNA polymerase.

In general, seven out of eight restriction enzymes tested were unable to cleave the modified DNA containing N^4-acyl-functional groups in either one (acetyl, hexanoyl, nicotinoyl, acetylbenzoyl and benzoylbenzoyl) or both (acetyl) strands (Figure 8) (Paper III, Figure 3, 5). Interestingly, Figure 9 shows that the only exception was XbaI, which tolerated modified DNA fragments containing dC^{Ac}s in one strand of the recognition sequence, yet was inhibited by the presence of the functional groups on the both DNA strands (Paper III, Figure 3, 5). Such a discrimination by XbaI could become useful for the separation of the dC^{Ac}-DNAs containing either one or both modified strands by a simple linearization procedure.

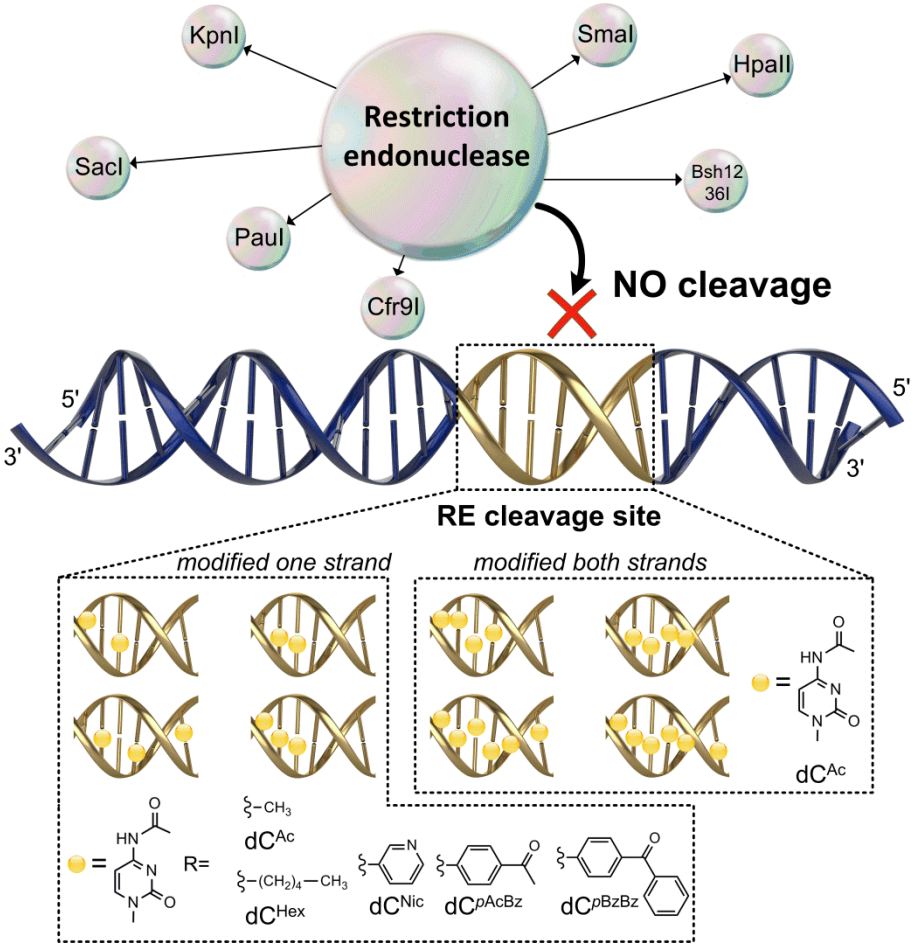


Figure 8. Resistance of N^4-acyl-dC-modified DNA against the cleavage by restriction endonucleases. The restriction endonucleases are indicated at the top of the figure, the N^4-modifications suitable for the protection of DNA are listed at the bottom of the figure.

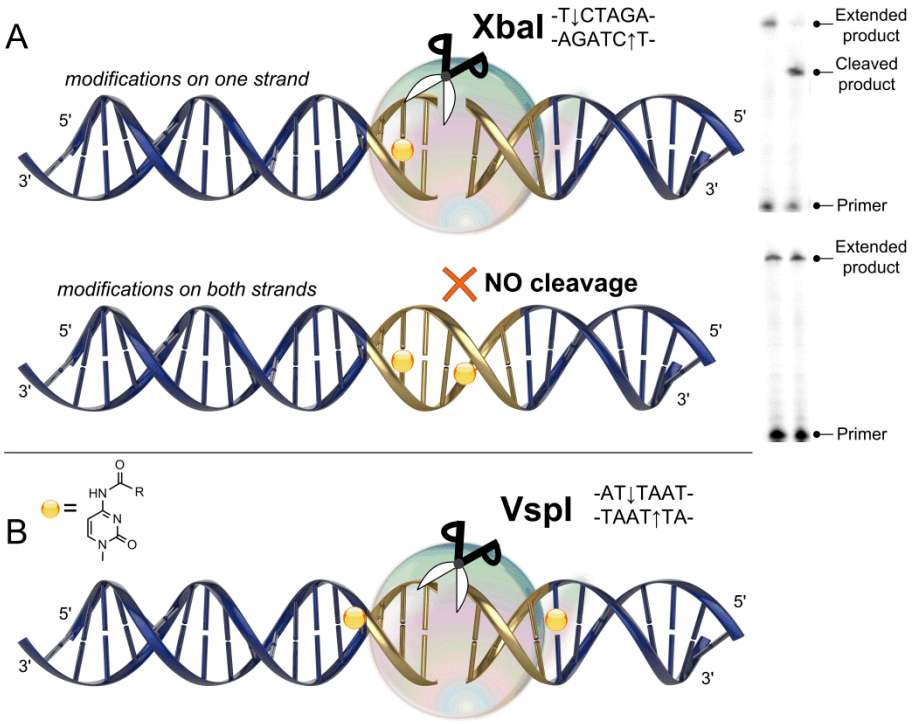


Figure 9. Activity of XbaI (**A**) and VspI (**B**) restriction endonucleases on the N^4-acyl-dC-modified DNA.

Importantly, the activity of VspI that recognizes and cleaves the AT-containing sequence remained unaffected even when the recognition sequence was surrounded with dC^{Acyl}s (Figure 9) (Paper III, Figure 3, 5). Notably, both strands of the modified DNA were treated equally (Figure S11). These findings reveal that the modified DNA is prone to degradation by one enzyme, yet the activity of the others remain unaffected. As such, the protection of DNA using N^4-substituted cytidines can be controlled by simply selecting the right restriction enzyme.

3.1. Competitive primer extension in the presence of dC^{Acyl}TPs and natural dNTPs

Enzymatic synthesis of functionalized DNA using a particular modified dNTP is usually performed in the absence of its natural counterpart. To obtain a proper degree of specific labeling or to understand the kinetics of the nucleotide incorporation by DNA polymerases the ratio of the

chemically-modified nucleotides to the native ones must be carefully tuned. This can be accomplished via the competitive nucleotide incorporation studies using a mixture of modified and natural nucleotides. Based on the several studies on the competitive primer extension using a variety of exonuclease-deficient DNA polymerases and C5/C7 modified nucleotides (124, 125), it was interesting to examine whether $dC^{Acyl}TPs$ would be able to compete with dCTP for the incorporation by a proofreading phi29 DNA polymerase.

Protection of the N^4-modified DNA sequences against the cleavage by specific REs enabled the study of the competitive incorporations by an elegant competition assay developed and described earlier (Paper III, Scheme 1) (124, 125). Using equimolar (1:1) mixtures of modified and natural nucleotides, phi29 DNA polymerase was found to either incorporate dC^{Acyl} and dC quite similarly or give the preference towards the modified nucleotide (Paper III, Table 1). Contrary, in the case of an excessive amount of $dC^{Acyl}TPs$ (10:1), a large proportion of the modified DNA was observed. The poorest competitiveness of $dC^{Ac}TP$ might be reasoned by the fact that due to the best similarity to dCTP, phi29 DNA polymerase was incapable of discriminating between $dC^{Ac}TP$ and dCTP to the high extent. Otherwise, complex factors including the chemistry of a functional group of the modified nucleotide and the characteristics of DNA polymerase determine the outcome of the incorporation. Still, these findings highlight the fact that various DNA polymerases are suitable for the competitive incorporations whether or not exonuclease activity is present.

4. Demodification of the N^4-acylated-ONs

To utilize the benefits of a site-specific DNA protection in the everyday laboratory routines, ONs with removable functional groups are highly desired. A precise control over the demodification process is required indicating that only labile modifications could be attached to the predetermined positions of a nucleobase leaving the rest of the nucleic acid molecule intact. One of the well-known approaches of the reversible DNA protection is DNA caging, which utilizes light-removable protecting groups that render chemically modified DNA temporary inactive (126). Removal of a caging group and restoration of the active DNA is brought about by the irradiation with UV light that works as a switch to turn on or off biological processes. The caging groups can be installed at either phosphodiester backbone or the nucleobase, the latter being superior in terms of controlling

the exact location and the number of protecting groups (127–135). A variety of caged nucleobases are known bearing photolabile groups on different positions of pirimidines (O4, N3, N4,) and purines (N3, O6, C8,), where the base pairing properties as well as the decaging procedure mainly depend on the specific position of a caging group. There is quite a few studies on the applications and the effects of photocaged nucleobases, nonetheless the data regarding the influence of these groups on the activity of restriction enzymes is obscure (136–138). Although it has been shown that photocaging the DNA with 5-[(2-nitrobenzyl(oxymethyl)]-dU results in a transient protection of DNA against the cleavage by several REs, photodecaging releases the 5-hydroxymethyluracil-containing DNA that is susceptible to REs attack, yet might be invalid for the downstream applications (66). A similar effect has been demonstrated with other than caged DNA, where (triethylsilyl)ethynyl modification of 7-deazaadenine, which provides a temporary immunity to REs, cannot be removed completely, and leaves the DNA with an acetylene residue at the position 7 of 7-deazaadenine (115). This, in turn, suggests that the most common nucleobase modifications that are usually anchored with a rather chemically inert bond at the C5/C7 of pyrimidines and 7-deazapurines, respectively, are not the most suitable for this type of temporary DNA protection. Instead, more reactive nucleobase positions as well as labile protecting groups should be used.

As to the temporary modification, the strong potential of the N^4-amide-linked residues is evident. Indeed, it has been well adapted during an automated synthesis of ONs, where N^4-protected cytosine phosphoramidites are the common building blocks for the synthetic ONs. Accordingly, the scope of this thesis was to find out whether $dC^{Acyl}TPs$ can actually be used for the transient DNA protection as well as to adjust the demodification process to a practicable level for the molecular biology purposes.

Once again, protection of the N^4-modified DNA sequences against the cleavage by specific REs enabled the study of demodification by a very facile and straightforward procedure (Figure 10). Notably, the focus was the one-pot procedures only, since simplicity and the minimal amount of purification steps are often of primary importance. Two deblocking agents namely ethanolamine (EA) and ammonium hydroxide were tested, since only these two were shown to be active not only in an anhydrous organic solvent or neat but also in the presence of water (139, 140). It was shown that performing a one-pot elongation, demodification and restriction test, EA appeared to be an effective deblocking tool and, most importantly, all manipulations could be performed in a single mixture (Paper III, Figure 7).

This, in turn, represented a rather simple, fast, effective and lower-cost approach that is a golden standard these days.

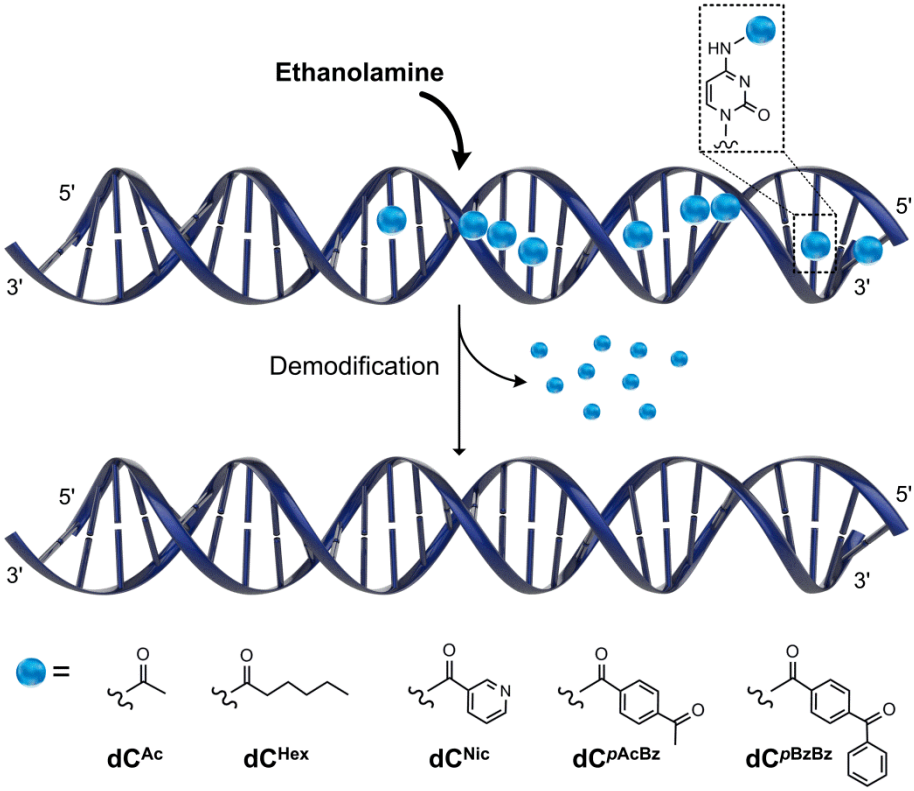


Figure 10. Demodification of N^4-acyl-dC-containing DNA using ethanolamine. The used N^4-groups are indicated on the bottom of the figure.

Also, it was essential to known whether a single round of modification-demodification can cause any mistakes or mutations within the targeted sequence and if the treated DNA can be further used for a variety of purposes. To find out the potential for an error-free cloning, the amplified dC^{Ac}-containing pUC19 DNA was demodified and inserted into *E. coli* cells. The sequences of ten random clones were compared (Figure S12). No discrepancies between the tested sequences were detected revealing two major findings. First, it was obvious that the demodification did not cause any damage to the DNA. Second, since the modified DNA was generated using phi29 DNA polymerase, these results confirmed that no misincorporation during the rolling circle amplification was made and $dC^{Ac}TP$ was used strictly instead of dCTP. Altogether, acyl-modifications at a position N^4 of a cytosine base were demonstrated to act as a transient and

easily removable DNA modification suitable for the protection against the cleavage by restriction enzymes as well as for other DNA manipulations.

5. Photochemical cross-linking of dU^{4S}, dC^{AcBz}- or dC^{BzBz}-containing ONs

Photochemical cross-linking is a powerful strategy to study biomolecule interactions as well as to anchor a variety of ligands on different surfaces. A photo-induced cross-linking can be achieved by either using an external photolinker or introducing a photoactive group into a targeted molecule, thus minimizing a cross-linking heterogeneity (141, 142). Among a variety of photoreactive groups used for the photo cross-linking, an aryl ketone benzophenone (BP) is the most widely used and versatile photophore in bioorganic chemistry and material science. BP and its derivatives find applications in a wide spectrum of fields and are used for the photo-cross-linking of polymers, modification of two- or three-dimensional solid supports, photochemical immobilization, grafting and patterning, construction of diverse molecular diagnostic tools, etc (reviewed in 143).

It is known that in its reactive radical state BP and other aryl ketones (e.g. acetophenone (AP)) abstract an aliphatic hydrogen atom from a donor molecule, depending on H steric accessibility and close proximity to AP/BP (143). Contrary, the thiol-based conjugation strategy relies on either the good nucleophilicity of the sulphur atom or the ability to participate in radical reactions (144). Specifically, the latter prevails considering RNA-protein or RNA-RNA cross-linking, where thionucleotides (4-thiouridine or 6-thioguanosine) are used as photoreagents (145, 146). In addition, the photoactive moiety is attached directly to the nucleobase ensuring the covalent formation of the photoadducts that are in a close proximity.

Both AP/BP-containing substrates and photoactivable thionucleotides are excited with long-wavelength UV (~365 nm) avoiding protein and nucleic acid damage. As a result, due to a great number of cross-linking applications, a considerable emphasis must be placed on the development of a simple as well as flexible approach for the functionalization of interacting partners.

Among all modified pyrimidine nucleotides tested in this thesis (Figure 1), dU^{4S}TP and several dC^{Acyl}TPs namely *ortho*, *meta* and *para* isomers of dC^{AcBz}TP and dC^{BzBz}TP contained photoactive groups. Important to note, that acetylbenzoyl and benzoylbenzoyl functionalities can be also referred to as acetophenone and benzophenone modifications, respectively. Hereafter,

to emphasize the photoactive characteristics of dC^{AcBz} and dC^{BzBz} the latter two will be used as well.

5.1. UVA-induced photo-cross-linking of dC^{AcBz}- or dC^{BzBz}-bearing ONs with interacting proteins

It has been shown that the interaction between proteins can be detected using a BP-containing amino acid, whereas protein-DNA complexes can be identified using a BP-bearing peptide (147–149). Although a similar effect could be achieved by using a photoactive nucleic acid molecule rather than a labeled protein, it was previously overlooked.

To demonstrate that BP-containing DNA can be used to capture the interacting proteins, single-stranded photoactive ONs were generated by TdT-mediated 3′-elongation. To simplify the process, the cross-linking step was performed immediately after the elongation reactions meaning that, in this case, TdT itself was the protein of interest.

It was shown that upon exposure to a 365 nm UV light, DNA-TdT covalent conjugates were successfully formed (Figure 11) (Paper IV, Figure 2). The yield of the cross-linking strongly depended on the number of photoreactive groups present on a DNA molecule, making *ortho*-AcBz/BzBz the least, while *para*-AcBz/BzBz isomers the most effective cross-linking agents (Paper IV, Figure 2). To confirm that the cross-linking occurred with interacting proteins only, a single-stranded DNA binding protein (SSB) was added after the removal of TdT. The formation of several dC^{pBzBz}-ON:SSB cross-linked products that can be explained by the heterogeneity of the modified DNA as well as the multimericity of SSB was revealed (Figure S13). In addition, no covalent adducts with other than DNA-binding proteins (i.e. BSA) were detected verifying the specificity of AP/BP-bearing photoprobes (Figure S13).

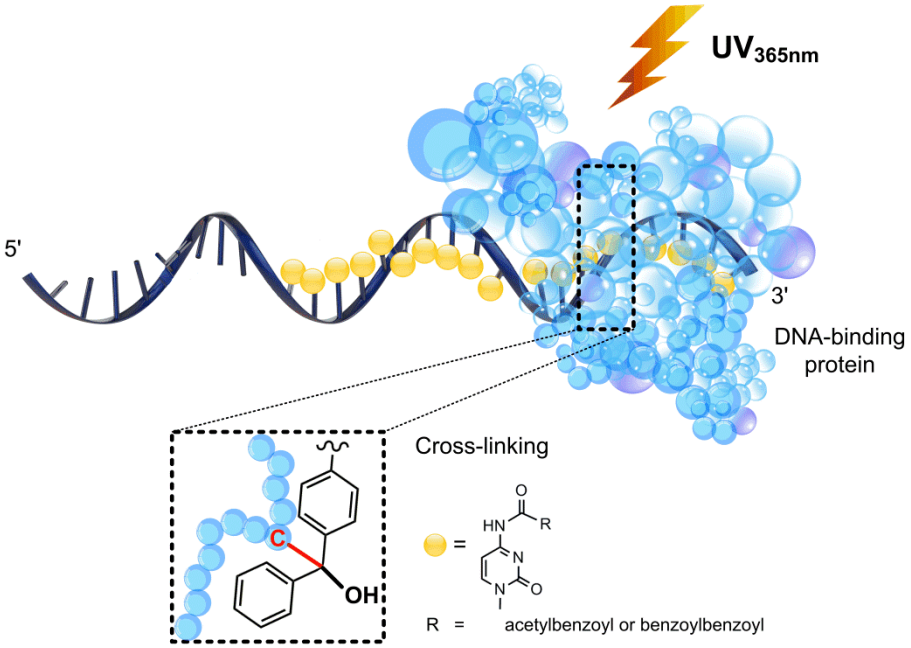


Figure 11. UVA-induced photo cross-linking between N^4-acetylbenzoyl-dC or N^4-benzoylbenzoyl-dC containing DNA with interacting protein.

Notably, after UV irradiation a considerable part of material was unable to enter the gel, particularly in the case of *p*AcBz or *p*BzBz modifications (Paper IV, Figure 2B). To determine its origin, the irradiated samples were further treated with protease. The disappearance of all the bands with the lower mobility (including the ones at the wells) demonstrated that all covalent adducts formed were DNA-protein rather than DNA-DNA conjugates (Paper IV, Figure 2B). Due to a high number of photoreactive as well as highly hydrophobic groups present in a nucleic acid molecule, formation of multiple covalent bonds or specific nanostructured aggregates, or both, could occur. Accordingly, these results highlight that AP/BP-bearing DNAs generated by TdT-mediated tailing can become promising photoprobes for studying DNA-protein interactions without a direct damage to biological material.

5.2. UVA-induced photo-cross-linking of dU^{4S}-ONs with interacting proteins

Similarly to the aforementioned photo cross-linking of AP/BP-containing ONs with interacting proteins, dU^{4S}-DNA was tested as a photoprobe as well. Single-stranded dU^{4S}-bearing ONs were generated by TdT-mediated 3′-elongation. A successful cross-linking between dU^{4S}-DNA and TdT was observed with a majority of 1:1 ratio of DNA and protein (Paper II, Figure 3). It was also shown that the dose of UVA irradiation was detrimental to the formation of covalent adducts, since no cross-links were detected exposing to UV light of lower intensity.

dU^{4S} and its halogenated analogues are known as DNA UVA sensitizers (150, 151) while 4-thiothymidine is easily incorporated into DNA during replication (152). Both dU^{4S} and 4-thiothymidine have been reported as novel potential anti-cancer drug (152,153). Nevertheless, thiodeoxyribonucleotides receive much less attention compared to ribo variants that are extensively studied and used. In the context of this thesis, it was demonstrated that 3′-dU^{4S}-DNA can be easily cross-linked with its interacting proteins and therefore can be used for identifying contacts between nucleic acids and proteins (*in vitro* and *in vivo*) without perturbing its interface.

5.3. Photoimmobilization of N^4-modified DNA onto solid supports

The most common approach to a BP-mediated photoimmobilization is the application of BP as a discrete photoinitiator (154). As such, it renders BP-induced immobilization strategies restricted since the solid support itself must be coated, chemically activated or otherwise pre-treated. To overcome this limitation, a straightforward photoimmobilization approach using AP/BP-containing DNAs was attempted.

Single stranded 3′-modified DNAs were immobilized via its AP or BP-containing tail upon UV irradiation. Remarkably, the greater number of modified residues were present, the more efficiently immobilization took place (Paper IV, Figure 3). Comparing immobilization effectiveness between the different modifications, BP appeared to be superior.

A variety of intact solid substrates such as polystyrene (PS), polypropylene (PP), polylactate (PLA), polydimethylsiloxane (PDMS), borosilicate (BS) glass and sol-gel based composites were selected and tested for the photoimmobilization (Paper IV, Figure 3, 4). Although these

relatively inert (PS, PP, BS) as well as biodegradable (PLA, PDMS) polymer substrates are especially widely used, they usually require a complicated and time consuming surface pre-treatment. Nonetheless, it was shown that all the above-mentioned polymers served as good supports for the UVA-induced photoimmobilization of AcBz/BzBz-containing DNAs (Paper IV, Figure 4). Owing to the low cost, simple design and biocompatibility of such polymers, the functionalization presented in this thesis will allow to expand their utilization.

5.3.1. Detection of DNA targets using photoimmobilized dC^{BzBz}-containing DNA probes

To obtain a functional DNA-based nanodevice, such as DNA biosensor, recognition of complementary DNA targets and convertion into a quantified signal must take place (155, 156). Needless to say, the DNA hybridization events have become one of the main concerns where all the challenges and drawbacks should be acknowledged carefully. Therefore, to verify that the immobilized 3′-dC^{BzBz}-tailed DNA probes are properly oriented for a successful detection of complementary targets, a simple hybridization procedure was performed (Paper IV, Figure 5). To examine the hybridization specificity a few hybridization parameters such as temperature and salt concentration were varied. High stringency hybridization conditions (high temperature, low salt concentration) permit hybridization between highly similar nucleic acid molecules, whereas low stringency conditions (low temperature, high salt) allow unspecific hybridization.

Remarkably, regardless of the previously mentioned conditions hybridization occurred between complementary DNA sequences only, meaning that the presented detection system is highly selective (Figure 12). Moreover, the detection of complementary DNA targets was possible in a wide temperature range (20–55 °C) as well as within short time periods (2 hours) suggesting a versatile and timesaving approach (Paper IV, Figure 5B).

To examine the potential of the presented photoimmobilization strategy for the construction of DNA microarrays, a simple DNA denaturation procedure was carried out. To make the process as simple as possible and to avoid harsh denaturation methods, such as alkaline treatment, or denaturation in nonaqueous solvents (e.g. dimethyl sulfoxide), thermal denaturation was tested. The results indicated that a full DNA melting was achieved after heating the sample for a certain period of time (Paper IV,

Figure 5C). Importantly, thermal denaturation being the most frequent and simple method of DNA denaturation suggests the immobilized N^4-acyl-dC-DNA probes as promising alternatives for DNA hybridization based assays.

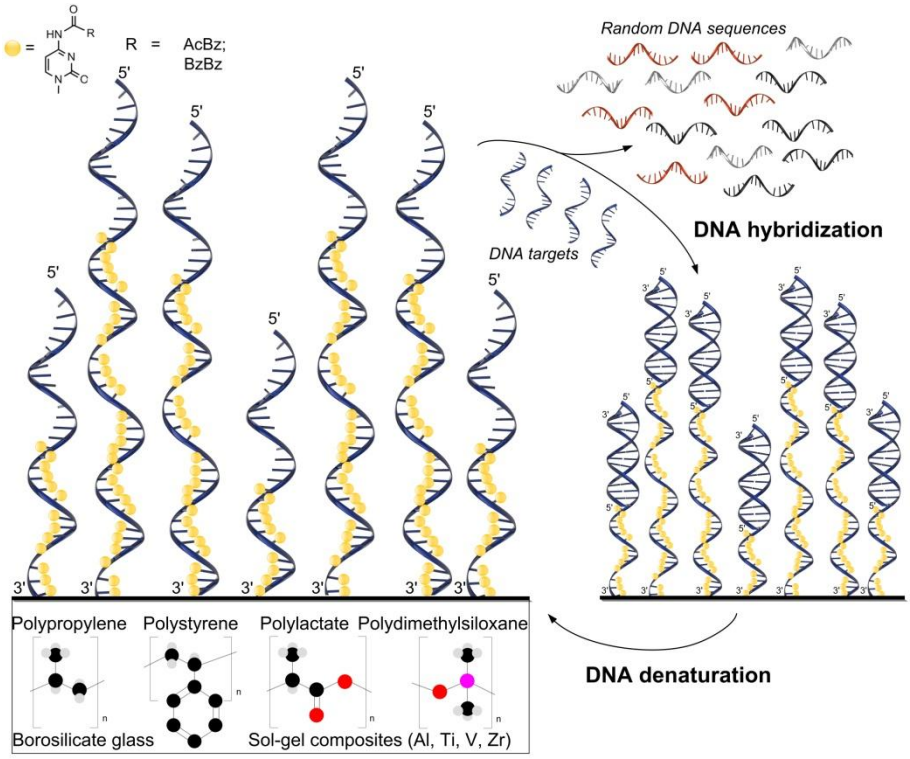


Figure 12. A photoimmobilization, hybridization and denaturation approach using N^4-acetylbenzoyl-dC or N^4-benzoylbenzoyl-dC DNA. The appropriate solid supports for photoimmobilization are indicated at the bottom of the figure.

Taken together these findings show that a novel photoimmobilization method (i) does not require any prior chemical derivatization of solid supports, (ii) is suitable for a broad range of nucleic acid molecules (single-stranded or double-stranded DNA/RNA) as well as the most classic hybridization-denaturation procedures, and (iii) may become suitable for the reuse due to the possibility for the rapid detachment of N^4-acyl-modified DNA from the support.

Final remarks

Data presented in this thesis propose novel modified pyrimidine nucleotides as promising building blocks for the enzymatic synthesis of functionalized DNA. A set of new N^4-acylated cytidine derivatives were shown to be good substrates for the template-dependent as well as template-independent DNA polymerases. Also, various pyrimidine nucleotide analogues including pyridone-based, halogenated and C5-modified nucleotides were demonstrated to be suitable for TdT-mediated 3′-elongation. Importantly, N^4-acyl-modification has a great potential to become a perfect pioneer for the transient and programmable DNA modification era. Moreover, protection of N^4-acyl-DNA against the cleavage by REs suggests its utilization in DNA manipulation routines. Nucleotides bearing photoactive functional groups, such as thio or benzoylbenzoyl, appear to be suitable for the UVA-induced covalent cross-linking with interacting proteins and a variety of solid supports. A straightforward photoimmobilization, hybridization and denaturation was shown indicating that N^4-acyl-functionalization could serve as an efficient alternative for the construction of DNA-based molecular tools. Novel modified pyrimidine nucleotides are proposed to expand the toolbox of nucleotide derivatives for the synthesis of functionalized DNA considerably.

CONCLUSIONS

1. Novel modified pyrimidine nucleotides – dC^{Ac}, dC^{Hex}, dC^{Bz}, dC^{Nic}, dC^{AcBz}, dC^{BzBz}, dPyr, $dPyr^{4OH}$, $dPyr^{4Cl(Br)}$, dU^{4S}, N^4-amino-dC and dU^{5CN} – are suitable building blocks for the construction of functionalized DNA via enzymatic approaches.

2. Klenow Fragment (exo–), *Taq*, KOD XL, Bsm and phi29 DNA polymerases accept $dC^{Ac}TP$, $dC^{Hex}TP$, $dC^{Bz}TP$, $dC^{Nic}TP$, $dC^{AcBz}TP$ and $dC^{BzBz}TP$ as substrates.

3. dC^{Ac}, dC^{Hex}, dC^{Bz}, dC^{Nic}, dC^{AcBz}, dC^{BzBz}, dPyr, $dPyr^{4OH}$, $dPyr^{4Cl(Br)}$, dU^{4S}, N^4-amino-dC and dU^{5CN} are suitable for the template-independent synthesis of funcionalized DNA using terminal deoxynucleotidyl transferase.

4. In addition to the formation of complementary C^{Acyl}•G pair, a strong base-pairing between N^4-acyl-cytosine and adenine takes place when *Taq*, Klenow fragment (exo–), Bsm, and KOD XL DNA polymerases are used for the primer extension reactions.

5. N^4-modified DNA containing acetyl, hexanoyl, nicotinoyl, acetylbenzoyl or benzoylbenzoyl groups is protected against the cleavage by selected type-II restriction endonucleases.

6. Modified DNA bearing photoreactive thio, acetylbenzoyl or benzoylbenzoyl residues is an excellent photochemical cross-linking agent for a covalent conjugation with interacting proteins.

7. N^4-modified DNA is demodified using aqueous ethanolamine without causing any damage to the sequence of DNA.

8. Modified DNA bearing acetylbenzoyled or benzoylbenzoyled bases at the 3'-end is easily photoimmobilized onto untreated polymeric solid substrates and is suitable for the detection of complementary DNA targets.

FUTURE RESEARCH

There is no doubt that further questions need to be addressed to deepen the knowledge on the effects of these nucleobase functionalities to the properties of DNA. To precisely control the enzymatic synthesis of modified DNA a more detailed investigation is required explaining the exact nucleotide selection by a variety of DNA polymerases. A comprehensive study on the competitive incorporations could help to delve deeply into the mechanism of polymerase incorporation of modified substrates. To move the enzymatic DNA modification far beyond, combinations of differently modified nucleotides as well as engineered and improved polymerases should be used more frequently.

More attention should be paid on the transient nucleobase modifications since they offer a greater variety of applications. N^4-cytidine modification was shown to be a promising alternative to the already known transient DNA protection using photocaging strategy. However, its potential in the regulation of gene activity or expression is yet unknown. Further interesting questions on this topic might concern the interactions of the modified DNA with a wide spectrum of DNA-binding proteins such as DNA ligases, methyltransferases or transcription factors. Transient DNA protection along with the knowledge on modified DNA interacting proteins could assist in monitoring or regulating the activity of targeted enzymes and proteins.

Since these novel modified nucleotides could be used for the development of aptamers, it is essential to learn more in the context of the selection techniques. The prerequisite for the modified nucleotides to be applicable in selection experiments is the conversion of modified ONs to corresponding unmodified DNA which is often performed by PCR. Therefore, it should be studied. Alternatively, a single-round selection approaches could be examined, particularly in the case of photoSELEX using photoactive N^4-acetylbenzoyl or benzoylbenzoyl groups. In addition, potential of N^4-benzoylbenzoyl-dC modified ONs for the DNA microarrays was presented however additional studies should be carried out taking possible technical problems into the considerations.

Studies in the above-mentioned directions will allow to expand utilization of these novel modified pyrimidine nucleotides.

SUMMARY/SANTRAUKA

Modifikuotą nukleobazę turintys nukleotidai yra plačiai naudojami nukleorūgščių žymėjimui, biokonjugacijai, aptamerų ir biojutiklių srityje, bei nanoįrankių ir nanomedžiagų kūrimui. Modifikuotų oligonukleotidų sintezė gali būti atliekama vienu iš trijų būdų: chemine sinteze, fermentine sinteze arba post-sintetine modifikacija. Dažniausiai oligonukleotidai sintetinami ant kieto paviršiaus naudojant paruoštus fosforamiditus. Vis dėlto, dėl įvairių trūkumų (cheminis nesuderinamumas su sintezės sąlygomis, ribotas fosforamiditų pasirinkimas, sudėtinga ilgų oligonukleotidų sintezė) vis plačiau taikomi kiti sintezės metodai. Post-sintetinė modifikacija remiasi jau paruošto oligonukleotido modifikavimu, kai prie nedidelės, bet reaktyvios pastarojo grupės (pvz., amino, etinil ar azido) yra prikabinamos žymos. Deja, šis metodas pasižymi prastu chemoselektyvumu bei itin komplikuotu sudėtinių žymų prijungimu.

Alternatyvus ir labai perspektyvus metodas yra fermentinė modifikuotų oligonukleotidų sintezė. Šis būdas remiasi DNR/RNR polimerazių gebėjimu naudoti modifikuotus (2'-deoksi)ribonukleozidų 5'-trifosfatus kaip substratus įjungiant juos į sintetinamą grandinę. Dažniausiai yra modifikuojamos pirimidinų C5 ir 7-deazapurinų C7 padėtys, nes tokie nukleotidų atitikmenys įprastai tinka įvairiems nukleorūgščių dauginimo metodams. Yra žinoma didžiulė C5/C7 modifikuotų nukleotidų įvairovė, turinčių fluorescencines, redokso, fotojautrias, reaktyvias, apsaugines ar kitokias funkcines grupes. Šios grupės gali pagerinti esamas arba suteikti naujų pageidaujamų savybių modifikuotoms nukleorūgštims, tokiu būdu praplečiant jų pritaikymo sritis.

Norint padidinti modifikuotų nukleobazių gausą būtina pabrėžti ne tik cheminio funkcinės grupės sąstato, bet ir kitų nukleobazės padėčių keitimo svarbą. Nepaisant to, kitos nukleobazių padėtys yra mažai tiriamos. Yra žinomi C6 pirimidinų ir C2/C8 purinų modifikuoti nukleotidai, tačiau šie yra prastesni polimerazių substratai. O^4 ir N^4 nukleobazių padėtys yra tyrinėjamos dar mažiau. Taigi, siekiant praplėsti jau žinomų modifikuotų nukleotidų rinkinį, **šio darbo tikslas** buvo ištirti naujų modifikuotų pirimidino nukleotidų panaudojimo galimybes. Tikslui pasiekti buvo iškelti šie uždaviniai:

- Ištirti modifikuotų pirimidino nukleotidų naudojimą fermentinei modifikuotos DNR sintezei.
- Nustatyti DNR polimerazes, naudojančias šiuos nukleotidus kaip substratus.

- Nustatyti N^4-acil-citozino bazių porų sudarymo ypatybes.
- Įvertinti N^4-acil-modifikacijų, esančių DNR molekulėje, įtaką restrikcijos endonukleazėms.
- Ištirti N^4-acil-modifikacijų pašalinimą.
- Patikrinti fotoaktyvuojamus pirimidino nukleotidus turinčios DNR taikymą nuo UVA priklausomam kryžminiam sujungimui.

Šio darbo metu buvo tiriamas modifikuotų pirimidino nukleotidų rinkinys, kurį sudarė įvairūs N^4-modifikuoti 2′-deoksicitidino 5′-trifosfatai, piridono nukleobazę turintys nukleotidai, C5-pakeisti bei tio grupę turintys uridino analogai. Siekiant įvertinti šių nukleotidų panaudojimo galimybes fermentinei modifikuotos DNR sintezei buvo atliekama nuo matricos priklausoma ir nepriklausoma DNR sintezė. Tiriant nuo matricos priklausomą modifikuotos DNR sintezę buvo naudojamos keturios egzonukleazinio aktyvumo neturinčios DNR polimerazės – Klenow fragmentas (exo–) (KF (exo–)), *Taq*, KOD XL ir Bsm. Taip pat, buvo naudojama itin tiksli ir procesyvi egzonukleazinį aktyvumą turinti phi29 DNR polimerazė. Nustatyta, kad visi N^4-acil-modifikuoti 2′-deoksicitidino 5′-trifosfatai ($dC^{Acil}TP$) yra geri KF (exo–)), *Taq*, KOD XL ir Bsm DNR polimerazių substratai. Paaiškėjo, kad šios DNR polimerazės geba naudoti $dC^{Acil}TP$ vietoj natūralaus dCTP, ir prijungti modifikuotus citidino nukleotidus išsidėsčiusius tiek iš eilės, tiek atskirai. Be to, egzonukleazinio aktyvumo neturinčios DNR polimerazės poravo dC^{Acil} ne tik su G, bet ir su adenino baze, gebėdamos naudoti $dC^{Acil}TP$ vietoj dTTP. Tokiu būdu, naudojant šias DNR polimerazes ir $dC^{Acil}TP$, galima modifikuoti tiek GC-, tiek AT-turtingas sekas.

Priešingai, egzonukleazinį aktyvumą turinti phi29 DNR polimerazė toleravo ne visas N^4-padėties modifikacijas, ir pradmens ilginimo metu efektyviai naudojo $dC^{Acil}TP$, turinčius acetil, heksanoil, nikotinoil, acetilbenzoil ir benzoilbenzoil pakaitus. Taip pat, phi29 DNR polimerazė išskirtinai poravo dC^{Acil} tik su G. Atliekant izoterminę amplifikaciją bei dauginant plazmidinę DNR, iš visų $dC^{Acil}TP$ phi29 DNR polimerazė naudojo tik N^4-acetil-modifikuotą nukleotidą. Pabrėžtina, kad, šiuo atveju, phi29 DNR polimerazė sintetino dvigrandininę N^4-acetil-modifikuotą DNR. Taigi, phi29 DNR polimerazė galėtų būti naudojama siekiant labai tikslios modifikuotos DNR sintezės.

Nuo matricos nepriklausomos modifikuotos DNR sintezės metu buvo remiamasi terminalinės deoksinukleotidil transferazės (TdT) aktyvumu prijungti įvairius nukleotidus prie oligonukleotido 3′-galo. Paaiškėjo, kad

TdT gebėjo naudoti beveik visus modifikuotus pirimidino nukleotidus iš tiriamos kolekcijos. Karboksi grupę turintys analogai nebuvo TdT substratai. Įdomu, kad TdT buvo itin aktyvi naudojant acetilbenzoil ar benzoilbenzoil turinčius $dC^{Acil}TP$, ir sintetino DNR, kurio 3′-modifikuotą galą sudarė iki kelių šimtų pakeistų nukleotidų. Kadangi ši modifikuota DNR netiko įprastiems DNR frakcionavimo metodams, buvo pasiūlyta, jog hidrofobinės modifikacijos galėjo sukelti specifinių erdvinių struktūrų susidarymą. Tai galėtų būti pritaikyta DNR-grįstų nanomedžiagų ir nanostruktūrų kūrimui.

Išsiaiškinta, kad N^4-acil-modifikacijas turinti DNR yra atspari tirtų restrikcijos endonukleazių (KpnI, SmaI, Crf9I, HpaII, SacI, XbaI, PauI and Bsh1236I) vykdomai hidrolizei. Parodyta, kad DNR, kurios viena arba abi grandinės yra modifikuotos, nėra hidrolizuojama. Vis dėlto, buvo pastebėtas išskirtinis XbaI endonukleazės elgesys, kuri hidrolizavo DNR, turinčią vieną modifikuotą grandinę, tačiau neveikė pilnai modifikuotos DNR. Svarbu paminėti, jog restrikcijos endonukleazė, atpažįstanti AT-seką, toleravo šalia esančius dC^{Acil}. Dėl to, N^4-acil-modifikacija galėtų būti naudojama kaip DNR apsauga tik nuo tam tikrų restrikcijos endonukleazių. Be to, tokia DNR apsauga sudarė sąlygas atlikti pradmens ilginimo reakcijas mišinyje esant modifikuotiems ($dC^{Acil}TP$) ir natūraliems (dCTP) nukleotidams. Savo ruožtu, konkurencinio pradmens ilginimo rezultatai gali suteikti žinių apie naudojamos polimerazės substrato pasirinkimo mechanizmą.

Norint turėti universalią, patogią naudoti ir plačiai taikomą DNR modifikaciją, ši turėtų būti ne tik įjungiama į DNR, bet ir pašalinama esant poreikiui. Dažniausiai naudojami nukleotidų analogai turi nepaslankią ir tvirtą C5/C7 padėties modifikaciją, kuri negali būti visiškai pašalinta. Priešingai, tirti $dC^{Acil}TP$ pasižymėjo sąlyginai lengvai kintančio N^4-amidinio ryšio savybe, kuri yra taikoma fosforamiditų chemijoje. Parodyta, kad N^4-acil-modifikuota DNR gali būti demodifikuota naudojant vandeninį etanolamino tirpalą. Ypač svarbu yra tai, kad modifikacijos įjungimas, pašalinimas ir rezultato įvertinimas remiantis restrikcijos endonukleazėmis yra atliekamas tame pačiame mėgintuvėlyje, be jokių papildomų gryninimo žingsnių. Be to, buvo nustatyta, kad modifikacijos-demodifikacijos ciklas nesukuria DNR mutacijų, todėl tokia DNR gali būti sėkmingai naudojama genų inžinerijos tikslais. Tai atskleidžia pristatyto demodifikavimo metodo paprastumą bei N^4-modifikacijų taikomumą.

Toliau buvo parodyta, jog fotoreaktyvias grupes turintys nukleotidai yra tinkami tiesioginiam kovalentiniam sujungimui su įvairiais substratais. Kovalentinių kompleksų sudarymas gali būti vykdomas naudojant DNR, turinčią 4-tiouridino atitikmenį arba acetilbenzoil/benzoilbenzoil

modifikuotus dC^{Acil}. Šiuo atveju, kovalentinis sujungimas yra įmanomas dėl minėtų modifikacijų fotoaktyvavimo apšviečiant UVA (365 nm) šviesa. Pabrėžtina, kad apšvietimas UVA šviesa nesukelia biologinių molekulių pažaidų, todėl yra patrauklus būdas nagrinėti DNR-DNR, DNR-baltymų ar baltymų-baltymų kompleksus. Taigi, buvo nustatyta, kad 3′-modifikuota DNR, turinti tio, acetilbenzoil arba benzoilbenzoil modifikacijas gali būti kovalentiškai sujungta su sąveikaujančiais baltymais. Be to, N^4-acetilbenzoil arba benzoilbenzoil modifikuota DNR yra tinkama fotoimobilizavimui ant įvairių chemiškai neapdorotų paviršių, pvz., polistireno, polipropileno, borosilikatinio stiklo, polilaktato ar polidimetilsiloksano polimerų. Svarbu, kad šiuo atveju sudėtingas paviršių paruošimas ir padengimas nereikalingas. Parodyta, kad imobilizuota N^4-acetilbenzoil arba benzoilbenzoil modifikuota DNR yra tinkamai išsidėsčiusi hibridizacijai su komplementariomis DNR molekulėmis. Paprasto denatūracijos karščiu suderinamumas suteikia dar daugiau patrauklumo, todėl N^4-acetilbenzoil arba benzoilbenzoil modifikuota DNR siūloma kaip nesudėtinga, bet kartu ir efektyvi alternatyva komplementarių DNR molekulių aptikimui.

Apibendrinant, galima teigti, kad buvo ištirtas ir parodytas naujų modifikuotų pirimidino nukleotidų naudojimas fermentinei oligonukleotidų sintezei bei apžvelgtos kelios modifikuotų oligonukleotidų taikymo sritys. Nustatytos DNR polimerazės, naudojančios modifikuotus nukleotidus kaip substratus. Įvertintos N^4-acil-citozino bazių porų sudarymo ypatybes bei N^4-acil-modifikacijų įtaka restrikcijos endonukleazėms. Parodyta, kad N^4-acil-modifikacijos gali būti naudojamos kaip laikinos apsauginės grupės. Nustatytas fotoaktyvias grupes turinčių nukleotidų pritaikomumas nuo UV priklausomam kovalentiniam sujungimui su sąveikaujančiais baltymais ir kitais substratais. Suformuluotos darbo **išvados**:

1. Nauji modifikuoti pirimidino nukleotidai – dC^{Ac}, dC^{Hex}, dC^{Bz}, dC^{Nic}, dC^{AcBz}, dC^{BzBz}, dPyr, $dPyr^{4OH}$, $dPyr^{4Cl(Br)}$, dU^{4S}, N^4-amino-dC ir dU^{5CN} – yra tinkami fermentinei modifikuotos DNR sintezei.

2. Klenow fragmentas (exo–), *Taq*, KOD XL, Bsm ir phi29 DNR polimerazės naudoja $dC^{Ac}TP$, $dC^{Hex}TP$, $dC^{Bz}TP$, $dC^{Nic}TP$, $dC^{AcBz}TP$ ir $dC^{BzBz}TP$ kaip substratus.

3. dC^{Ac}, dC^{Hex}, dC^{Bz}, dC^{Nic}, dC^{AcBz}, dC^{BzBz}, dPyr, $dPyr^{4OH}$, $dPyr^{4Cl(Br)}$, dU^{4S}, N^4-amino-dC ir dU^{5CN} yra tinkami nuo matricos nepriklausomai DNR sintezei naudojant terminalinę deoksinukleotidil transferazę.

4. Klenow fragmentas (exo–), *Taq*, KOD XL ir Bsm DNR polimerazės sudaro ne tik komplementarią C^{Acil}•G, bet ir C^{Acil}•A bazių porą.

5. N^4-modifikuota DNR, turinti acetil, heksanoil, nikotinoil, acetilbenzoil arba benzoilbenzoil grupes, yra atspari kai kurioms II-tipo restrikcijos endonukleazėms.

6. N^4-modifikuota DNR yra demodifikuojama naudojant vandeninį etanolamino tirpalą, nepažeidžiant DNR sekos.

7. Modifikuota DNR, turinti fotoaktyvias tio, acetilbenzoil ar benzoilbenzoil grupes, yra efektyvi fotocheminio kryžminio sujungimo su sąveikaujančiais baltymais priemonė.

8. 3′-Modifikuota DNR, turinti acetilbenzoil ar benzoilbenzoil grupes, yra nesudėtingai imobilizuojama ant neapdirbtų polimerinių paviršių ir tinkama komplementarių DNR taikinių paieškai.

SCIENTIFIC PARTICIPATION

Patent and patent application

- Meškys, R., **Jakubovska, J.**, Tauraitė, D. N^4-Modifikuoti citidino nukleotidai ir jų panaudojimas. Lithuanian patent No. 6615.

- Meškys, R., **Jakubovska, J.**, Tauraitė, D. N^4-Modified cytidine nucleotides and their use. International application No. PCT/IB2018/056961.

Conference presentations

Poster presentations:

- **Jakubovska, J**., Tauraitė, D., Birštonas, L., Meškys, R. (2018) Modified nucleotides as UVA-induced cross-linking agents. Vita Scientia, Vilnius, Lithuania.
- **Jakubovska, J.**, Tauraitė, D., Meškys, R. (2018) Cleavage of N^4-acetyl-cytidine-modified DNA by type II restriction endonucleases. The XVth International conference of the Lithuanian biochemical society, Dubingiai, Lithuania.
- Tauraitė, D., **Jakubovska, J.**, Meškys, R. Synthesis of N^4-modified deoxycytidine nucleotides as substrates for enzymatic synthesis of DNA. The Xth International conference Balticum Organicum Syntheticum, Tallinn, Estonia.
- **Jakubovska, J.**, Tauraitė, D., Birštonas, L., Meškys, R. (2018) Novel N^4-acyl-2'-deoxycytidine-5'-triphosphates for the enzymatic synthesis of modified DNA. The 43^{red} FEBS Congress, Prague, Czech Republic.

Oral presentation:

- **Jakubovska, J.** (2018) N^4-acil-modifikuoti nukleotidai funkcionalizuotos DNR sintezei. 11-oji Jaunųjų mokslininkų konferencija „Bioateitis: gamtos ir gyvybės mokslų perspektyvos“, Vilnius, Lithuania.

Scientific awards

- The Lithuanian Academy of Sciences Young scientist and Doctoral student award (2018) in Technological sciences, Chemistry Engineering (T 005) for the study "Modified N^4-acyl-cytidine nucleotides for the synthesis of functionalized DNA".

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Darbo patirtis

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COPIES OF PUBLICATIONS

Paper I

N^4-acyl-2′-deoxycytidine-5′-triphosphates for the enzymatic synthesis of modified DNA

Jakubovska, J., Tauraitė, D., Birštonas, L., Meškys, R.

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Paper II

Modified nucleotides as substrates of terminal deoxynucleotidyl transferase

Tauraitė, D., Jakubovska, J., Dabužinskaitė, J., Bratchikov, M., Meškys, R.

Molecules 2017, **22**, 672

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Paper III

Transient N^4-acyl-DNA protection against the cleavage by restriction endonucleases

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Paper IV

A versatile method for the UVA-induced cross-linking of acetophenone-or benzophenone-functionalized DNA

Jakubovska, J., Tauraitė, D., Meškys, R.

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SUPPLEMENT

Table S1. Sequences of the primers and templates used in this study [Paper I, Supplementary Data p. 12].

Oligo	Sequence
Temp^{A}	5'-CCGGAATTAAAATCTCCCTATAGTGAGTCGTATTA-3'
Temp^{G}	5'-CCGGAATTGGGGTCTCCCTATAGTGAGTCGTATTA-3'
Temp^{C}	5'-CCGGAATTCCCCTCTCCCTATAGTGAGTCGTATTA-3'
Temp^{T}	5'-CCGGAATTTTTTTCTCCCTATAGTGAGTCGTATTA-3'
Temp^{47}	5'-CTAGTTATTGCTCAGCGGTGACATCTCCCTATAGTGAGTC GTATTA-3'
Temp^{58}	5'-TATGCGCGCTCTAGATTAATGAGCTCCCGGGTACCTCTCC CTATAGTGAGTCGTA-3'
Temp^{4A}	5'-AAAATCTCCCTATAGTGAGTCGTATTA-3'
Temp^{4G}	5'-GGGGTCTCCCTATAGTGAGTCGTATTA-3'
P1	5'-TAATACGACTCACTATAGGGAGA-3'
P1^{res}	5'-TAATACGACTCACTATAGGG*A*G*A-3'

* – *phosphorothioate modification*.

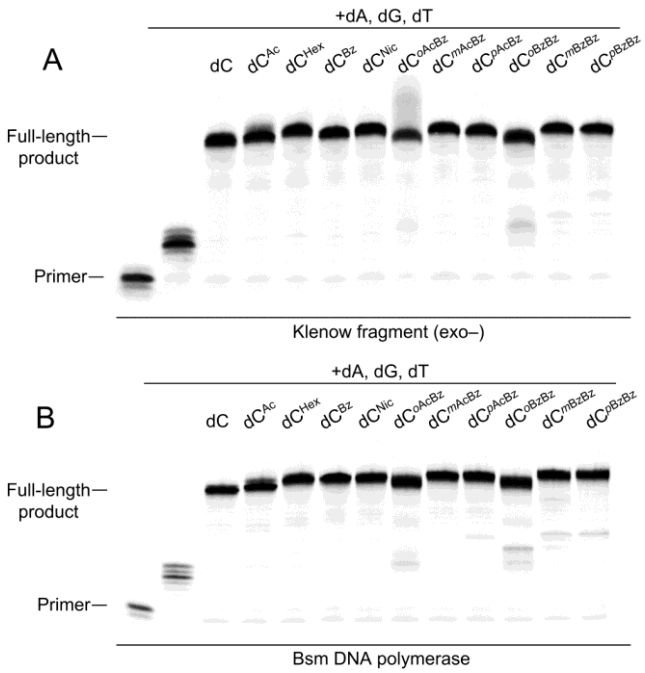

Figure S1. Autoradiograms of denaturing polyacrylamide gels showing primer extension using **Temp^{47}**, KF (exo–) or Bsm DNA polymerase and dC^{Acyl}TP instead of dCTP. The used canonical or modified dNTPs and DNA polymerases are indicated above and below the lanes, respectively [Paper I, Supplementary Data p. 17].

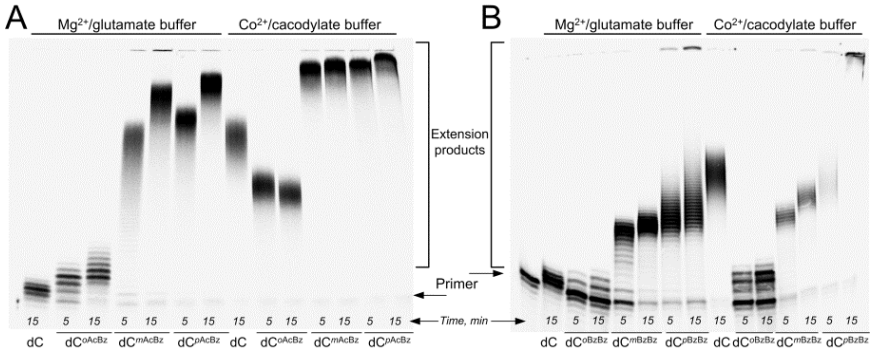

Figure S2. Autoradiograms of denaturing polyacrylamide gels showing primer 3′-elongation using different buffers, TdT and dNTPs. The used buffers and nucleotides are indicated above and below the lanes, respectively. The duration of reactions (min) is indicated on the bottom of the lanes [Paper I, Supplementary Data p. 20].

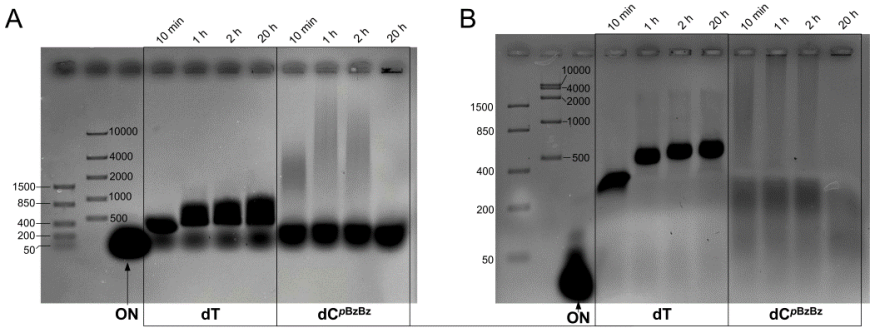

Figure S3. Fluorescence images of agarose gels showing electrophoretic migration of a 3'-elongated ON using TdT and dTTP or dC^{pBzBz}TP. (**A**) 0.8 % agarose gel, TBE buffer; (**B**) 2.5 % agarose gel, TAE buffer. The elongation time and used dNTPs are indicated above and below the lanes, respectively. DNA ladders used (kb): FastRuler^{TM} DNA ladder, low range (Thermo Scientific) (left); FastRuler^{TM} DNA ladder, high range (Thermo Scientific) (right) [Paper IV, Supplementary Data p. 3].

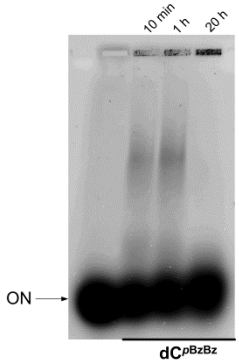

Figure S4. A fluorescence image of a pulsed-field agarose gel showing electrophoretic migration of a 3'-elongated ON using TdT and dC^{pBzBz}TP. The elongation time is indicated above the lanes [Paper IV, Supplementary Data p. 4].

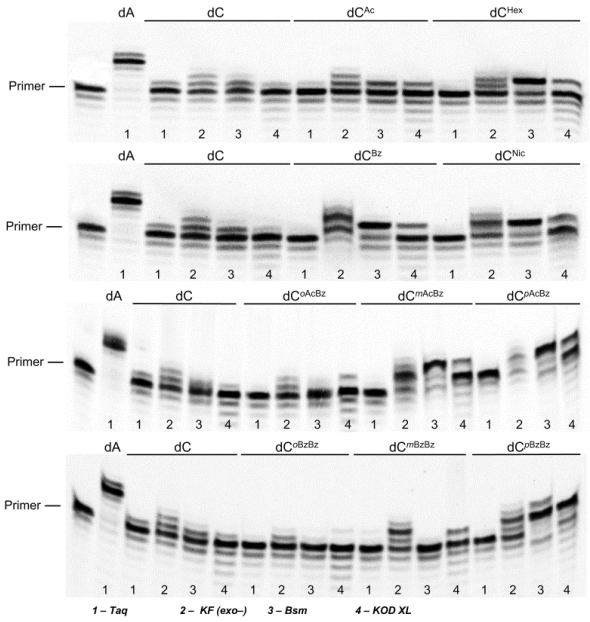

Figure S5. Autoradiograms of denaturing polyacrylamide gels showing primer extension using 35-mer **Temp^{T}**. The used modified dC^{Acyl}TPs and DNA polymerases are indicated above and below the lanes, respectively [Paper I, Supplementary Data p. 15].

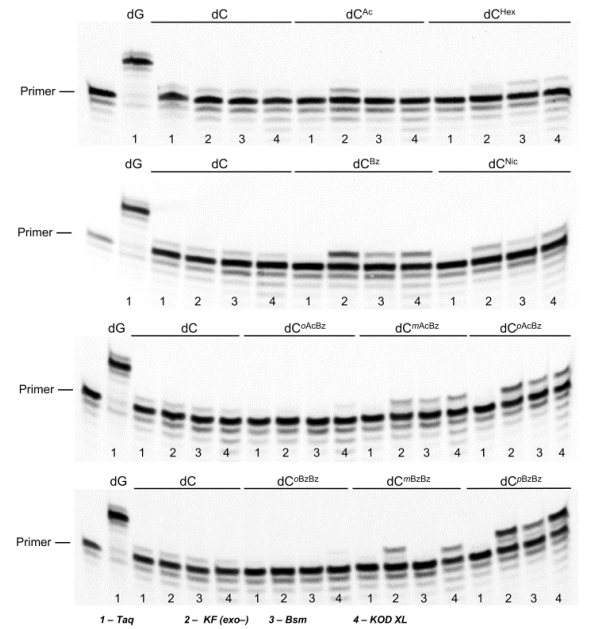

Figure S6. Autoradiograms of denaturing polyacrylamide gels showing primer extension using 35-mer **Temp^{C}**. The used modified dC^{Acyl}TPs and DNA polymerases are indicated above and below the lanes, respectively [Paper I, Supplementary Data p. 16].

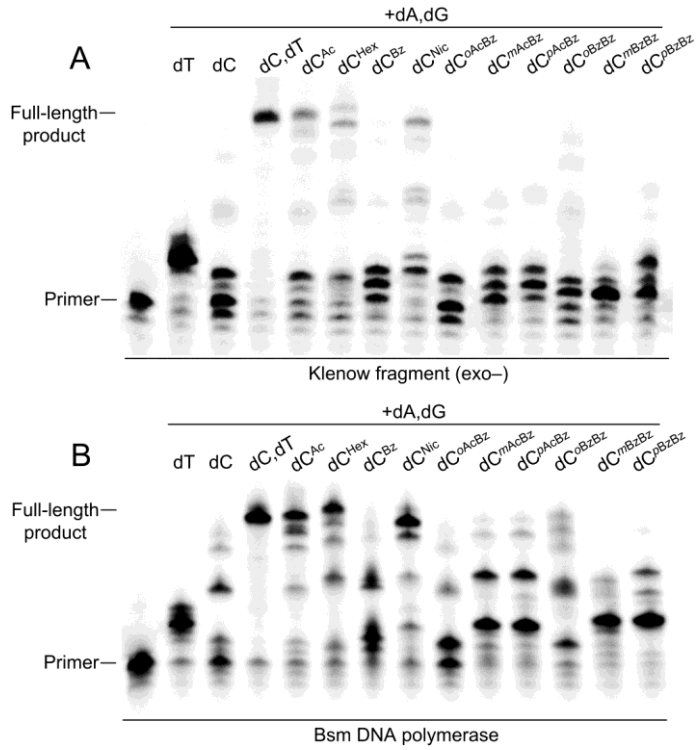

Figure S7. Autoradiograms of denaturing polyacrylamide gels showing primer extension using **Temp**[47], KF (exo–) or Bsm DNA polymerase and a set of three nucleotides only. The used canonical or modified dNTPs and DNA polymerases are indicated above and below the lanes, respectively [Paper I, Supplementary Data p. 18].

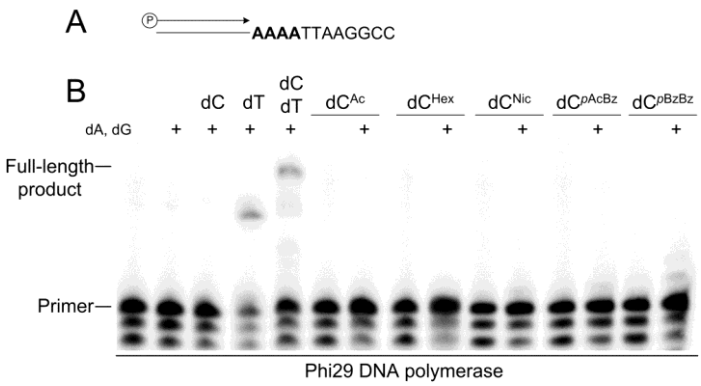

Figure S8. An incorporation of modified dC^{Acyl} nucleotides by a proofreading phi29 DNA polymerase. (**A**) A scheme of the primer-template sequence. (**B**) An autoradiogram of a denaturing polyacrylamide gel showing primer extension using phi29 DNA polymerase, **Temp**^{A} and a set of three nucleotides only. The used canonical or modified dNTPs are indicated above the lanes [Paper I, Supplementary Data p. 19].

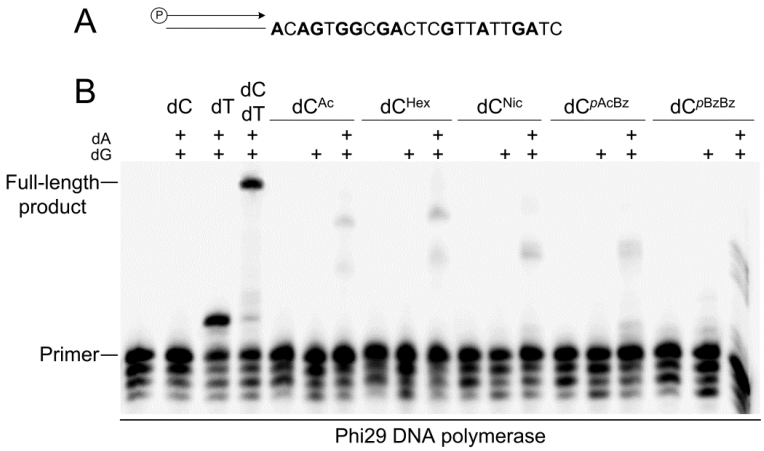

Figure S9. An incorporation of modified dC^{Acyl} nucleotides by a proofreading phi29 DNA polymerase. (**A**) A scheme of the primer-template sequence. (**B**) An autoradiogram of a denaturing polyacrylamide gel showing primer extension using phi29 DNA polymerase, **Temp**[47] and a set of three nucleotides only. The used canonical or modified dNTPs are indicated above the lanes [Paper I, Supplementary Data p. 19].

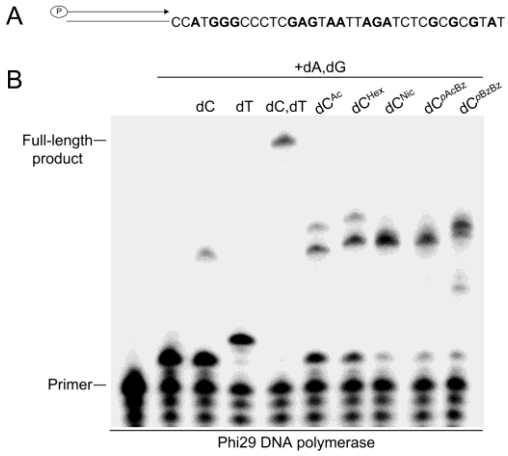

Figure S10. An incorporation of modified dC^{Acyl} nucleotides by a proofreading phi29 DNA polymerase. (**A**) A scheme of the primer-template sequence. (**B**) An autoradiogram of a denaturing polyacrylamide gel showing primer extension using phi29 DNA polymerase, **Temp**[58] and a set of three nucleotides only. The used canonical or modified dNTPs are indicated above the lanes [Paper I, Supplementary Data p. 20].

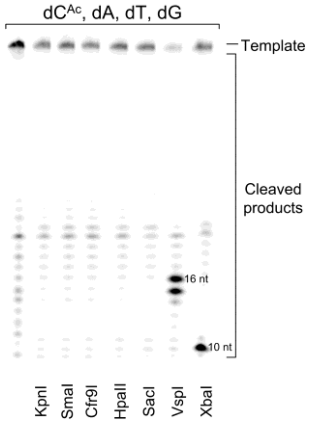

Figure S11. An autoradiogram of a denaturing polyacrylamide gel showing the cleavage of a template DNA by restriction endonucleases. PEX was performed using dC^{Ac}TP instead of dCTP. The used nucleotides and restriction endonucleases are indicated at the top and the bottom of the figure, respectively [Paper III, Supplementary Data. p. 1].

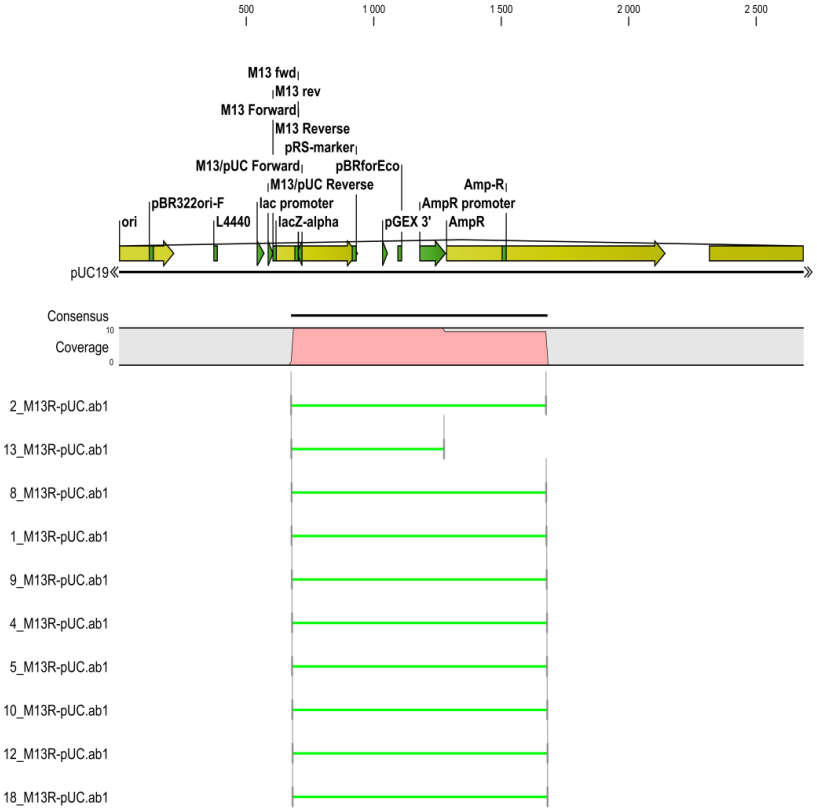

Figure S12. Alignment of the ten sequences and comparison to the pUC19 plasmid DNA. The sequences were subjected to the modification-demodification procedure following the transformation of the *E. coli* cells. Modification was performed using dC^{Ac}TP instead of dCTP [Paper III, Supplementary Data p. 2].

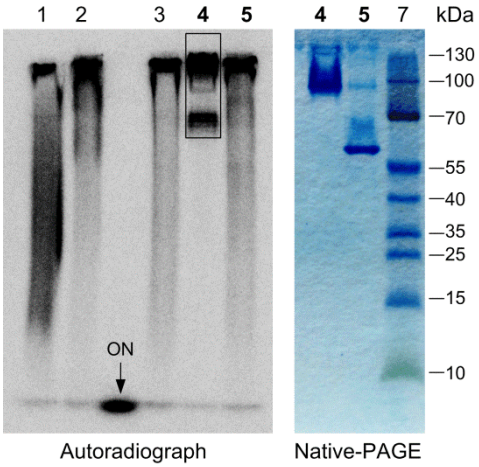

Figure S13. An autoradiogram of the native polyacrylamide gel (left) and Coomassie staining of the same gel (right) showing UVA-induced cross-linking of dC^{pBzBz}-containing DNA to proteins. Lane 1, dC^{pBzBz}-ON:TdT UV-free control; lane 2, UV cross-linked complexes of dC^{pBzBz}-ON:TdT; lane 3, UV exposed control sample after removing TdT; lane 4, UV cross-linked complexes of dC^{pBzBz}-ON:SSB; lane 5, UV exposed dC^{pBzBz}-ON:BSA control; lane 7, molecular mass marker (kDa) – PageRuler^{TM} prestained protein ladder (Thermo Scientific) [Paper IV, Supplementary Data p. 2].

NOTES

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