**ABSTRACT**

A huge diversity of modified nucleobases is used as a tool for studying DNA and RNA. Due to practical reasons, the most suitable positions for modifications are C5 of pyrimidines and C7 of purines. Unfortunately, by using these two positions only, one cannot expand a repertoire of modified nucleotides to a maximum. Here, we demonstrate the synthesis and enzymatic incorporation of novel N4-acylated 2′-deoxycytidine nucleotides (dCAcyl). We find that a variety of family A and B DNA polymerases efficiently use dCAcylTPs as substrates. In addition to the formation of complementary CCAcyl*G pair, a strong base-pairing between N4-acyl-cytosine and adenine takes place when Taq, Klenow fragment (exo−), Bsm and KOD XL DNA polymerases are used for the primer extension reactions. In contrast, a proofreading phi29 DNA polymerase successfully utilizes dCAcylTPs but is prone to form CCAcyl*A base pair under the same conditions. Moreover, we show that terminal deoxy nucleotidyl transferase is able to incorporate as many as several hundred N4-acylated-deoxycytidine nucleotides. These data reveal novel N4-acylated deoxycytidine nucleotides as beneficial substrates for the enzymatic synthesis of modified DNA, which can be further applied for specific labelling of DNA fragments, selection of aptamers or photoimmobilization.

**INTRODUCTION**

Nucleoside 5′-triphosphates bearing nucleobase modifications are widely used for specific labelling of nucleic acids (1–3) and for the development of aptamers (4–6), DNAzymes (7), biosensors (8) and therapeutics (9,10). In general, the most favoured positions for modifications are position 7 and 8 of purines as well as position 5 and 6 of pyrimidines, since they do not alter correct base-pairing. Specifically, the most popular modifications are anchored at the C5 position of pyrimidines and C7 position of 7-deazapurines (2,11,12). Since C5/C7 positions orient the modified residue toward the major groove of the double helix, this arrangement is commonly considered to minimize interference with enzymatic activity. Therefore, such nucleotide analogues serve as good to excellent substrates for various DNA and RNA polymerases and can be recruited for the successful amplification (13–15). Remarkably, several aryl group-bearing C5/C7-modified dNTPs have actually been demonstrated to prevail over natural nucleotides due to their higher affinity for a variety of polymerases (16,17). A great number of novel C5-modified pyrimidines are reported to carry redox labels for electrochemical detection (18,19), environment-sensitive fluororescent labels (20,21), photocleavable groups (22), reactive groups for bioconjugation (23,24) and labels for tracking protein binding (25). These modifications are very diverse, varying from small substituents (e.g. 5-ethyl-, 5-iodo-, 5-bromo-) to medium size residues (e.g. 5-pentynyl-, 5-tyrosyl-, 5-imidazole-) and to considerably large modifications, such as indole-, naphthylaminocarbonyl or adenylyl groups (26,27). Indeed, C5-modified uridines containing various functional moieties that mimic amino acid side chains (e.g. -benzyl-, -naphthyl-, -indolyl-) are the most commonly used nucleotide analogues in SOMAmers (Slow Off-rate Modified Aptamers) technology (28).

Sterically demanding groups, such as bulky fluorescent labels (i.e. cyanines, fluorescein) or affinity tags (biotin), are often anchored to the C5 position of pyrimidines through a flexible linker (29). Remarkably, the optimization of both composition and length of a flexible tether has led to the successful enzymatic synthesis of artificial DNA bearing extremely bulky C5-substituents, such as grafted organic chains (e.g. -benzyl-, -naphtyl-, -indolyl-) or the most commonly used nucleotide analogues in SOMPAsmers (Slow Off-rate Modified Aptamers) technology (28).

1To whom correspondence should be addressed. Tel: +370 5 223 4383; Email: jevgenija.jakubovska@bcnli.vu.lt

© The Author(s) 2018. Published by Oxford University Press on behalf of Nucleic Acids Research. This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com
lize pyrimidines substituted at C-5 with massive cargos that exceed the size of the polymerase itself.

Efficient incorporation of unnatural nucleotides is often essential due to the necessity for an amplification step required in various in vitro selection techniques (e.g. SELEX, Systematic evolution of ligands by exponential enrichment) for the development of functional nucleic acids, such as aptamers (34), ribozymes (35) or DNAzymes (36). To expand the nucleotide repertoire and forgo the search for or genetic engineering of novel polymerases with a broad substrate specificity, Click (copper(I)-catalysed azide-alkyne cycloaddition) chemistry may be applied (34). However, since in Click-SELEX the base analogue C5-ethyl-UTP is being used as a substrate for DNA polymerase, neither purines nor pyrimidines modified at other positions are applicable.

Whilst a broad spectrum of nucleobase analogue incorporations has already been designed, little attention has been paid to altering other positions. Although C6- or 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 (37–39). In fact, it has been shown that nucleotide analogues with small substituents at positions other than C5/C7 (e.g. 8-bromo-, 8-methyl-dATP or 2-methyl-, 2-vinyl-dATP) serve as good substrates, whilst the respective phenyl modification is too bulky to be incorporated (40,41). Other positions, such as O4 of purines/pyrimidines or N4 of pyrimidines, have been exploited even less. For example, O2-alkylated thymidine and guanosine are considered as the inducers of mutagenic DNA lesions that are formed due to exposure to alkylating agents (42). N4-acetyctydine is a naturally occurring minor nucleoside found in several tRNAs and rRNAs (43). Hydroxylamine and O-methylhydroxylamine are mutagenic agents that convert cytidine to N4-hydroxycytidine and N4-methoxycytidine, respectively, causing misincorporation during DNA synthesis, and thus, C→T transitions (44). Chemical synthesis, thermal stabilities and hybridization capabilities of ONs containing various cytidine nucleotides with N4-acetyl, N4-alkoxycarbonyl and N4-carbamoyl residues have been previously described (45). Moreover, it is known that N4-acetyl-CTP is efficiently used as a substrate in a T7 RNA polymerase-catalysed in vitro transcription (46), whilst N4-alkyl-deoxycytidines have been tested for polymerase chain reaction (PCR) amplification of GC-rich DNA regions (47). Altogether, it is obvious that by gaining more details about this type of modification as well as by screening for polymerases that would use such nucleotides, it would be possible to deepen our understanding of a biological role of N4-modified-cytidine.

Here, we report on the synthesis and properties of various N4-acetylated 2′-deoxyctydine 5′-triphosphates. To investigate the influence of a bulkiness of the N4-substituents on DNA polymerases, we present a series of aliphatic and aromatic N4-modifications, ranging from nucleotides bearing a small acetyl moiety to a bulky benzoylbenzoyl residue. The study is mainly focused on benzoyl-modified nucleotides and their more sterically demanding derivatives (N4-acetylbenzoyl- and N4-benzoylbenzoyl-dCTP). We find that different types of DNA polymerases are able to incorporate N4-acetyl-deoxycytidine nucleotides, and pair the N4-acylated cytosine base with adenine to a great extent. We further show that phi29 DNA polymerase successfully uses N4-modified deoxycytidine nucleotides despite its proofreading activity. Our findings indicate that, during the template-independent DNA synthesis using terminal deoxynucleotidyl transferase (TdT), as many as several hundred consecutive monomers are incorporated. We suggest that 3′-tailed DNA containing acetylbenzoyl or benzoylbenzoyl functional groups folds into a specific tertiary structure. Overall, our results provide a set of novel N4-acetyldexoxyctydine nucleotides that can significantly expand the toolbox for the enzymatic synthesis of modified DNAs.

MATERIALS AND METHODS

Primer extension (PEX) using a 35-mer template

Primer extension (PEX) reactions with natural and modified 2′-deoxynucleoside triphosphates as substrates were performed in the presence of DNA polymerase (Taq, KF (exo−), Bsm, KOD XL or phi29). Primer was 5′-radio-labelled by using T4 polynucleotide kinase in the presence of [γ-32P]-ATP. The 5′-labelled primer was desalted using Zeba spin desalting columns (7K MWCO). Primer (100 nM) and appropriate template (110 nM) were annealed by heating to 95°C and then gradually cooling to room temperature. PEX was performed in the following buffers: 10 mM Tris–HCl, 50 mM KCl, 1 mM MgCl2, 0.08% (v/v) Nonidet P40, (pH 8.8) (Taq DNA polymerase), 20 mM sodium glutamate, 20 mM NaCl, 10 mM DTT, 0.5% Triton X-100, 1 mM MgCl2 (pH 8.2) (KF (exo−)), 20 mM Tris–HCl, 10 mM KCl, 10 mM (NH4)2SO4, 2 mM MgSO4, 0.1% (v/v) Tween 20 (pH 8.8) (Bsm DNA polymerase), 33 mM Tris-acetate, 10 mM Mg-acetate, 66 mM K-acetate, 0.1% (v/v) Tween 20, 1 mM DTT (pH 7.9) (phi29 DNA polymerase) or buffer supplied by the manufacturer for KOD XL DNA polymerase. The reaction mixture (10 μl) contained Taq DNA polymerase (2.5 U), KF (exo−) (0.5 U), Bsm DNA polymerase (0.8 U), KOD XL DNA polymerase (0.25 U) or phi29 DNA polymerase (3.5 U), dCTP or dCAcylTP (10 μM), 5′-33P-labelled primer P1/P1′exo (P1′exo for phi29 DNA polymerase) and template (TempA, TempB, TempC or TempD) hybrid (5 nM) and an appropriate buffer. Reaction mixtures were incubated at 37°C (Taq DNA polymerase, KF (exo−), 60°C (Bsm DNA polymerase), 75°C (KOD XL DNA polymerase) or 30°C (phi29 DNA polymerase) temperature for 5 or 15 min. Reactions using phi29 DNA polymerase were supplemented with inorganic pyrophosphate (0.01 U). Reactions were quenched by the addition of 2× loading solution (95% (v/v) formamide, 20 mM ethylenediaminetetraacetic acid (EDTA), 0.03% (w/v) bromophenol blue, 0.03% (w/v) xylene cyanol) and heated at 95°C for 5 min. Reaction mixtures were subjected to gel electrophoresis in denaturing polyacrylamide gel (PAGE, 15%) containing TBE (Tris/Borate/EDTA) buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) and urea (8 M). Visualization was carried out by phosphorimaging using the FLA-5100 imaging system (FUJIFILM, Tokyo, Japan). The intensity of the bands representing the extension products was determined using OptiQuant analysis software (version 03.00, Packard Instrument Company Inc., Meriden, CT, USA).
USA). Sequences of the ONs used in this study can be found in the Supplementary Data (p. 11).

PEX using a 47-mer template

PEX reactions with 47-mer template (Temp⁴) were performed in the same way as described above using KF (exo–) (0.5 U), Bsm (0.8 U) or phi29 DNA polymerase (3.5 U), four dNTPs (10 μM each), 5'-33P-labelled primer P1/P1* (P1* for phi29 DNA polymerase) and template (Temp⁴) hybrid (5 nM) and an appropriate buffer. dATP, dGTP, dTTP and dCTP or dCAcTP nucleotide combination was used to monitor dCTP/dCAcTP interchange, whilst to examine dTTP/dCAcylTP replacement combination of dATP, dGTP, dCTP and dTTP/dCAcylTP was used.

TdT-catalysed 3'-elongation

The primer P1 was 5'-33P-labelled as described above. The reaction mixtures were prepared in a total volume of 10 μl and consisted of TdT (0.5 U), 5'-33P-labelled P1 (5 nM), natural or modified dNTP (10 μM) and glutamate reaction buffer (20 mM sodium glutamate, 20 mM NaCl, 10 mM DTT, 0.5% Triton X-100, 1 mM MgCl₂/MnCl₂ (pH 8.2)) or buffer supplied by manufacturer for TdT. The reaction mixtures were incubated for 5 min at 37°C, quenched by the addition of 2 × loading solution and heated at 95°C for 5 min. Reaction products were resolved in denaturing PAGE (15%) containing TBE buffer and urea (8 M). Visualization was performed by phosphorimaging.

Monitoring the dependence of TdT-catalysed primer elongation on the reaction time

The primer P1 was 5'-33P-labelled as described above. The reaction mixtures (150 μl) consisted of TdT (7.5 U), 5'-33P-labelled P1 (5 nM), dCTP, dCAcylTP or dCAcylTP (10 μM) and buffer supplied by manufacturer for TdT. Reactions proceeded from 10 s to 60 min at 37°C, and 10 μl samples were taken out from the reaction mixture and quenched with 2 × loading solution at predetermined times (10, 15, 30, 45 s and 1, 2, 5, 10, 15, 30 and 60 min). Elongation products were analysed by denaturing PAGE (15%, 8 M urea) and autoradiography. Graphical visualization was generated by the data extraction from the gel image using an online data extraction tool WebPlotDigitizer (https://automeris.io/WebPlotDigitizer/).

RESULTS

Synthesis of N⁴-modified 2'-deoxycytidine triphosphates

Pyrimidines modified at N⁴ position of the heterocyclic base were synthesized by acylation of 2'-deoxycytidine with an appropriate activated ester of carboxylic acid. The synthesized nucleosides were purified by column chromatography, and N⁴-acyl-2'-deoxycytidines (1–8) were isolated in 40–84% yields. The synthesized nucleosides were further converted to nucleotides following the one-step phosphorylation method with modifications (48). This method allowed to obtain the modified nucleotide from the appropriate nucleoside in 29–48% yields (Figure 1). The synthesized nucleotides were isolated from the crude reaction mixture by ion exchange chromatography using diethylaminoethyl Sephadex A-25 column with a gradient of LiCl, followed by precipitation of modified nucleotides from acetone/methanol mixtures. Modified nucleoside triphosphates were once again purified by reverse phase chromatography using C-18 flash cartridges. The one-step phosphorylation procedure and two chromatography purifications allowed us to readily obtain the nucleoside triphosphates (9–18) with >95% purity, and applicable for further studies. The structures of new compounds were proved by nuclear magnetic resonance spectroscopy and high-performance liquid chromatography-mass spectrometry (HPLC-MS) analysis (Supplementary Data).

Incorporation of N⁴-acyl-2'-deoxyctydine nucleotides by exonuclease-deficient polymerases

To determine whether N⁴-acyl-modified deoxycytidine nucleotides can be incorporated into DNA, we first examined the ability of several DNA polymerases to perform PEX in the presence of a dCAcTP. KF (exo–) of Escherichia coli (family A), thermostable Thermus aquaticus Taq DNA polymerase (family A), Thermococcus kodakaraensis KOD XL (family B) as well as Bsm DNA polymerase (family A) featuring strong strand displacement activity were tested. Four different templates were used to examine whether dCAcyl behaves as a single complementary nucleotide. To make PEX more challenging and to test if these modified nucleotides can be incorporated one after the other, templates contained four identical nucleobases (A, G, C or T) in a consecutive order (for sequences, see Supplementary Table S1). PEX experiments using Temp⁴ revealed that all exo– DNA polymerases tested were able to incorporate three to four N⁴-acyl-modified deoxcytidine nucleotides in a row (Figure 2). The smaller modification-bearing nucleotides (i.e. dCAc, dCHex, dCBr, dCNic) were incorporated with comparable efficiency irrespectively of a polymerase used. In contrast, the data presented in Figure 2 indicated that dCAcBr, dCyAcBr, dCAcBr and dCyBBr were better substrates for KF (exo–) and Bsm than for Taq or KOD XL, hence the bulkiness of the modification predetermined the elongation performance of individual DNA polymerases. Nevertheless, all N⁴-acyl-modified nucleotides had similar pairing efficiencies with guanine, and showed nearly the same pairing efficiency as their natural counterpart. These results suggest that modification at N⁴-position of cytosine does not interfere with the formation of correct hydrogen bonds with the guanine base.

Surprisingly, PEX experiments using Temp⁴ revealed a strong complementarity between adenine base and N⁴-acyl-modified cytosine (Figure 3 and Supplementary Figure S1). As seen in Figure 3 and Supplementary Figure S1, all DNA polymerases tested much more efficiently incorporated N⁴-acyl-deoxycytidine nucleotides than their natural counterparts. KF (exo–) and Bsm were found to be the best polymerases, which gave the longest modified products, whereas the other two enzymes formed the truncated DNA fragments. In general, the three polymerases (except for Taq) generated DNA products containing two deoxycytidine analogues mostly. Moreover, it could be noticed
Figure 1. Synthesis of \( N^4 \)-modified 2′-deoxycytidine-5′-triphosphates. Reagents and conditions: (i) NHS, DCC, ethyl acetate, rt, 24 h; (ii) 2′-deoxycytidine, DMF, 25–30 °C, 24–48 h; (iii) POCl₃, TBA, trimethyl phosphate, 0–20 °C, 1–2 h; then TBA, \((NHBu_3)_2H_2P_2O_7/CH_3CN\), rt, 10–15 min.

Incorporation of deoxycytidine analogues using templates bearing pyrimidine bases (TempT and TempC) was more complicated, and only some combinations of modified nucleotides and polymerases exhibited a slightly enhanced incorporation compared to 2′-deoxycytidine (Supplementary Figures S4 and 5). Yet the results seemed somewhat extraordinary, as it appeared that only the bulkiest modification-containing nucleotides (acetylbenzoyl and benzoylbenzoyl) were incorporated better than both cytosine and thymine bases. This may indicate to an additional stabilization of a DNA duplex architecture or stacking interactions provided by the modification group.

Next, we studied the synthesis of longer DNA products in a PEX reaction using a 47-mer template Temp47. Such PEX reaction should lead to a 47-mer DNA containing either five or six modified deoxycytidine nucleotides opposite to adenine or guanine at multiple positions, respectively. PEX experiments were conducted using KF (exo−) and Bsm DNA polymerases. It was demonstrated that in the case of both polymerases, replacing dCTP with dCAcylTP generated rather clean bands of the 47-mer ON products (Supplementary Figure S6). These results were consistent with the data presented in Figure 2.

By substituting TTP with modified deoxycytidine analogues, a larger amount of truncated products was generated, though several modifications did not interfere with an elongation efficiency (Figure 4). Data presented in Figure 4 revealed that acetyl, hexanoyl and nicotinoyl group-bearing nucleotides were proved to be the best substrates for KF (exo−) and Bsm during incorporation and pairing with adenine (Figure 3 and Supplementary Figure S1). Remarkably, as for Bsm DNA polymerase, PEX reactions using three nucleotides only (dCAcylTP, dATP and dGTP) resulted in a quite similar accumulation of the full-length products containing acetyl, hexanoyl and nicotinoyl groups as using a full set of nucleotides (Supplementary Figure S7). On the other hand, KF (exo−) struggled to elongate in the absence of one nucleotide, though a small portion of the 47-mer ON product was still generated using dCAcTP, dCHexTP and dCNicTP. These results revealed rather dual behaviour of dCAcylTPs (as dCTP or TTP), and a promising possibility for a considerably successful elongation despite the lack of nucleotides.

Incorporation of \( N^4 \)-acyl-modified deoxycytidine nucleotides by a proofreading polymerase

Next, we tested \( N^4 \)-acyl-modified deoxycytidine nucleotides in a PEX reaction using a highly processive phi29 DNA polymerase (family B) with strong strand displacement activity that allows for the efficient isothermal DNA amplification (49). Phi29 DNA polymerase also possesses a \( 3' \rightarrow 5' \)
exonuclease (proofreading) activity, and therefore performs a very accurate DNA synthesis (50).

First, four different DNA templates (TempG, TempA, TempC, TempT) were used to investigate the substrate specificity as well as the proofreading activity of phi29 DNA polymerase. As seen in Figure 5, PEX using TempG gave positive results, whereas utilization of other templates resulted in no extension (data not shown). Notably, phi29 DNA polymerase selectively paired guanine with several modified deoxycytidine analogues, namely those bearing acetyl, hexanoyl, nicotinoyl, p-acetylbenzoyl and p-benzoylbenzoyl groups (Figure 5).

Modified nucleotides demonstrating the best results were then subjected to a PEX reaction for the synthesis of a 47-bp long DNA fragment. Here, all four dNTPs were used, yet dCTP was replaced with one of the modified analogues. Despite the fact that phi29 DNA polymerase is known to discriminate between correct and incorrect nucleotides, Figure 6 revealed that full-length products containing six modifications in total were generated. Moreover, data presented in Figures 5 and 6 indicated that the processivity of phi29 prevented the generation of truncated DNA products, thus the majority of products were of full length.

To examine the accuracy of phi29 DNA polymerase for the synthesis of DNA with modified nucleotides, we further performed PEX using three nucleotides—dC\(^{\text{Acyl}}\)TP, dATP and dGTP. Here, three different DNA templates (TempA, Temp\(^{7}\), Temp\(^{8}\)) were used to test the impact of the adjacent nucleobase positions on the incorporation of modified nucleotides by phi29 DNA polymerase. In general, phi29 DNA polymerase failed to generate full-length products in the absence of TTP (Supplementary Figures S8–10). Yet, we noticed that utilization of templates with discrete A sites, particularly Temp\(^{7}\) and Temp\(^{8}\), caused a small portion of truncated products to appear (Supplementary Figures S9 and 10). Such a phenomenon, however, was completely not relevant when using Temp\(^{5}\) that started with four adenine bases straight (Supplementary Figure S8). In either way, phi29 DNA polymerase was unsuccessful to initiate elongation in the presence of dC\(^{\text{Acyl}}\)TP and dGTP or dC\(^{\text{Acyl}}\)TP only.

**TdT-mediated 3′-end tailing using dC\(^{\text{Acyl}}\)TPs**

To expand the utilization of novel dC\(^{\text{Acyl}}\) nucleotides for the enzymatic synthesis of modified DNA, we applied a template-independent 3′-tailing based on TdT. The latter is an X family DNA polymerase that catalyses the repetitive addition of random nucleotides to the 3′-OH terminus of DNA (51,52). Among all DNA polymerases, TdT is unique for its broad utilization of divalent metal ions such as Mg\(^{2+}\), Zn\(^{2+}\), Co\(^{2+}\), Mn\(^{2+}\) (53). Therefore, to monitor the discrimination between the incorporation efficiencies of modified nucleotides by TdT, two buffer systems were used. The first system was based on sodium glutamate containing Mg\(^{2+}\), which could likely be the basic enzyme cofactor \textit{in vivo}. The other buffer system, referred to as an optimal buffer for TdT, was based on potassium cacodylate and Co\(^{2+}\), which was absolutely artificial, hence incompatible with downstream applications.

Figure 7 illustrates a non-templated 3′-end elongation by TdT using all dC\(^{\text{Acyl}}\)TPs in Mg\(^{2+}\)-containing and Co\(^{2+}\)-containing (optimal) buffer systems. It could be noticed right away, that the majority of modified nucleotides were better substrates for TdT than the natural ones. In addition, the Mg\(^{2+}\)-containing buffer mostly yielded uneven products, ranging from several to several hundred nucleotides in length, whilst using the Co\(^{2+}\)-containing system resulted in the generation of more homogenous DNA products. Not surprisingly, the utilization of optimal buffer for TdT resulted in a more efficient incorporation of all nucleotides, though Co\(^{2+}\)/cacodylate barely improved the incorporation of dC\(^{\text{Hex}}\)TP and dC\(^{\text{BzBz}}\)TP. These two nucleotides were then tested in a Mn\(^{2+}\)-containing buffer, which is known to modulate the substrate specificity of numerous polymerases including TdT (54). We found however, that manganese ions had very little effect on TdT with dC\(^{\text{Hex}}\)TP or dC\(^{\text{BzBz}}\)TP as a substrate, and the incorporation efficiency of these nucleotides, in the presence of Mn\(^{2+}\), was higher than in Mg\(^{2+}\)-containing but lower than in Co\(^{2+}\)-containing buffer (data not shown).

To evaluate a characteristic efficiency of 3′-elongation using modified nucleotides, we monitored the reactions over time by removing aliquots during tailing reaction,
and analysing products by the denaturing PAGE. We used acetylbenzoyl and benzoylbenzoyl-bearing nucleotides since they exhibited a pronounced difference in TdT-mediated DNA polymerization reactions with regard to acetyl or benzoyl residue position (i.e. ortho, meta, para) (Figure 7). We found that despite of prolonged reaction time, both ortho isomers were poorly used by TdT though dC<sub>ortho</sub>BzTP was preferred over dC<sub>ortho</sub>BzBzTP (Supplementary Figure S11). On the other hand, the polymerization of the meta and para isomers, using both buffer systems, clearly depended on the duration of the reaction (Supplementary Figure S11). Indeed, we found it impressive that 3' -tailing using benzoylbenzoyl-bearing nucleotides in a Co<sup>2+</sup>-containing buffer was so effective that, in the case of dC<sub>meta</sub>BzBzTP and dC<sub>para</sub>BzBzTP, a considerable portion of the radioactive material was unable to enter the gel (Figure 7 and Supplementary Figure S11). Identical accumulation of the radioactive material near the gel wells was also observed in agarose (0.8%) gels (data not shown).
To investigate the dependency of the TdT-catalysed tailing on the duration of the reaction in more detail, we examined 3’-elongation using dCmAcBzTP as the best representative (Figure 8). Based on the data analysis, TdT demonstrated considerably high reaction rate by adding one modified dCmAcBzTP per second (1 nt × s⁻¹), compared to a 6-fold lower reaction rate using dCTP (~0.17 nt × s⁻¹). Moreover, TdT catalysed a step-wise addition of the mAcBz-deoxycytidine nucleotides until a certain level of elongation was achieved. After that point, even prolonged reaction did not improve the efficiency of 3’-tailing (Figure 8). Overall, these results indicate that N⁴-acylated nucleotides are excellent substrates for TdT, and the desired results can be readily obtained through the optimization of reaction conditions.

**DISCUSSION**

Recent investigations indicate that the enzymatic synthesis of the base-modified DNA is almost exclusively performed by using pyrimidines modified at C5, and 7-deazapurines modified at C7 (2,11,12). This is mainly due to the excellent substrate properties shown by these analogues not only in PEX but also in PCR. In contrast, nucleotides modified at other positions of pyrimidines/purines are less favoured by DNA polymerases (39). It has been discovered that in terms of incorporation of modified nucleotides, family B DNA polymerases are superior to the enzymes belonging to the family A (13). Here, we show that both family A and B DNA polymerases readily incorporate N⁴-acylated cytidine nucleotides and pair with guanine. Small as well as large moieties bearing nucleotides are good or even greater substrates than the natural dCTP, depending upon a DNA polymerase. In fact, we find that dC⁴AcTPs are suitable for
Figure 5. An incorporation of modified dC\textsubscript{Acyl} nucleotides by a proofreading phi29 DNA polymerase. (A) A scheme of the PEX experiment using phi29 DNA polymerase and Temp\textsuperscript{Ex}. (B) An autoradiogram of a denaturing polyacrylamide gel showing PEX using phi29 DNA polymerase and modified dC\textsubscript{Acyl}TPs. The used modified dC\textsubscript{Acyl}TPs are indicated above the lanes.

Figure 6. An autoradiogram of a denaturing polyacrylamide gel showing PEX using Temp\textsuperscript{47}, phi29 DNA polymerase and dC\textsubscript{Acyl}TP instead of dCTP. The used nucleotide combinations are indicated above the lanes.

We notice, however, that the major differences in the incorporation efficiencies are caused by either the presence or the lack of the 3’-exonuclease activity. It is acknowledged that the exonuclease and polymerase domains utilize distinct mechanisms of substrate discrimination (55). Depending upon the type of DNA polymerase, in general, the accuracy of the enzyme is determined by the geometric discrimination of base pairs within the active site. In contrast, the exonuclease uses a thermodynamic mechanism of the substrate recognition. Here, we show that Taq, KF (exo–), Bsm and KOD XL DNA polymerases exhibit similar substrate specificity towards dC\textsubscript{Acyl}TPs, whilst phi29 DNA polymerase with 3’-exonuclease activity acts differently. Moreover, exo– DNA polymerases succeed to incorporate dC\textsubscript{Acyl} against adenine at a quite high level, whilst phi29 is prone to directly pair C\textsubscript{Acyl} with A. Our results suggest that mismatched C\textsubscript{Acyl} • A, C\textsubscript{Acyl} • T or C\textsubscript{Acyl} • C pairs fail to maintain an appropriate geometry in the active site of phi29, therefore successful incorporation is restricted. The C\textsubscript{Acyl} • G base pair is more likely to simulate a correct alignment, though these DNA duplexes differ in terms of thermodynamic stability. Upon detection of an unstable duplex variant, a proofreading activity of phi29 prevails and no incorporation is detected. However, considering the template sequence and extreme shortage of specific nucleotides, phi29 DNA polymerase somehow manages to pass through a C • A or C\textsubscript{Acyl} • A mispair to a certain degree, if the next correct nucleotide is immediately incorporated. So it seems that a certain catalytic competition between nucleotide itself, its incorporation (polymerase activity) and the removal of mispairs (exonuclease activity) regulates the outcome. Nevertheless, complementing with all the necessary nucleotides would easily solve such an insignificant misincorporation process and offer an opportunity for the application of N\textsuperscript{4}-modified nucleotides for the isothermal amplification during SELEX. Although phi29 DNA polymerase has been applied in a variety of DNA amplification procedures, only few cases have been reported regarding the use of non-natural substrates (56,57). Here, we demonstrate that phi29 DNA polymerase is able to incorporate N\textsuperscript{4}-acylated cytidine analogues resulting in the synthesis of functionalized DNA. Modification of DNA with a variety of diverse residues by the means of isothermal amplification would allow a great improvement in diagnostics, biosensing, bioanalysis and therapeutic applications (58).

Next, we demonstrate a successful base-pairing between N\textsuperscript{4}-acylated cytosine and adenine. Although very few studies have been reported on natural A • C mispairing (59,60), the experimental data suggest that two possible A • C structures involving single or double hydrogen bonds coexist in the DNA duplex (60). In contrast, the A • C structures with strictly two N–H–N hydrogen bonds (i.e. reverse wobble and reverse Hoogsteen) are calculated to be more energetically favourable using the theoretical modelling methods.
Figure 7. TdT-catalysed 3′-end tailing using dC\textsuperscript{Ac\textsubscript{m}BzTP} as substrates. (A) Scheme of tail-labelling of the ON probe with \textsuperscript{N\textsuperscript{4}}-acylated deoxycytidine markers and TdT. (B and C) Autoradiograms of denaturing polyacrylamide gels showing primer 3′-elongation. The used nucleotides and buffers are indicated above and below the lanes, respectively.

Figure 8. Dependence of the efficiency of TdT-catalysed 3′-tailing on the reaction duration, using dCTP or dC\textsuperscript{Ac\textsubscript{m}BzTP} as substrates. (A) An autoradiogram of a denaturing polyacrylamide gel showing primer 3′-elongation using TdT. The elongation time is indicated above each lane. (B) Fit of the elongation process with dC\textsuperscript{Ac\textsubscript{m}BzTP} using the data extracted from the gel image (A).
During the studies on N4-modified 2′-deoxyctydine nucleotides, such as N4-acyl-dC or N4-alkoxybenzoyl-dC, extraordinary strong base-pairing with adenine has been also detected (62, 63). For example, N4-alkoxybenzoyl-dC forms a base pair with adenine in a different geometry from the naturally occurring A•C mismatched pair of Wobble type, including two unique hydrogen bonds (C-4NH•••A-•N and C-O\textsuperscript{ester}•••N-A,\textsuperscript{dNH}) (63). Nevertheless, it is more complicated than an unusual mismatched base-pairing. For instance, it has been determined that N4-acyl groups are oriented in a geometrically fixed manner that makes the formation of conventional Watson-Crick type base pairs with the guanine residue, owing to an intramolecular hydrogen bond between the carbonyl oxygen atom and the 5-vinyl proton of the cytosine ring (64). In contrast, N4-carbamoyl-dC•G base-pairing occurs only in a form of ONs, suggesting that geometry of N4-substituents strongly depends on the salination and intramolecular hydrogen bonds (64). Although there are several reports on the decreased stability of DNA duplexes upon increasing alkyl chain length in the N4-acyl group of cytidine (45), we have failed to notice such tendency during the PEX experiments. Based on our results, the bulkiness of a group attached to N4 position of the cytosine ring does not impede the incorporation of a forthcoming nucleotide, since in some cases the incorporation of benzoylbenzoyl-containing nucleotides has been comparable to or even better than that of smaller acetylbenzoyl-analogues. It seems likely that even large N4-substituents, such as C\textsubscript{8}-aliphatic or benzoylbenzoyl, do not disturb the hydrogen bond network structure around the major groove of the DNA duplex, hence resulting in the base-pairing with both guanine and adenine.

The larger part of studies on N4-acylated deoxyctydine-containing ONs is based on a solid-phase synthesis using phosphoramidite method. In contrast, our data rely only on the enzymatic synthesis of N4-acyl-modified DNA, and thus we imply that a number of factors play a major role in performing the enzymatic DNA synthesis. These are: the substrate specificity of different polymerases, the elongation of DNA next to the modification, polymerase translocation across modified DNA, the nature of modification (size, charge, hydrophobicity) and the formation of intramolecular bonds, duplex stability of modified DNA, the formation of tertiary structures, etc. Nevertheless, these are, beyond doubt, remarkable results on the modification of DNA in several aspects. First, one can design the template for just a single or several modifications that can be either isolated or at adjacent positions. Second, N4-modified deoxyctydine nucleotides not only may be used instead of natural ones, but may also offer limitless possibilities in modifying AT-rich DNA regions. In addition, N4-acyl-nucleotides are suitable for the enzymatic synthesis of DNA using a variety of DNA polymerases, if the specific conditions for the catalysis (depending on the presence or the absence of an exonuclease activity) are fulfilled.

As to the template-independent DNA synthesis, TdT is known to use a wide variety of nucleotide analogues, hence it may be applied for an efficient tail-labelling without the necessity for a precise quantification of the labels. TdT uses different base-modified nucleotides for the enzymatic synthesis of artificial DNA (65-68), nucleotides bearing steric aromatic pyrene residues for the generation of fluorescent oligomers (69,70) or pyridone/imidazole-based analogues for the specific metal coordination and immobilization (71-73). Our experiments confirm that TdT utilizes dC\textsuperscript{Acyl}TPs to a similar or even greater extent than natural nucleotides. We also demonstrate a possible 3′-tailing dependency on the size of a substituent at N4 position of deoxyctydine. As such, with several exceptions, by increasing the size of a functional group attached to the position N4 of cytidine (acetyl<hexanoyl>benzoyl<nicotinyl>acytelybenzoyl), the elongation efficiency is being increased regardless of a buffer used. Considering the length of the aliphatic acyl group of a modified nucleotide (i.e. acetyl/hexanoyl), the Co\textsuperscript{3+}-containing buffer demonstrates slightly different results than the Mg\textsuperscript{2+}-based buffer. It is known that different divalent metal ions required for the enzymatic reaction catalysed by TdT contribute to the kinetics of nucleotide incorporation differently (74). Thus, it might be reasoned that due to the unfavourable environment (e.g. Co\textsuperscript{2+}, cacodylate), dC\textsuperscript{Hex} is poorly incorporated.

In the case of dC\textsuperscript{AcBz} and dC\textsuperscript{BzBz} isomers, an obvious improvement in the 3′-tailing can be noticed in the order of ortho<meta<para positions in both buffer systems. It is known that TdT requires at least three deoxynucleotide residues on the primer strand for the efficient catalysis of the tailing reaction (75). Subsequently, when polymerase reaches the extended section, 3′-end modifications may block the entrance of an incoming dNTP by enhancing primer affinity to TdT and the polymerisation reaction may terminate. This would explain why only a limited number of residues were appended in the case of, e.g. dC\textsuperscript{CoAcBz} and dC\textsuperscript{CoBzBz}.

Since our data show an extremely effective 3′-tailling using benzoylbenzoyl-bearing nucleotides, it may be assumed that 3′-modified tail folds into a specific tertiary structure that is supported by the stacking of additional aromatic rings of modified nucleobases. Thus, intramolecular or intermolecular interactions as well as hydrophobic nature of benzoylbenzoyl modification may stimulate the assembly of modified DNA, and cause the abnormal electrophoretic mobility. A proposed theory on the specific steric structure of 3′-elongated DNA is also supported by the fact that TdT terminates the synthesis after the incorporation of a certain number of acetylbenzoyl-containing nucleotides. Considering the fact that neither the enzyme nor dC\textsuperscript{Acyl}TP concentration limit the reaction, TdT seems to be forced to dissociate from 3′-modified ON, rather than reversibly bind to it and continue the elongation. As a result it supports the formation of unfavourable tertiary structures for TdT-catalysed elongation using dC\textsuperscript{Acyl}TPs. Such a scenario, however, cannot be applied in the case of dCTP. Moreover, as seen in Figure 8, the longest dC\textsuperscript{AcBz}-containing DNA fragments are of very similar molecular weight suggesting that the dC\textsuperscript{Acyl} nucleotides described in this study may become very promising in generating the functionalized DNA-based nanomaterials. Compared with synthetic polymeric materials, DNA possesses many unique properties, such as its biological function, biocompatibility, biodegradability, nanoscale geometry or molecular recognition. Subsequently, the modified DNA may contribute not only to
the development of the three-dimensional DNA-ordered assemblies but also to the construction of multifunctional architectures for nanoelectronics, intelligent sensing and targeted drug delivery (76).

In summary, novel N4-modified nucleotides significantly expand the toolbox for the enzymatic synthesis of modified DNA. Further studies are needed to understand the subtle details of the interaction between various DNA polymers and dCAc3′TPs. Further exploitation of these modified nucleotides for the development of novel aptamers, particularly using a photoSELEX approach, is in progress.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS
We would like to thank Dr Laura Kaliniene for critical reading of the manuscript and Audrone Rukšėnaitė for assistance with MS analysis.

FUNDING
European Union’s Horizon 2020 Research and Innovation Program [BlueGrowth: Unlocking the potential of Seas and Oceans] (INMARE) [634486]. Funding for open access charge: European Union’s Horizon 2020 Research and Innovation Program [BlueGrowth: Unlocking the potential of Seas and Oceans] (INMARE) [634486].

Conflict of interest statement. J.J., D.T. and R.M. declare potential financial interests in the future development and commercialization of the N4-acetyl-2′-deoxyctydines. Vilnius University has filed a Lithuanian patent application (LT2017523).

REFERENCES
1. Xu, W., Chan, K.M. and Kool, E.T. (2017) Fluorescent nucleobases as tools for studying DNA and RNA. Nat. Chem., 9, 1043–1055.
2. Hocek, M. (2014) Synthesis of base-modified 2′-deoxyribonucleoside triphosphates and their use in enzymatic synthesis of modified DNA for applications in bioanalysis and chemical biology. J. Org. Chem., 79, 9914–9921.
3. Tauraitė, D., Ražanaš, R., Mikalkėnas, A., Serva, S. and Meškys, R. (2016) Synthesis of pyridone-based nucleoside analogues as substrates or inhibitors of DNA polymerases. Nucleosides Nucleotides Nucleic Acids, 35, 163–177.
4. Lapa, S.A., Chudnov, A.V. and Timofeev, E.N. (2016) The toolbox for modified aptamers. Mol. Biotechnol., 58, 79–92.
5. Kuwahara, M. and Sugimoto, N. (2010) Molecular evolution of functional nucleic acids with chemical modifications. Molecules, 15, 5423–5444.
6. Renders, M., Miller, E., Lam, C.H. and Perrin, D.M. (2017) Whole cell-SELEX of aptamers with a tyrosine-like side chain against live bacteria. Org. Biomol. Chem., 15, 1980–1989.
7. Hollenstein, M. (2015) DNA catalysis: the chemical repertoire of DNAzymes. Molecules, 20, 20777–20804.
8. Hollenstein, M., Hipolito, C., Lam, C., Dietrich, D. and Perrin, D.M. (2008) A highly selective DNAzyme sensor for mercuric ions. Angew. Chem. Int. Ed., 47, 4346–4350.
9. Wilson, C. and Keefe, A.D. (2006) Building oligonucleotide therapeutics using non-natural chemistries. Curr. Opin. Chem. Biol., 10, 607–614.
10. Mikalkėnas, A., Ravoitytė, B., Tauraitė, D., Servienė, E., Meškys, R. and Serva, S. (2017) Conjugation of phosphonoacetic acid to nucleobase promotes a mechanism-based inhibition. J. Enzyme Inhib. Med. Chem., 33, 384–389.
11. Hocek, M. and Fojta, M. (2008) Cross-coupling reactions of nucleoside triphosphates followed by polymerase incorporation. Construction and applications of base-functionalized nucleic acids. Org. Biomol. Chem., 6, 2233–2241.
12. Hollenstein, M. (2012) Nucleoside triphosphates—building blocks for the modification of nucleic acids. Molecules, 17, 13569–13591.
13. Sawai, H., Nagashima, J., Kuwahara, M., Kitagata, R., Tamura, T. and Matsui, I. (2007) Differences in substrate specificity of C (5)-substituted or C (5)-unsubstituted pyrimidine nucleotides by DNA polymerases from thermophilic bacteria, archaea, and phages. Chem. Biodivers., 4, 1979–1995.
14. Liu, E., Lam, C.H. and Perrin, D.M. (2015) Synthesis and enzymatic incorporation of modified deoxyuridine triphosphates. Molecules, 20, 13591–13602.
15. Raindlrová, V., Janoušková, M., Slavičková, M., Perlíková, P., Boháčová, Š., Milasiovječ, N., Sanderová, H., Benda, M., Barvík, I., Kráiný, L. et al. (2016) Influence of major-groove chemical modifications of DNA on transcription by bacterial RNA polymerases. Nucleic Acids Res., 44, 3000–3012.
16. Cahová, H., Panattoni, A., Kielkiewski, P., Fanfrikli, J. and Hocek, M. (2016) 5-Substituted pyrimidines and 7-substituted 7-deazapurine dNTPs as substrates for DNA polymerases in competitive primer extension in the presence of natural dNTPs. ACS Chem. Biol., 11, 3163–3171.
17. Kielkiewski, P., Fanfrikli and Hocek, M. (2014) 7-Aryl-7-deazaadenine 2′-deoxyribonucleoside triphosphates (dNTPs): better substrates for DNA polymerases than dATP in competitive incorporations. Angew. Chem. Int. Ed., 53, 7552–7555.
18. Macíčková-Cahová, H., Pohl, R., Horáková, P., Havran, L., Špaček, J., Fojta, M. and Hocek, M. (2011) Alkylsulfanylnaphthyl derivatives of cytosine and 7-deazaadenine nucleotides, nucleotides and nucleoside triphosphates: synthesis, polymerase incorporation to DNA and electrochemical study. Chem. Eur. J., 17, 5833–5841.
19. Cahová, H., Havran, L., Brázdilová, P., Pivońková, H., Pohl, R., Fojta, M. and Hocek, M. (2008) Aminophenyl- and nitrophenyl-labeled nucleoside triphosphates: synthesis, enzymatic incorporation, and electrochemical detection. Angew. Chem. Int. Ed., 120, 2089–2092.
20. Riedl, J., Pohl, R., Rulišek, L. and Hocek, M. (2011) Synthesis and photophysical properties of biaryl-substituted nucleos(t)ides. Polymerase synthesis of DNA probes bearing solvatochromic and pH-sensitive dual fluorescent and 19F NMR labels. J. Org. Chem., 77, 1026–1044.
21. Riedl, J., Pohl, R., Ernsting, N.P., Orság, P., Fojta, M. and Hocek, M. (2012) Labelling of nucleosides and oligonucleotides by solvatochromic 4-aminothialmidé fluorophore for studying DNA–protein interactions. Chem. Sci., 3, 2797–2806.
22. Boháčová, Š., Ludvíková, L., Slavětinská, L.P., Vaníková, Z., Klán, P. and Hocek, M. (2018) Protected 5-hydroxymethyl uracil nucleotides bearing visible-light photoactive organic subunits as building blocks for polymerase synthesis of photocaged DNA. Org. Biomol. Chem., 16, 1527–1535.
23. Raindlrová, V., Pohl, R., Šanda, M. and Hocek, M. (2010) Direct polymerase synthesis of reactive aldehyde-functionalized DNA and its conjugation and staining with hydrazines. Angew. Chem. Int. Ed., 49, 1064–1066.
24. Raindlrová, V., Pohl, R. and Hocek, M. (2012) Synthesis of aldehyde-linked nucleotides and DNA and their bioconjugations with lysine and peptides through reductive amination. Chem. Eur. J., 18, 4080–4087.
25. Vaníková, Z. and Hocek, M. (2014) Polymerase synthesis of photocaged DNA resistant against cleavage by restriction endonucleases. Angew. Chem. Int. Ed., 126, 6852–6855.
26. Imazumi, Y., Kasahara, Y., Fujita, H., Kitadume, S., Ozaki, H., Endoh, T., Kuwahara, M. and Sugimoto, N. (2013) Efficacy of base-modification on target binding of small molecule DNA aptamers. J. Am. Chem. Soc., 135, 9412–9419.
27. Hollenstein, M. (2012) Synthesis of deoxyribonucleoside triphosphates that include proline, urea, or sulfamide groups and their polymerase incorporation into DNA. Chem. Eur. J., 18, 13320–13330.
side chains: modified aptamers and their use as diagnostic and therapeutic agents. Mol. Ther. Nucleic Acids, 3, e201.

29. Kuwahara,M., Nagashima,J.I., Hasegawa,M., Tamura,T., Kitagata,R., Hanawa,K., Hososhima,S.I., Kasamatsu,T., Ozaki,H. and Sawai,H. (2006) Systematic characterization of 2′-deoxynucleoside-5′-triphosphate analogs as substrates for DNA polymerases by polymerase chain reaction and kinetic studies on enzymatic production of modified DNA. Nucleic Acids Res., 34, 5383–5394.

30. Baccaro,A. and Marx,A. (2010) Enzymatic synthesis of organic-polymer-grafted DNA. Chem. Eur. J., 16, 218–226.

31. Baccaro,A., Steck,A.L. and Marx,A. (2012) Barcoded nucleotides. Angew. Chem. Int. Ed., 51, 254–257.

32. Verga,D., Welter,M., Steck,A.L. and Marx,A. (2015) DNA polymerase-catalyzed incorporation of nucleotides modified with a G-quadruplex-derived DNAzyme. Chem. Commun., 51, 7379–7381.

33. Welter,M., Verga,D. and Marx,A. (2016) Sequence-specific incorporation of enzyme-nucleotide chimera by DNA polymerases. Angew. Chem. Int. Ed., 55, 1031–1033.

34. Tolle,F., Brändle,G.M., Matzner,D. and Mayer,G. (2015) DNA polymerase. J. Phys. Chem. A, 119, 1751–1757.

35. Kohler,K., Erschler,J. and Eckert,K.A. (2002) The proofreading 3′→5′ exonuclease activity of DNA polymerases: a kinetic barrier to translesion DNA synthesis. Mutat. Res., 510, 45–54.

36. Linck,L., Reiß,E., Bier,F. and Resch-Genger,U. (2012) Direct labeling rolling circle amplification as a straightforward signal amplification technique for biodetection formats. Anal. Methods, 4, 1215–1220.

37. Balasubramanian,B. and Meier,J.L. (2017) Profiling cytidine acetylation with specific affinity enrichment and analysis. Nucleic Acids Research, 45, 5710–5721.

38. Smirnova,F.A., Sekine,M., Srinivasan,B. and Baccaro,A. (2012) An improved protection-free on-pot chemical synthesis of 2′-deoxynucleoside-5′-triphosphates. Nucleosides Nucleotides Nucleic Acids, 31, 423–431.

39. Balboa,L., Bernad,A., Lazaro,J.M., Martin,G., Garmendia,C. and Salas,M. (1989) Highly efficient DNA synthesis by the phage phi 29 DNA polymerase. Symmetrical mode of DNA replication. J. Biol. Chem., 264, 8935–8940.

40. Esteban,J.A., Salas,M. and Blanco,L. (1993) Fidelity of phi 29 DNA polymerase. Comparison between protein-primed initiation and DNA polymerization. J. Biol. Chem., 268, 2719–2726.

41. Fowler,J.D. and Suo,Z. (2006) Biochemical, structural, and physiological characterization of terminal deoxynucleotidyl transferase. Chem. Rev., 106, 2092–2110.

42. Motea,E.A. and Berdis,A.J. (2010) Terminal deoxynucleotidyl transferase: The story of a misguided DNA polymerase. Biochim. Biophys. Acta, 1804, 1151–1166.

43. Johnson,D. and Morgan,A.R. (1976) The isolation of a high molecular weight terminal deoxynucleotidyl transferase from calf thymus. Biochem. Biophys. Res. Commun., 72, 840–849.

44. Kato,K.I., Goncalves,J.M., Houts,G.E. and Bollum,F.J. (1967) Deoxynucleotide-polymerizing enzymes of calf thymus gland. II. Properties of the terminal deoxynucleotidyltransferase. J. Biol. Chem., 242, 2780–2789.

45. Khare,V. and Eckert,K.A. (2002) The proofreading 3′→5′ exonuclease activity of DNA polymerases: a kinetic barrier to translesion DNA synthesis. Mutat. Res., 510, 45–54.

46. Proctor,R.K., Clark,R.T. and Thomas,K.J. (1999) An efficient method for the preparation of 5′-deoxynucleoside-5′-triphosphates and their effects on primer extension by DNA polymerases. ChemBiochem, 16, 2046–2053.

47. Böge,N., Jacobsen,M.I., Szombati,Z., Baerns,S., Di Pasquale,F., Marx,A. and Meier,C. (2008) Synthesis of DNA strands site-specifically damaged by 8-arylamino purine adducts and effects on various DNA polymerases. Chem. Eur. J., 14, 11194–11208.

48. Cahova,H., Pohl,R., Bednarova,L., Novakova,K., Cvacka,J. and Hocek,M. (2008) Synthesis of 8-bromo-, 8-methyl- and 8-phenyl-dATP and their polymerase incorporation into DNA. Org. Biomol. Chem., 6, 3657–3660.

49. Matyasovský,J., Peršliková,P., Malnut,V., Pohl,R. and Hocek,M. (2016) 2′-Substituted dATP derivatives as building blocks for polymerase-catalyzed synthesis of DNA modified in the minor groove. Angew. Chem. Int. Ed., 128, 16088–16091.

50. Basu,K. and Essigmann,J.M. (1990) Site-specifically alkylated oligodeoxynucleotides: Probes for mutagenesis, DNA repair and the structural effects of DNA damage. Mutat. Res., 233, 189–201.

51. Thomas,G.E., Gordon,J.U. and Rogg,H.A. (1978) N4-Acetylcytidine. A previously unidentified labile component of the small subunit of eukaryotic ribosomes. J. Biol. Chem., 253, 1101–1105.

52. Shugar,D., Huber,C.P. and Birnbaum,G.I. (1976) Mechanism of hydrolysis of nucleoside monophosphates. Biochim. Biophys. Acta, 447, 274–284.

53. Wada,T., Kobori,A., Kawahara,S. and Sekine,M. (2001) Synthesis and hybridization ability of oligodeoxynucleotides incorporating N-acycloxydeoxycytidine derivatives. Eur. J. Org. Chem., 2001, 4583–4593.

54. Sinclair,W.R., Arango,D., Shrimp,J.H., Zeng,Y.T., Thomas,J.M., Montgomery,D.C., Fox,S.D., Andressen,T., Oberdoerffer,S. and Meier,J.L. (2017) Profiling cytidine acetylation with specific affinity and reactivity. ACS Chem. Biol., 12, 2922–2926.

55. Flores-Juárez,C.R., González-Jasso,E., Antaramian,A. and Pless,R.C. (2016) PCR amplification of GC-rich DNA regions using the nucleotide analog N4-methyl-2′-deoxycytidine 5-triphosphate. Biotechniques, 61, 175–182.

56. Korze,A.R., Shankaragunduram,M., Senthivelu,A. and Srinivasan,B. (2012) An improved protection-free on-pot chemical synthesis of 2′-deoxynucleoside-5′-triphosphates. Nucleosides Nucleotides Nucleic Acids, 31, 423–431.
69. Cho, Y. and Kool, E.T. (2006) Enzymatic synthesis of fluorescent oligomers assembled on a DNA backbone. *ChemBiochem.*, 7, 669–672.
70. Hollenstein, M., Wojciechowski, F. and Leumann, C.J. (2012) Polymerase incorporation of pyrene-nucleoside triphosphates. *Bioorg. Med. Chem. Lett.*, 22, 4428–4430.
71. Kobayashi, T., Takezawa, Y., Sakamoto, A. and Shionoya, M. (2016) Enzymatic synthesis of ligand-bearing DNAs for metal-mediated base pairing utilising a template-independent polymerase. *Chem. Commun.*, 52, 3762–3765.
72. Takezawa, Y., Kobayashi, T. and Shionoya, M. (2016) The effects of magnesium ions on the enzymatic synthesis of ligand-bearing artificial DNA by template-independent polymerase. *Int. J. Mol. Sci.*, 17, 906–1015.
73. Röthlisberger, P., Levi-Acobas, F., Sarac, I., Baron, B., England, P., Marlèire, P., Herdewijn, P. and Hollenstein, M. (2017) Facile immobilization of DNA using an enzymatic his-tag mimic. *Chem. Commun.*, 53, 13031–13034.
74. Chang, L.M. and Bollum, F.J. (1990) Multiple roles of divalent cation in the terminal deoxynucleotidyltransferase reaction. *J. Biol. Chem.*, 265, 17436–17440.
75. Delarue, M., Boulé, J.B., Lescar, J., Expert-Bezançon, N., Jourdan, N., Sukumar, N., Rougeon, F. and Papanicolaou, C. (2002) Crystal structures of a template-independent DNA polymerase: murine terminal deoxynucleotidyltransferase. *EMBO J.*, 21, 427–439.
76. Yang, D., Hartman, M.R., Derrien, T.L., Hamada, S., An, D., Yancey, K.G., Cheng, R., Ma, M. and Luo, D. (2014) DNA materials: bridging nanotechnology and biotechnology. *Acc. Chem. Res.*, 47, 1902–1911.