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Polymerase incorporation of pyrene-nucleoside triphosphates

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ABSTRACT

Pyrene-deoxynucleoside triphosphates (dPTPs), varying by the positioning of the aromatic system, were synthesized. Their ability to function as substrates for the Klenow fragment of *Escherichia* coli DNA polymerase I and the TdT polymerase was assessed. The dPTPs are all equally well tolerated by the Klenow fragment, and lead to elongation of up to 5 extra nucleotides of a ssDNA primer in a TdT-mediated reaction. The tailing efficiency of the dPTPs compares favorably to other less drastically modified dNTPs.

Nucleoside triphosphates (dNTPs) have emerged as a versatile and wide-ranging platform for the generation of chemically modified oligonucleotides.[1–3] Indeed, a myriad of dNTPs has been generated to supplement the rather limited chemical arsenal of the nucleobases, especially in the context of in vitro selections for the development of DNAzymes.[4–10] Moreover, modified dNTPs have been designed for a wide-ranging palette of applications including electrochemical and fluorescent labeling of nucleic acids,[11–15,1,16] bar-coding of DNA,[17] and bioanalytical purposes.[18,2] In particular, modified dNTPs act as a pivotal tool for the discovery of unnatural base pair systems[19,20] and by the same token, for investigating the contribution of electrostatic forces, aromatic stacking, and H-bonding to the strength of the Watson–Crick base pairs.[21,22] In this context, 1-pyrene deoxynucleoside triphosphate d[1]PTP **1** (Fig. 1) was originally conceived to evaluate the importance of these factors on the fidelity of replication of DNA polymerases.[21] The modified triphosphate d[1]PTP revealed to be a potent substrate for the Klenow fragment of *Escherichia coli* DNA polymerase I, thus underscoring the importance of steric complementarity.

Terminal deoxynucleotidyl transferase (TdT) is a Co^{2+}-dependent DNA polymerase that catalyzes the stepwise homopolymerization of nucleoside triphosphates on single-stranded oligonucleotides.[23] Surprisingly, the potential of this polymerase to incorporate modified dNTPs is vastly unexplored and only a few scarce examples have been reported so far.[24,25]

Here, we report on the synthesis of two novel pyrene-deoxynucleoside triphosphates d[2]PTP **2** and d[4]PTP **3** (Fig. 1), which differ from the original d[1]PTP **1** by the position of the anchoring point connecting the aromatic residue to the sugar unit, thus causing a differential tilt of the pyrene, which in turn could possibly lead to altered stacking abilities. We then explored the polymerase uptake of these modified dNTPs with a special emphasis on the TdT enzyme.

The synthesis of the modified triphosphates **1**–**3** proceeded smoothly and is highlighted in Scheme 1. Briefly, the DMT-protected nucleoside precursors **4**–**6**[26] were 3′-*O*-acetylated and the masking trityl groups were removed by treatment with dichloroacetic acid (see Supplementary data). The resulting intermediates **10**–**12** were then converted to the corresponding triphosphates by application of the one pot-four steps procedure developed by Ludwig and Eckstein.[27] RP-HPLC purification led to isolation of the pure dNTPs in moderate yields (11–36%).

With the modified triphosphates in hand, we first investigated their uptake by the Klenow fragment of *Escherichia coli* DNA polymerase I (Fig. 2 and Fig. S1, Supplementary data). Indeed, using a 28-mer template containing a tetrahydrofuran abasic analogue (ϕ),[21] standing start (Fig. S1, Supplementary data) and running start (Fig. 2) primer extension reactions were carried out.

All the modified dNTPs are good substrates for the Klenow fragment, since both in the running start and the standing start experiments, efficient extensions could be observed, with the dPTPs placed opposite to the abasic sites. However, the position of the aromatic system on the deoxyribose has little impact on the uptake efficacy. Indeed, none of the triphosphates showed any differential propensity at incorporation and all the running start experiments stalled after incorporation of the dPMPs.

The dPTPs were then tested for their ability to act as substrates in TdT-catalyzed extension reactions.[28] In order to do so, a 15-mer

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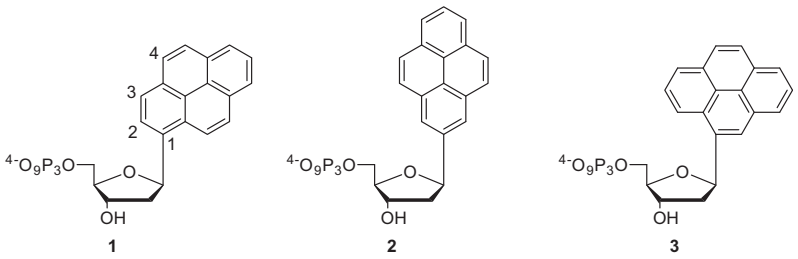
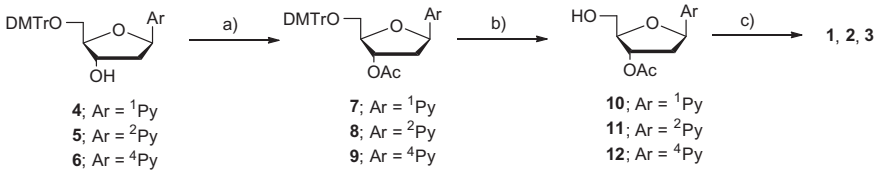


Figure 1. Chemical structures of the modified triphosphates d[1]PTP (**1**), d[2]PTP (**2**), and d[4]PTP (**3**). The pyrene numbering system is shown on dPTP **1**.



Scheme 1. Reagents and conditions: (a) Ac_2O, pyridine, 25 °C, 12 h, **7**: 99%, **8**: quant., **9**: 98%; (b) DCAA (1%), DCM, 25 °C, 40 min, **10**: 98%, **11**: 88%, **12**: 77%; (c) (i) 2-chloro-1,3,2-benzodioxaphosphorin-4-one, pyridine, dioxane, rt, 45 min, (ii) $(nBu_3NH)_2H_2P_2O_7$, DMF, nBu_3N, rt, 40 min, (iii) I_2, pyridine, H_2O, rt, 30 min, (iv) NH_3(aq), rt, 1 h, **1**: 11%, **2**: 32%, **3**: 36% (4 steps).

primer **P2** was 5′-32P-radiolabeled and incubated at 37 °C for 60 min in the presence of the various dPTPs and TdT (4 U/reaction). Reactions with the natural dTTP were also carried out in parallel for the sake of comparison, since this led to a nicely defined ladder (with the incorporation of up to 50 nucleotides). The resulting reaction products were then analyzed by gel electrophoresis (PAGE 20%), as shown in Figure 3.

For all the dPTPs, multiple (i.e. 3–5 nucleosides) incorporations were observed (lanes 2–4 and 6–8). Moreover, d[4]PTP **3** revealed to be the most proficient substrate for the TdT extension reaction. Indeed, at a concentration of 100 μM, mainly 4 additional nucleotides were incorporated (lane 8) with minor bands corresponding to 5 and 3 added residues. On the other hand, the reaction at a higher dPTP concentration (200 μM) led to the incorporation of 5 extra nucleotides (lane 2) with only a minor (~15%, see Table S1, Supplementary data) band corresponding to the primer extended with 4 d[4]Ps units. In addition, at a lower triphosphate concentration, d[1]PTP **1** mainly led to the addition of 4 residues (lane 6), while an uneven distribution pattern is observed for d[2]PTP **2** under the same conditions (lane 7 and Table S1). Moreover, higher triphosphate concentrations coerce a more even incorporation distribution for both d[1]P (lane 4) d[2]P (lane 3 and Table S1), with the main products corresponding to the addition of 4 and 5 extra residues. Finally, the terminal transferase necessitates at least three deoxynucleotide residues on the primer strand for an efficient catalysis of the tailing reaction.[23] Consequently, when the polymerase reaches the modified extended section, the reaction will stall, which might explain why no more than 5 residues are appended.

Therefore, the pyrene-deoxynucleoside triphosphates are much better substrates for the TdT polymerase than for the Klenow fragment. In addition, their tailing efficiencies compare favorably to that of 2′,4′-bridged nucleoside triphosphates, where only single

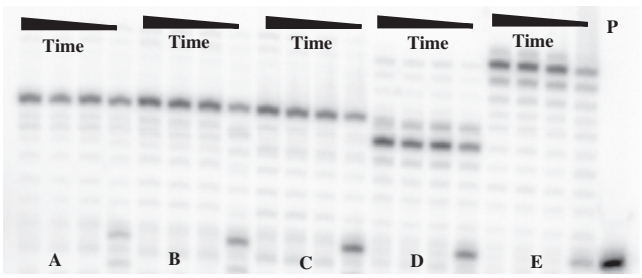


Figure 2. Gel image (PAGE 20%) of the running start primer-extension reactions: (A) d[4]PTP **3**, dATP, dCTP, dGTP, dTTP; (B) d[2]PTP **2**, dATP, dCTP, dGTP, dTTP; (C) d[1]PTP **1**, dATP, dCTP, dGTP, dTTP; (D) dATP, dCTP, dGTP, dTTP; (E) Control full length extension product with primer **P2**, template **T2** and only the natural dNTPs; **P**: [32]P-labeled primer **P2**; Time points (shown from right to left) were: 1, 30, 60, 120 min.

Figure 3. Gel image (PAGE 20%) of the TdT-mediated polymerization reactions. Lane 1: dTTP (200 μM); lane 2: d[4]PTP (200 μM); lane 3: d[2]PTP (200 μM); lane 4: d[1]PTP (200 μM); lane 5: [32]P-labeled primer; lane 6: d[1]PTP (100 μM); lane 7: d[2]PTP (100 μM); lane 8: d[4]PTP (100 μM); lane 9: dTTP (100 μM).

insertions were observed.[24] Surprisingly, the dPTPs are only slightly inferior TdT-substrates than some base modified dNTPs, where only in the case of some $dG^{X}TPs$, long tails of extra nucleotides were observed.[25] Finally, all these dPTPs had better tailing efficiencies than the α-anomer of $d^{1}PTP$.[28]

In summary, application of the Ludwig-Eckstein method led to pyrene-deoxynucleoside triphosphates. These dPTPs were then engaged in primer-extension reactions with the Klenow fragment. Despite a different tilt of the aromatic moiety, all the dPTPs behaved similarly well in both running start and standing start experiments. In addition, all the dPTPs proved to be good substrates for the TdT polymerase where the appendage of 3–5 extra nucleotides was observed. In this context, $d^{4}PTP$ arose as the better substrate since the extension reaction led to a major and clear product corresponding to the primer prolonged by 5 additional residues. The modified dPTPs compared favorably to the substrate ability of some bridged nucleoside triphosphates and even some base-altered dNTPs, despite being hydrogen-bond devoid entities. These results underscore the fact that the TdT polymerase is rather insensitive to the chemical nature of the triphosphate, albeit the accessibility of the 3′-OH seems to be a more crucial factor.[24] Finally, the use of TdT for the tail labeling of oligonucleotides with such fluorescent dPTPs bodes well for a facilitated access to the development of fluorescent chemosensors[29,30] or as molecular caps for the 3′-termini of oligonucleotides.[31]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.04.101.

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