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TERMINAL PHOSPHATE LABELED NUCLEOTIDES: SYNTHESIS, APPLICATIONS, AND LINKER EFFECT ON INCORPORATION BY DNA POLYMERASES

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▫ *A number of terminal phosphate-labeled nucleotides with three or more phosphates and with varied length linkers attached between the terminal phosphate and the dye have been synthesized. These nucleotides have been tested as substrates for different DNA and RNA polymerases. We have also explored their utility in DNA sequencing, SNP analysis, nucleic acid amplification, quantitative PCR, and other biochemical assays.*

Keywords Terminal Phosphate Labeled Nucleotides, Linkers, Dyes, DNA Polymerase Extension, Homogenous Assays

INTRODUCTION

Nucleotides are most commonly labeled with fluorescent dyes at the 5-position of pyrimidines or 7-position of 7-deazapurines for various genomic applications including DNA labeling and sequencing.[1–4] When incorporated by a DNA polymerase, these nucleotides add the labeled nucleotide base in the growing chain and the incorporated base can be read by determining the fluorescence of the dye attached to the base. However, incorporation of base-labeled nucleotides results in the modification of newly synthesized DNA structure. There has been tremendous interest in recent years in detecting the newly added base without modification to the native DNA structure in such applications as single-molecule DNA sequencing and SNP analysis.

We have developed a new series of nucleoside polyphosphates containing 3 or more phosphates with the fluorescent dye attached to the terminal phosphate. These nucleotides are efficient substrates of polymerases, particularly when more

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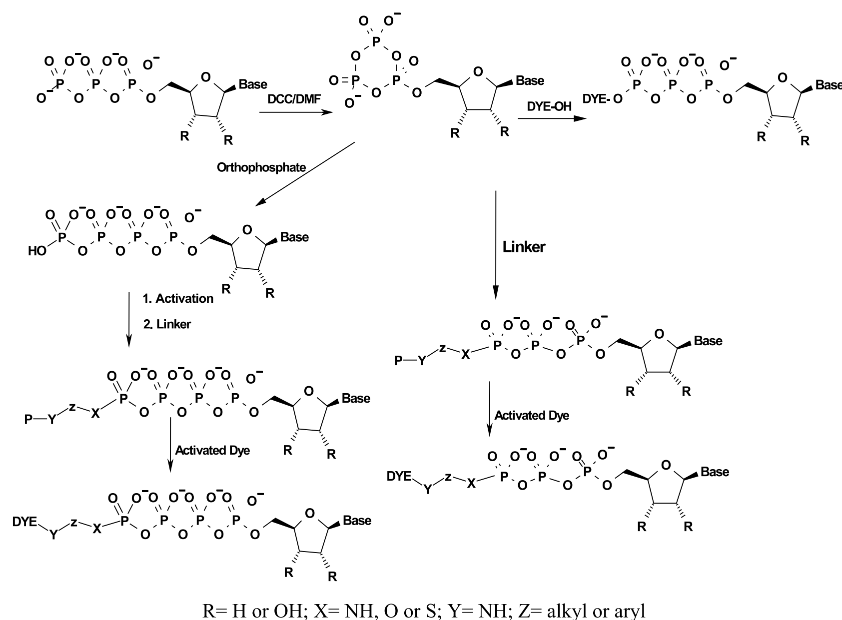
than three phosphates are present at the 5′-position of the nucleoside. We have also studied the effect of linker between the dye and terminal phosphate and their incorporation by different DNA polymerases. Their synthesis, incorporation by DNA polymerases, and utility in various genomic applications is presented here.

SYNTHESIS AND RESULTS

The synthesis of terminal phosphate labeled nucleotides was essentially carried out in the same manner as described by Arzumanov et al.[5] and Knorre et al.[6] with some modifications. Thus, for the synthesis of γ-phosphate modified triphosphates, the required triphosphate was reacted with DCC in DMF to give the cyclic trimetaphosphate, which was ring-opened with the desired nucleophilic linker to give γ-linker attached triphosphate. This linker-attached triphosphate was further reacted with Dye-NHS ester to provide γ-phosphate–labeled nucleoside-5′-triphosphate. The cyclic trimetaphosphate can also be reacted directly with dyes having a free OH group (such as resorufin, DDAO, coumarin, nitro-phenol, alkyl fluorescein, etc.) to give γ-phosphate–labeled triphosphates without linker (Scheme 1).

For the synthesis of tetra- and penta-phosphate–modified nucleotides, the cyclic trimetaphosphate was reacted with orthophosphate and pyrophosphate, respectively, to give tetra- or penta-phosphate, which was reacted with the appropriate



SCHEME 1 Synthetic scheme for the synthesis of terminal phosphate-labeled nucleotides.

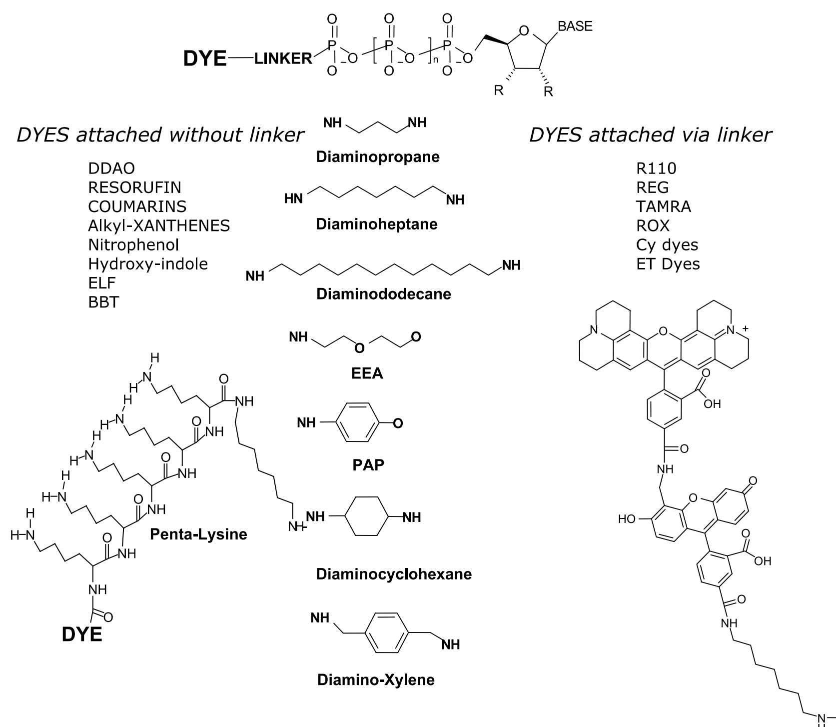


FIGURE 1 Structure of terminal phosphate labeled nucleotides with different linkers and dyes.

linker and finally with Dye-NHS ester to provide the required tetra- or penta-phosphate–modified nucleotides. The different types of linkers and dyes used in this study are shown in Figure 1.

Effect of Linker on the Incorporation Rate of Terminal Phosphate-Labeled Nucleoside Polyphosphates Containing a Linker Between the Label and the Terminal Phosphate

TAMRA-NH-linker-NH-dTTP (2′-deoxythymidine derivative) and REG-NH-linker-NH-dATP were investigated in a single base primer-extension reaction. Primer and template sequences used are shown below, the template base at the site of incorporation was varied depending on the base of the incoming nucleotide. Samples were prepared to contain 25 mM Tris, pH 8.0, 50 mM KCl, 5% glycerol, 1 mM beta-mercaptoethanol, 0.25 units shrimp alkaline phosphatase (SAP, added to digest any unlabeled triphosphate; terminal phosphate-labeled nucleotides are inert to the action of SAP), 0.5 mM $MgCl_2$, 0.125 mM $MnCl_2$, 100 nM primer/template, 1 μM nucleotide, and 150 nM ΔTts polymerase and were incubated at 30°C. Reaction mixtures were run on a gel at different times to determine the amount of incorporation. As can be seen from Figure 2, terminal phosphate-labeled nucleoside triphosphates with short linker (propyl) or alicyclic linker (cyclohexyl)

or very long linker (dodecyl) are not as well accepted by the DNA polymerases. The rest of the linkers are accepted to different degrees with heptyl ($(CH_2)_7$) being one of the best linkers.

5′ Cy5GTTCTCGGCATCACCATCCG-Primer

CAAGAGCCGTAGTGGTAGGC**T**GCTGTTGGTCTATTCCCAC-Template 1

CAAGAGCCGTAGTGGTAGGC**A**GCTGTTGGTCTATTCCCAC-Template 2

Effect of Adding Polypeptide (Charge) to the Linker Between the Terminal Phosphate and the Dye Moiety on the Incorporation Rate with Different DNA Polymerases

We have previously observed that adding charge on the linker between the dye and the nucleotide base influences the incorporation of dideoxynucleoside-5′-triphosphates by DNA polymerases.[7,8] The positive charge increases the substrate incorporation substantially, while the negative charge decreases the substrate incorporation by a number of different polymerases. To assess the effect of charge between the phosphates and the dye, we have synthesized terminal phosphate-modified nucleotides with penta-lysines attached between the optimal heptyl linker and the dye. Under the polymerase extension reaction conditions used, lysine residues are protonated. The rate of incorporation of the optimal linker attached dNTP ($NH(CH_2)_7NH$) between the terminal phosphate and the dye moiety was compared with the additional penta-lysine–attached dNTP. A number of different DNA polymerases were used in the study to ascertain the effect of lysine moieties in the linker on the incorporation of nucleotides. The reactions were carried out as outlined below and the results are presented in Table 1. The reaction mixture contained 10 nM Cy5-20-mer primer and 40-mer oligonucleotide template (shown above), 20 mM Tris-HCl, 0.01% Tween 20, 5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 1 mM

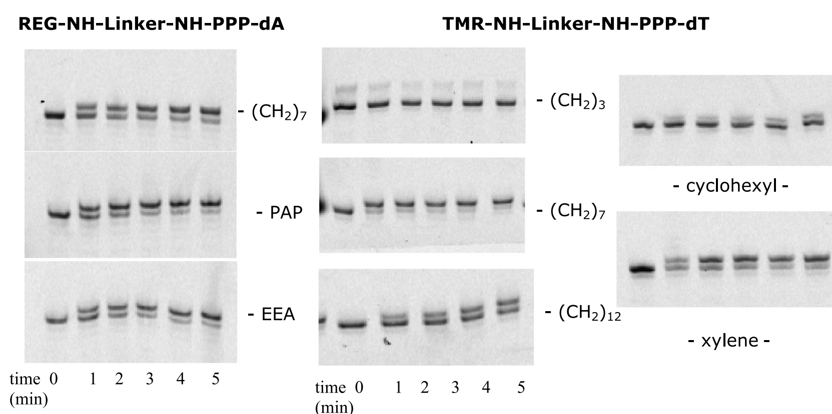


FIGURE 2 Gel pictures showing the single base extension using different length linkers between the dye and triphosphate on incorporation by DNA polymerase.

TABLE 1 Comparison of Rate of Incorporation of Heptyl- and Heptyl-Lys5-Linker Between the Dye and Triphosphate on Incorporation by Different DNA Polymerases

Enzyme (rates = nM/min)	dTTP-C7-TAMRA	dTTP-C7-Lys5-TAMRA
Thy B	1.29	17
Phi-29 D12A	2	0.66
Sequenase	0.2	3
Klenow exo-	0.2	1.6
ThermoSequenase I	0.2	9
AMV	0.2	0.2
9°N-exo- (NEN)	6	0.86
Tsp JS1	0.2	13
delta-T10	6.2	11
Pfu	4	2.6
Tsu	1.00	2
Tne	2.3	20
Ttm	1.2	4
Human pol beta	5.6	300

DTT, 0.25 units bacterial alkaline phosphatase (BAP), and 100 mM terminal phosphate-labeled nucleotide and DNA polymerase. All components with the exception of DNA polymerase were combined and incubated in the presence of 0.0015 U/μL of BAP for 5 min at 25°C to hydrolyze any unlabeled nucleotides. One microliter of the reaction mix was removed at a time = 0 and quenched in 4 μL of 95% formamide/25 mM EDTA. 1 μL of DNA polymerase was added to initiate the reaction. Two-microliter aliquots at different time points were taken (for rapid time courses at 15, 30, and 60 s) and were quenched in 8 μL 95% formamide/25 mM EDTA. Samples were denatured in a boiling water bath for 4 min, quick chilled on ice, and 2 μL was loaded on a 25% (19:1—acrylamide:bisacylamide), 8M Urea, 1 × TBE PAGE. Gels were run for approximately 1 h at 500 V. DNA products were visualized on Storm 860 and quantitated by calculating the fraction of the total 20-mer primer converted to 21-mer product.

It is clear from Table 1 below that penta-lysine–linked nucleotide is incorporated much more efficiently than the C7-linked nucleotide by the majority of polymerases.

Extended Synthesis Using All Four Terminal Phosphate-Labeled dNTPs with or without Linker Between the Dye and Phosphates

Using the same primer/template (shown above), two different types of modified nucleotides [optimized $(CH_2)_7$ linker or directly attached dye to the triphosphate] were tested for the extension reaction and formation of full-length product. Reaction mixture contained 25 mM Tris, pH 8.0, 50 mM KCl, 0.7 mM MnCl2, 0.5 mM beta-mercaptoethanol, 0.1 mg/mL BSA, 0.005 unit/μL SAP, 75 nM each primer and template, 1 μM each DDAO-dNTP (where N = A, T, G, C), or four different rhodamine dye–attached dNTPs with $(CH_2)_7$ linker, and 6 μL of 0.12 mg/mL Phi 29 exo-polymerase to start the reaction. Although the rate of

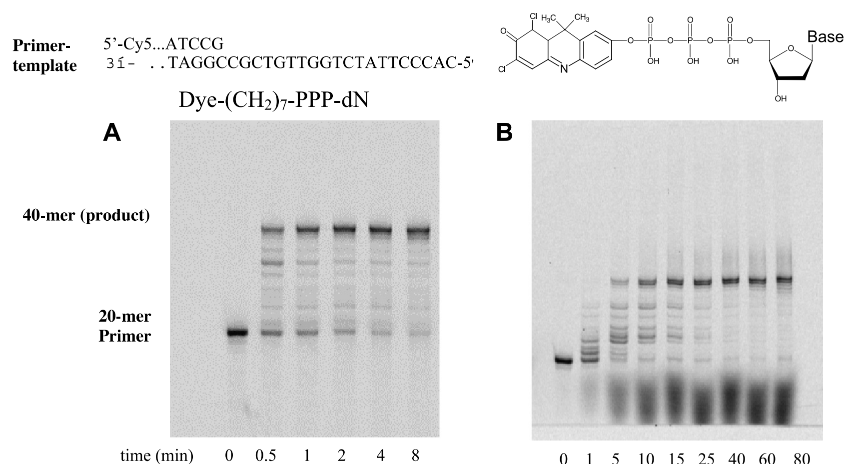


FIGURE 3 DNA synthesis using either linker based or directly attached dye to the terminal phosphate. The rate of synthesis is faster with Dye-$(CH_2)_7$-PPP-dN nucleotides.

synthesis is faster with Dye-$(CH_2)_7$-PPP-dN type of nucleotides, nucleic acid synthesis proceeds to completion with both types of modified nucleotides (Figure 3).

Phosphate-Labeled Nucleotides with More than Three Phosphates are Better Substrates for DNA Polymerases

Nucleoside-5′-triphosphates labeled on the terminal (γ) phosphate are relatively poor substrates for DNA and RNA polymerases. To assess the effect of extra phosphates on incorporation by DNA polymerases, we have synthesized and tested nucleotides with four or five phosphates. The rationale behind adding extra phosphate was to 1) move dye away from the enzyme binding site and/or 2) to compensate for the loss of charge on phosphates upon attachment of dye to the triphosphate. The synthesis of these nucleotides was carried out as shown in Scheme 1. These tetra- and penta-phosphate–labeled nucleotides are 1–2 orders of magnitude better substrates than the corresponding triphosphate as shown in Figure 4.

To assess the effect of extra phosphates, reactions containing 50 mM Tris, pH 8.0, 5% glycerol, 5 mM $MgCl_2$, 0.01% Tween 20, 0.21 units shrimp alkaline phosphatase (SAP), 100 nM primer annealed to template, and 1 μM ddT(P)n-DDAO were assembled in a quartz fluorescence ultra-microcuvet in an LS-55 Luminescence Spectrometer (Perkin Elmer), operated in time drive mode. Excitation and emission wavelengths are 620 nm and 655 nm, respectively. Slit widths were 5 nm for excitation slits, 15 nm for emission slits. The reaction was initiated by the addition of 0.35 μL (11 units) of a cloned DNA polymerase (Thy B) and 0.25 mM $MnCl_2$. As shown in Figure 4(A), both tetra- and penta-phosphates are significantly better substrates than triphosphates. Figure 4(B) shows the comparison of all four DDAO-labeled triphosphates with all four DDAO-labeled

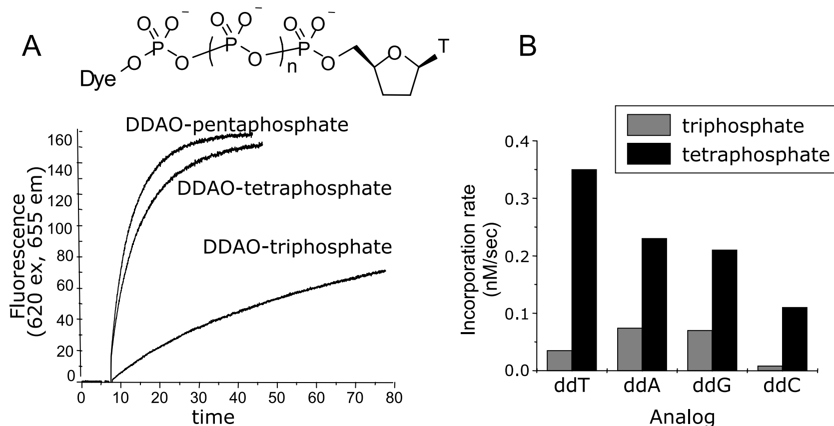


FIGURE 4 A) Effect of extra phosphate on incorporation by DNA polymerase. B) Comparison of all four dye-phosphate–labeled triphosphates with all four dye-phosphate–labeled tetraphosphates. Tetraphosphates are up to 50-fold better substrates than the corresponding triphosphates.

tetraphosphates. Again, tetraphosphates are up to 50-fold better substrates (depending on the nucleotide and polymerase used) than the corresponding triphosphates.

CONCLUSION

We have synthesized terminal phosphate-labeled nucleotides with three or more phosphates and with either dye attached directly to the terminal phosphate or with a linker between the phosphate and the dye. We have also shown that tetra- or higher phosphates are better substrates (up to 50-fold) for DNA polymerases than the corresponding triphosphates. The linker between the phosphate and dye also has significant effect on incorporation by DNA polymerase with intermediate length of alkyl, aryl, or positively charged linkers providing better substrate properties.

We have used these terminal phosphate-labeled nucleotides in a variety of applications including SNP detection, DNA sequencing, single molecule DNA sequencing, primer extension assays, analyte detection, quantitative PCR, RCA, and enzymatic assays.[9–12] We believe that these nucleotides will find use in designing biochemical and biological assays in many important fields.

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