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

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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 *N*⁴-acylated 2′-deoxycytidine nucleotides (dC^Acyl). We find that a variety of family A and B DNA polymerases efficiently use dC^AcylTPs as substrates. 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. In contrast, a proofreading phi29 DNA polymerase successfully utilizes dC^AcylTPs but is prone to form C^Acyl•A base pair under the same conditions. Moreover, we show that terminal deoxynucleotidyl transferase is able to incorporate as many as several hundred *N*⁴-acylated-deoxycytidine nucleotides. These data reveal novel *N*⁴-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 fluorescent 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-ethynyl-, 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-polymers ([30]), 40 nt long oligonucleotides (ONs) ([31]) or G-quadruplex derived DNAzymes ([32]). In addition, it has been demonstrated that even nucleotide chimeras bearing a 40 kDa horseradish peroxidase at C5 position, and being 100-fold larger than their natural counterparts, are readily incorporated by DNA polymerases ([33]). These examples prove the extraordinary ability of DNA polymerases to uti-

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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-ethynyl-dUTP 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 analogues has been already 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 O^4 of purines/pyrimidines or N^4 of pyrimidines, have been exploited even less. For example, O^4-alkylated thymidine and guanosine are considered as the inducers of mutagenic DNA lesions that are formed due to exposure to alkylating agents (42). N^4-acetylcytidine is a naturally occurring minor nucleoside found in several tRNAs and rRNAs (43). Hydroxylamine and *O*-methylhydroxylamine are mutagenic agents that convert cytidine to N^4-hydroxycytidine and N^4-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 N^4-acyl, N^4-alkoxycarbonyl and N^4-carbamoyl residues have been previously described (45). Moreover, it is known that N^4-acetyl-CTP is efficiently used as a substrate in a T7 RNA polymerase-catalysed *in vitro* transcription (46), whilst N^4-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 N^4-modified-cytidine.

Here, we report on the synthesis and properties of various N^4-acylated 2′-deoxycytidine 5′-triphosphates. To investigate the influence of a bulkiness of the N^4-substituents on DNA polymerases, we present a series of aliphatic and aromatic N^4-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 (N^4-acetylbenzoyl- and N^4-benzoylbenzoyl-dCTP). We find that different types of DNA polymerases are able to incorporate N^4-acyl-deoxycytidine nucleotides, and pair the N^4-acylated cytosine base with adenine to a great extent. We further show that phi29 DNA polymerase successfully uses N^4-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 N^4-acyl-deoxycytidine 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-^{33}P]-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 MgCl$_2$, 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 MgCl$_2$ (pH 8.2) (KF (exo–)), 20 mM Tris–HCl, 10 mM KCl, 10 mM (NH$_4$)$_2$SO$_4$, 2 mM MgSO$_4$, 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 dC^{Acyl}TP (10 μM), 5′-^{33}P-labelled primer **P1/P1^{res}** (**P1^{res}** for phi29 DNA polymerase) and template (**Temp^A**, **Temp^G**, **Temp^C** or **Temp^T**) hybdrid (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 pyrophosphatase (0.01 U). Reactions were quenched by the addition of 2 × loading solution (95% (v/v) formamide, 20 mM ethylendiaminetetraacetic 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). 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 (**Temp^{47}**) 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′-^{33}P-labelled primer **P1/P1^{res}** (**P1^{res}** for phi29 DNA polymerase) and template (**Temp^{47}**) hybrid (5 nM) and an appropriate buffer. dATP, dGTP, dTTP and dCTP or dC^{Acyl}TP nucleotide combination was used to monitor dCTP/dC^{Acyl}TP interchange, whilst to examine dTTP/dC^{Acyl}TP replacement combination of dATP, dGTP, dCTP and dTTP/dC^{Acyl}TP was used.

TdT-catalysed 3′-elongation

The primer **P1** was 5′-^{33}P-labelled as described above. The reaction mixtures were prepared in a total volume of 10 μl and consisted of TdT (0.5 U), 5′-^{33}P-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 MgCl$_2$/MnCl$_2$ (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′-^{33}P-labelled as described above. The reaction mixtures (150 μl) consisted of TdT (7.5 U), 5′-^{33}P-labelled **P1** (5 nM), dCTP, dC^{AcBz}TP or dC^{BzBz}TP (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 N^4-modified 2′-deoxycytidine triphosphates

Pyrimidines modified at N^4 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 N^4-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 N^4-acyl-2′-deoxycytidine nucleotides by exonuclease-deficient polymerases

To determine whether N^4-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 dC^{Acyl}TP. KF (exo–) of *Escherichia coli* (family A), thermostable *Thermus aquaticus Taq* DNA polymerase (family A), *Thermococcus kodakaraenis* 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 dC^{Acyl} 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 **Temp^G** revealed that all exo– DNA polymerases tested were able to incorporate three to four N^4-acyl-modified deoxycytidine nucleotides in a row (Figure 2). The smaller modification-bearing nucleotides (i.e. dC^{Ac}, dC^{Hex}, dC^{Bz}, dC^{Nic}) were incorporated with comparable efficiency irrespectively of a polymerase used. In contrast, the data presented in Figure 2 indicated that dC^{mAcBz}, dC^{pAcBz}, dC^{mBzBz} and dC^{pBzBz} 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 N^4-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 N^4-position of cytosine does not interfere with the formation of correct hydrogen bonds with the guanine base.

Surprisingly, PEX experiments using **Temp^A** revealed a strong complementarity between adenine base and N^4-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 N^4-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
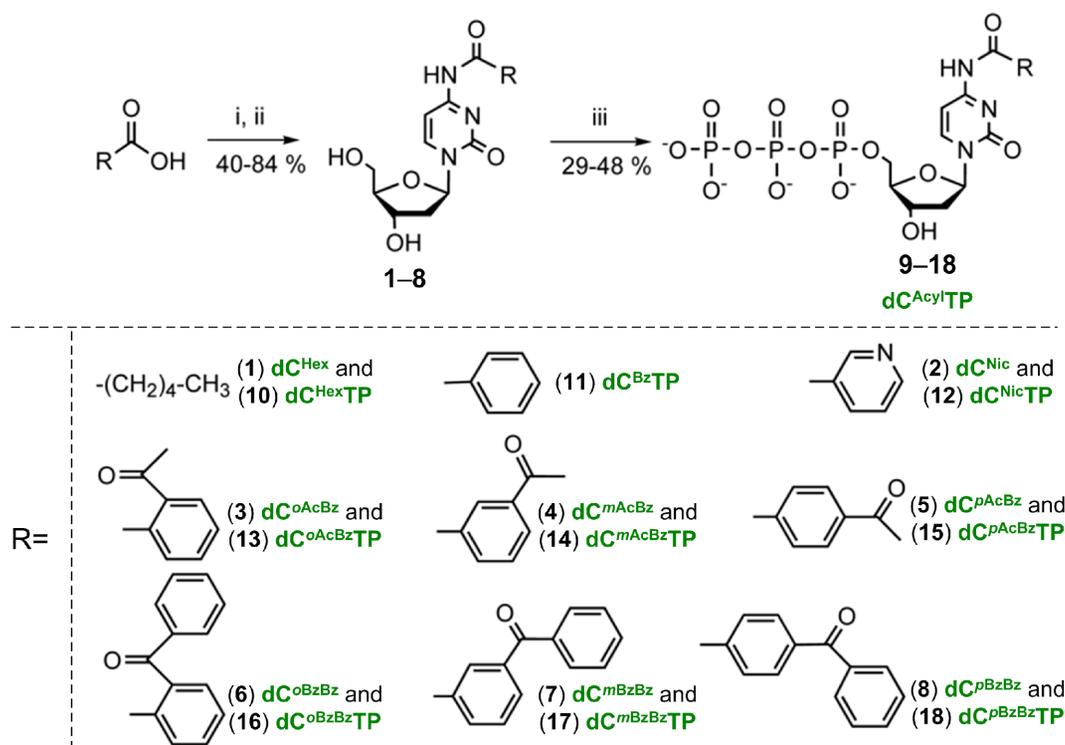
Figure 1. Synthesis of N^4-modified 2′-deoxycytidine-5′-triphosphates. *Reagents and conditions*: (i) NHS, DCC, ethyl acetate, rt, 24 h; (ii) 2′-deoxycytidine, DMF, 25–30°C, 24–48 h; (iii) POCl₃, TBA, trimethyl phosphate, 0–20°C, 1–2 h; then TBA, (NHBu₃)₂H₂P₂O₇/CH₃CN, rt, 10–15 min.

that *Taq*, KF (exo–) and Bsm showed the preference towards acetyl, hexanoyl and nicotinoyl groups resulting in the desired products with at least four modifications in a row within one DNA molecule (Figure 3 and Supplementary Figure S1). The extended modified ON products obtained by replacing dCTP and TTP by dC^{Bz}TP as a representative were confirmed by the electrospray ionization-mass spectrometry (ESI-MS) analysis showing masses corresponding the dC^{Bz}-containing ONs (Supplementary Figures S2 and 3).

Incorporation of deoxycytidine analogues using templates bearing pyrimidine bases (**Temp^T** and **Temp^C**) 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 **Temp^{47}**. 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 dC^{Acyl}TP generated rather clean bands of the 47-mer ON products (Supplemen-

tary 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 groups-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 (dC^{Acyl}TP, 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 dC^{Ac}TP, dC^{Hex}TP and dC^{Nic}TP. These results revealed rather dual behaviour of dC^{Acyl}TPs (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′→5′
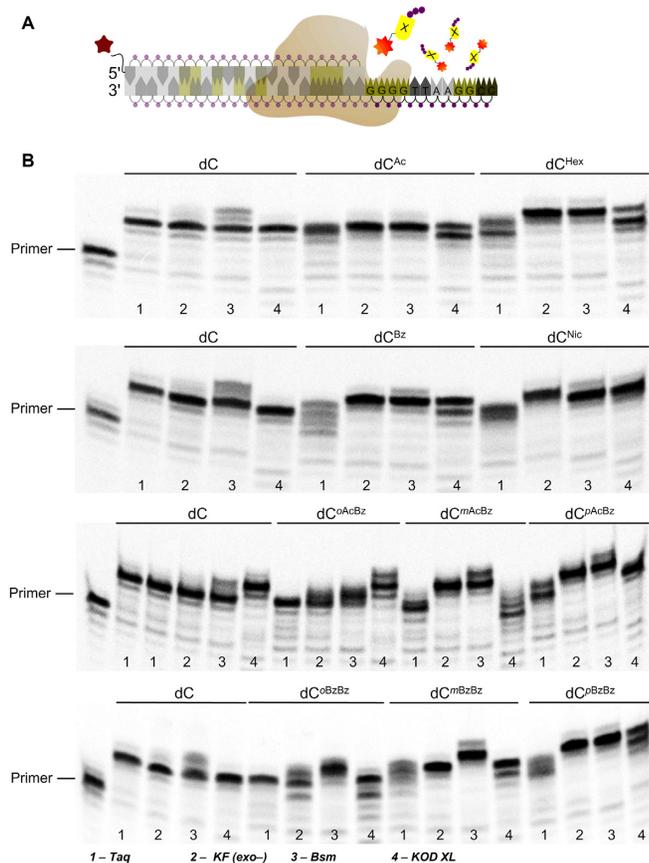
Figure 2. An incorporation of modified dC^{Acyl} nucleotides by exonuclease-deficient DNA polymerases. (**A**) A scheme of the PEX experiment. (**B**) Autoradiograms of denaturing polyacrylamide gels showing PEX using **Temp^G** and dC^{Acyl}TP instead of dCTP. The used modified dC^{Acyl}TPs and DNA polymerases are indicated above and below the lanes, respectively.

exonuclease (proofreading) activity, and therefore performs a very accurate DNA synthesis (50).

First, four different DNA templates (**Temp^G, Temp^A, Temp^C, Temp^T**) were used to investigate the substrate specificity as well as the proofreading activity of phi29 DNA polymerase. As seen in Figure 5, PEX using **Temp^G** 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^{Acyl}TP, dATP and dGTP. Here, three different DNA templates (**Temp^A**, **Temp^{47}**, **Temp^{58}**) 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^{47}** and **Temp^{58}**, 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^A** 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^{Acyl}TP and dGTP or dC^{Acyl}TP only.

TdT-mediated 3′-end tailing using dC^{Acyl}TPs

To expand the utilization of novel dC^{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 *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^{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^{Hex} and dC^{oBzBz}. 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^{Hex}TP or dC^{oBzBz}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,
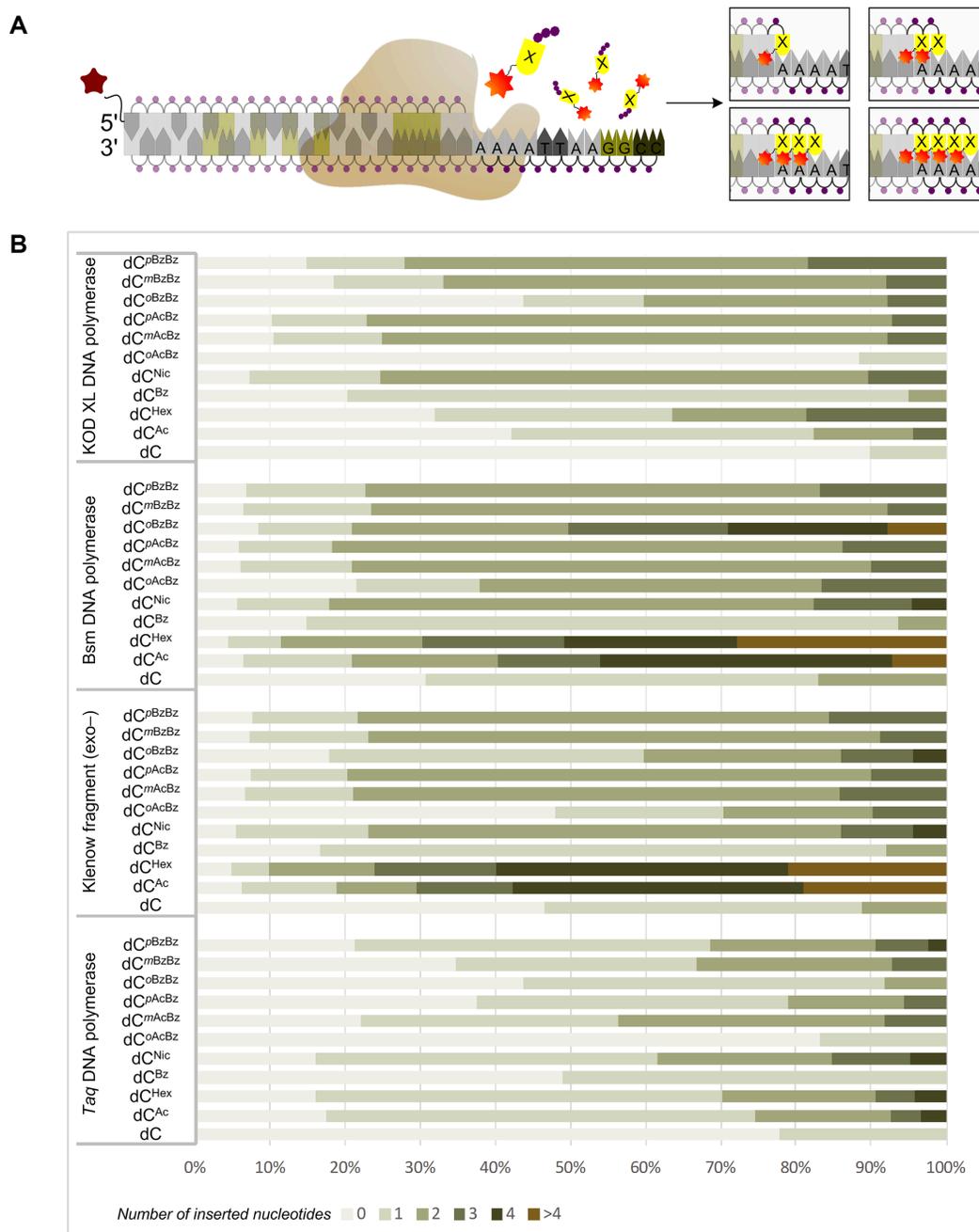
Figure 3. N^4-acyl-cytosine and adenine base-pairing during PEX performed by family A and B DNA polymerases. (**A**) A schematic representation of $C^{Acyl} \bullet A$ base-pairing experiment. (**B**) Multiple incorporation efficiencies and base-pairing of dC or dC^{Acyl} with dA by family A (*Taq*, KF (exo–), Bsm) and B (KOD XL) DNA polymerases.

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^{oAcBz}TP was preferred over dC^{oBzBz}TP (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^{2+}-containing buffer was so effective that, in the case of dC^{mBzBz}TP and dC^{pBzBz}TP, 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).
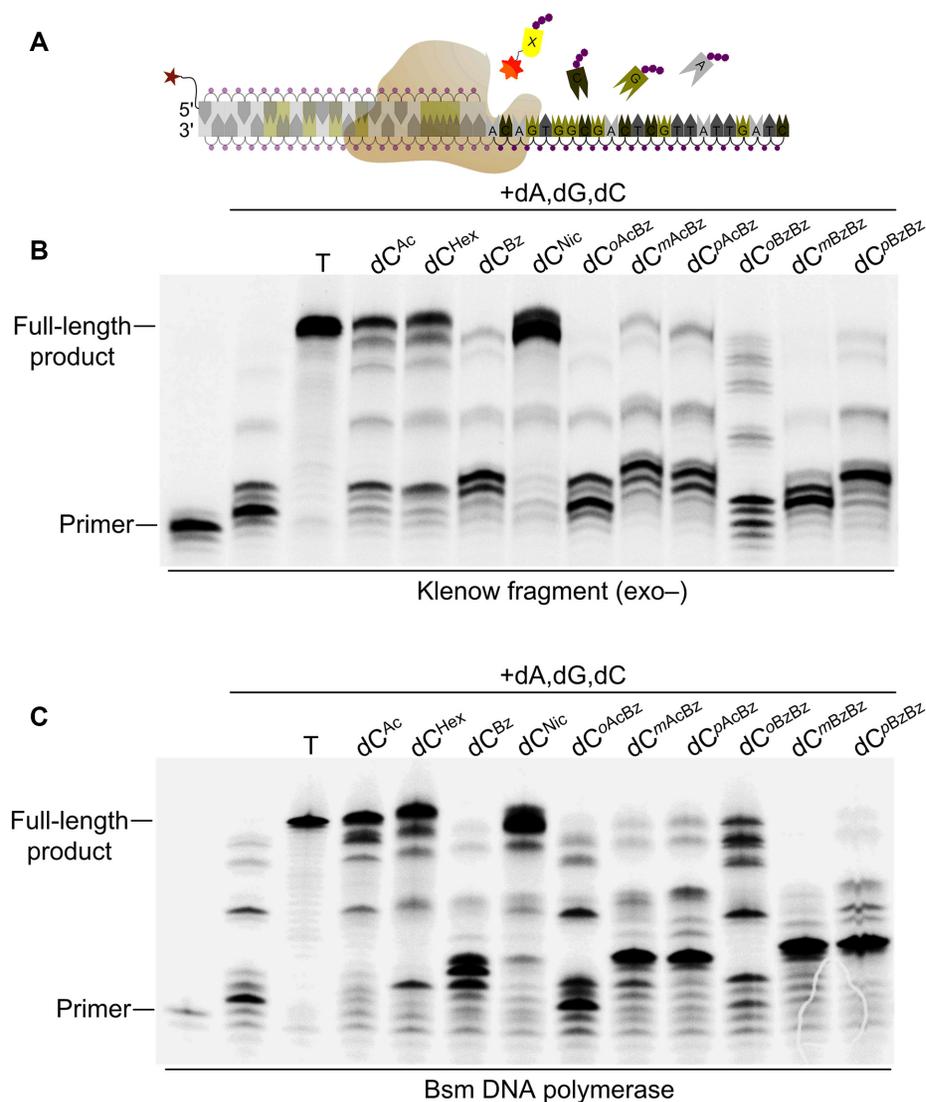
Figure 4. Substitution of TTP with dC^{Acyl}TP in PEX reactions. (**A**) A schematic representation of the TTP→dC^{Acyl}TP replacement experiment. (**B** and **C**) Autoradiograms of denaturing polyacrylamide gels showing PEX using **Temp^{47}**. DNA polymerases and nucleotide combinations are indicated at the bottom and the top of each figure, respectively.

To investigate the dependency of the TdT-catalysed tailing on the duration of the reaction in more detail, we examined 3′-elongation using dC^{mAcBz}TP as the best representative (Figure 8). Based on the data analysis, TdT demonstrated considerably high reaction rate by adding one modified dC^{mAcBz}TP 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 *m*AcBz-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 N^4-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 N^4-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 dC^{Acyl}TPs are suitable for
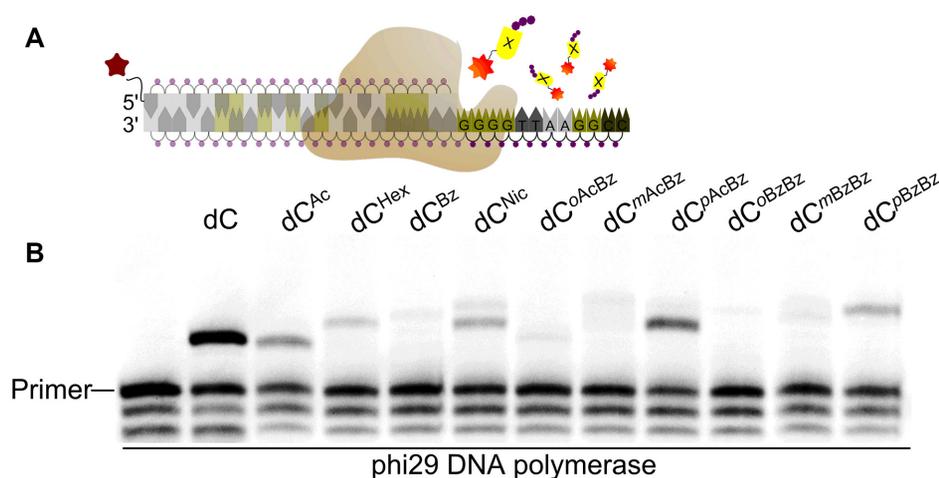
Figure 5. An incorporation of modified dC^{Acyl} nucleotides by a proofreading phi29 DNA polymerase. (**A**) A scheme of the PEX experiment using phi29 DNA polymerase and **Temp^G**. (**B**) An autoradiogram of a denaturing polyacrylamide gel showing PEX using phi29 DNA polymerase and modified dC^{Acyl}TPs. The used modified dC^{Acyl}TPs are indicated above the lanes.
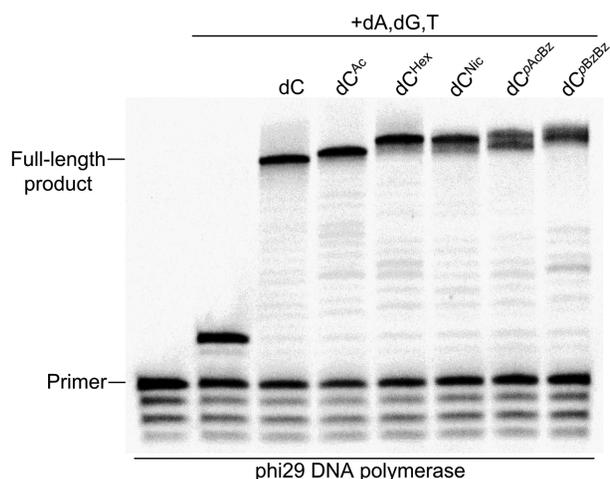

Figure 6. An autoradiogram of a denaturing polyacrylamide gel showing PEX using **Temp^{47}**, phi29 DNA polymerase and dC^{Acyl}TP instead of dCTP. The used nucleotide combinations are indicated above the lanes.

multiple insertions in the adjacent as well as discrete positions against guanine base, since it seems that N^4-acyl groups do not impair hydrogen bonding.

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^{Acyl}TPs, whilst phi29 DNA polymerase with 3′-exonuclease activity acts differently. Moreover, exo– DNA polymerases succeed to incorporate dC^{Acyl} against adenine at a quite high level, whilst

phi29 is prone to directly pair C^{Acyl} with A. Our results suggest that mismatched C^{Acyl}•A, C^{Acyl}•T or C^{Acyl}•C pairs fail to maintain an appropriate geometry in the active site of phi29, therefore successful incorporation is restricted. The C^{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^{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, supplementing with all the necessary nucleotides would easily solve such an insignificant misincorporation process and offer an opportunity for the application of N^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^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^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
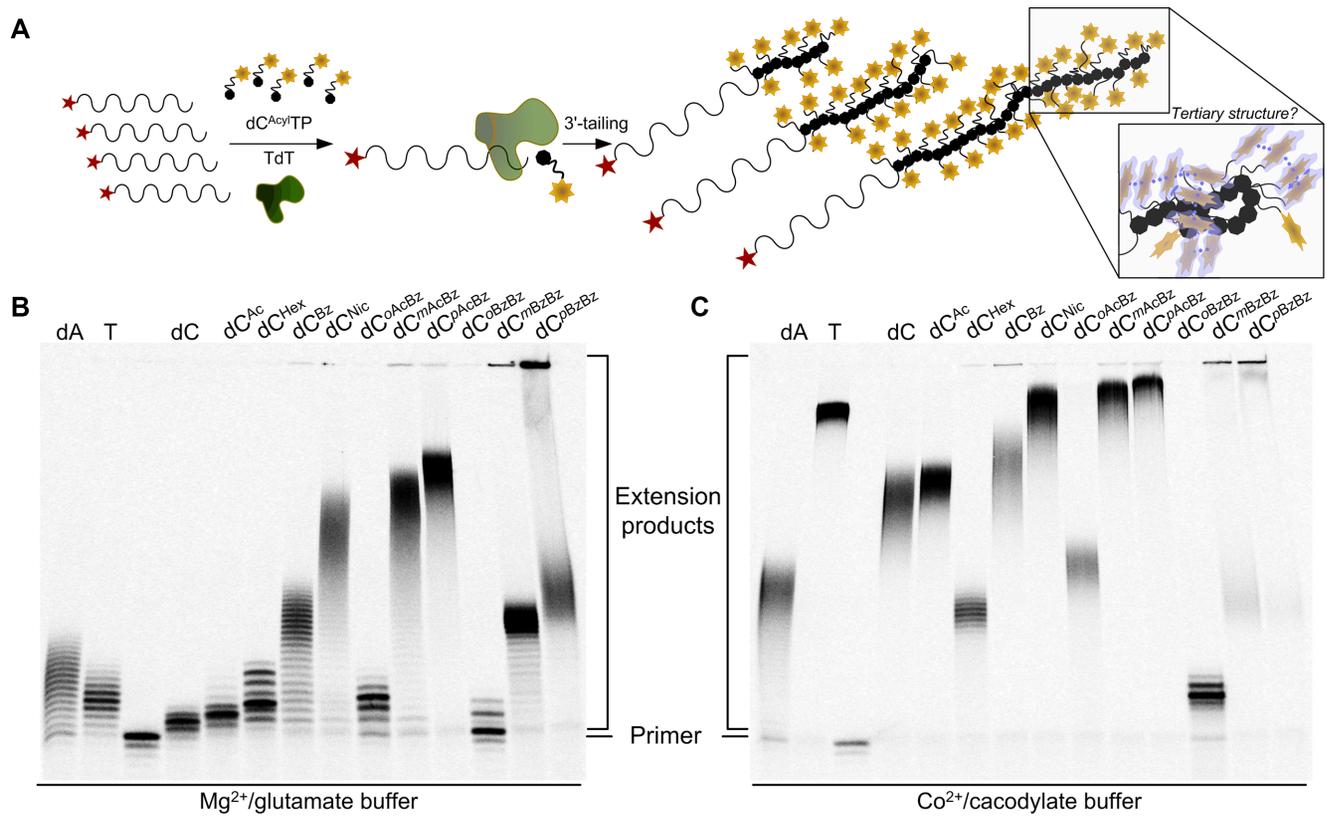
Figure 7. TdT-catalysed 3′-end tailing using $dC^{Acyl}TPs$ as substrates. (**A**) Scheme of tail-labelling of the ON probe with N^4-acylated deoxycytidine markers and TdT. (**B** and **C**) Autoradiograms of denaturing polyacrylamide gels showing primer 3′-elongation. The used nucleotides and buffers are indicated above and below the lanes, respectively.
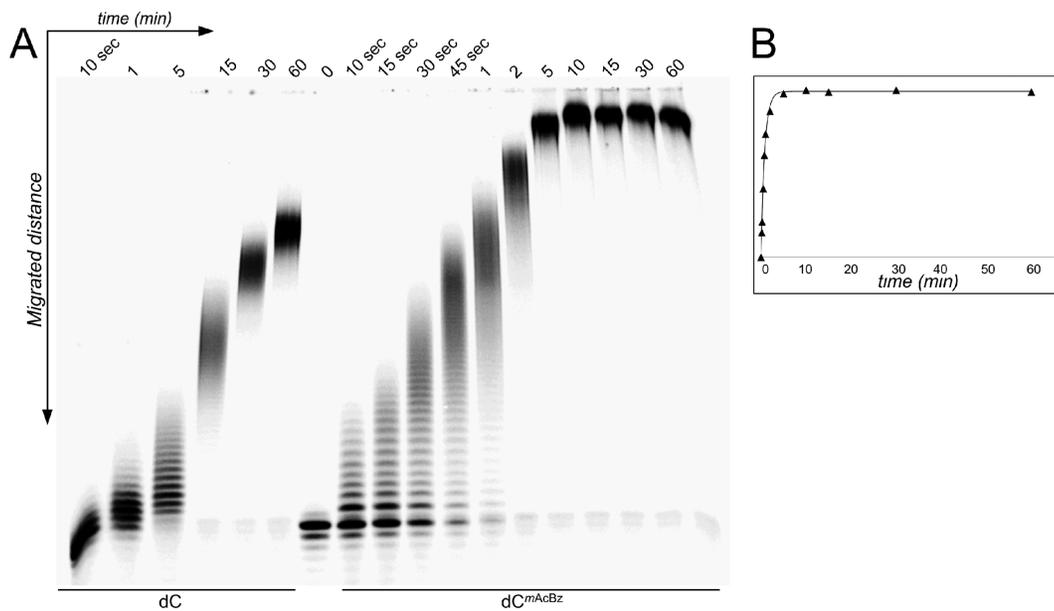

Figure 8. Dependence of the efficiency of TdT-catalysed 3′-tailing on the reaction duration, using dCTP or $dC^{mAcBz}TP$ as substrates. (**A**) An autoradiogram of a denaturing polyacrylamide gel showing primer 3′-elongation using TdT. The elongation time is indicated above each lane. (**B**) Fit of the elongation process with $dC^{mAcBz}TP$ using the data extracted from the gel image (**A**).

(61). During the studies on N^4-modified 2′-deoxycytidine nucleotides, such as N^4-acyl-dC or N^4-alkoxycarbonyl-dC, extraordinary strong base-pairing with adenine has been also detected (62,63). For example, N^4-alkoxycarbonyl-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-^{4}NH•••A-^{1}N and C-O^{ester}•••A-^{6}NH) (63). Nevertheless, it is more complicated than an unusual mismatched base-pairing. For instance, it has been determined that N^4-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, N^4-carbamoyl-dC•G base-pairing occurs only in a form of ONs, suggesting that geometry of N^4-substituents strongly depends on the salvation and intramolecular hydrogen bonds (64). Although there are several reports on the decreased stability of DNA duplexes upon increasing alkyl chain length in the N^4-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 N^4 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 N^4-substituents, such as C_6-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 N^4-acylated deoxycytidine-containing ONs is based on a solid-phase synthesis using phosphoramidite method. In contrast, our data rely only on the enzymatic synthesis of N^4-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, N^4-modified deoxycytidine nucleotides not only may be used instead of natural ones, but may also offer limitless possibilities in modifying AT-rich DNA regions. In addition, N^4-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 a robust tail-labelling without the necessity for a precise quantification of the labels. TdT uses different base-modified nucleotides for the enzymatic syn-thesis 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^{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 N^4 position of deoxycytidine. As such, with several exceptions, by increasing the size of a functional group attached to the position N^4 of cytidine (acetyl<hexanoyl<benzoyl/nicotinoyl<acetylbenzoyl), the elongation efficiency is being increased regardless of a buffer used. Considering the length of the aliphatic alkyl group of a modified nucleotide (i.e. acetyl/hexanoyl), the Co^{2+}-containing buffer demonstrates slightly different results than the Mg^{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^{2+}, cacodylate), dC^{Hex} is poorly incorporated.

In the case of dC^{AcBz} and dC^{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^{oAcBz} and dC^{oBzBz}.

Since our data show an extremely effective 3′-tailing 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^{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^{Acyl}TPs. Such a scenario, however, cannot be applied in the case of dCTP. Moreover, as seen in Figure 8, the longest dC^{mAcBz}-containing DNA fragments are of very similar molecular weight suggesting that the dC^{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 N^4-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 polymerases and dC^{Acyl}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.

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