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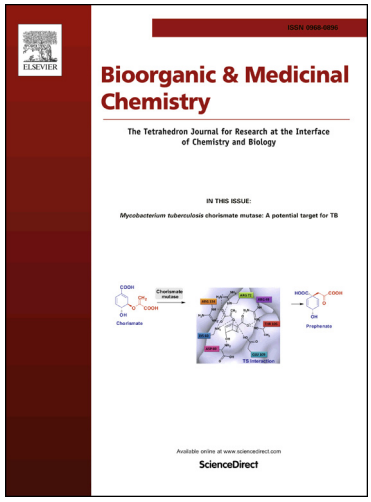

Diverse size approach to incorporate and extend highly fluorescent unnatural nucleotides into DNA

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Graphical Abstract

A series of size-diverse functional nucleotide triphosphates containing fluorescent units (**dApyrTP**, **dUpyrTP**, **dUantTP**, **dUthiTP**) and an azo quenching unit (**dUazoTP**) has been used to prepare DNA containing highly incorporated and extended fluorescent unnatural nucleotides.

Diverse Size Approach to Incorporate and Extend Highly Fluorescent Unnatural Nucleotides Into DNA

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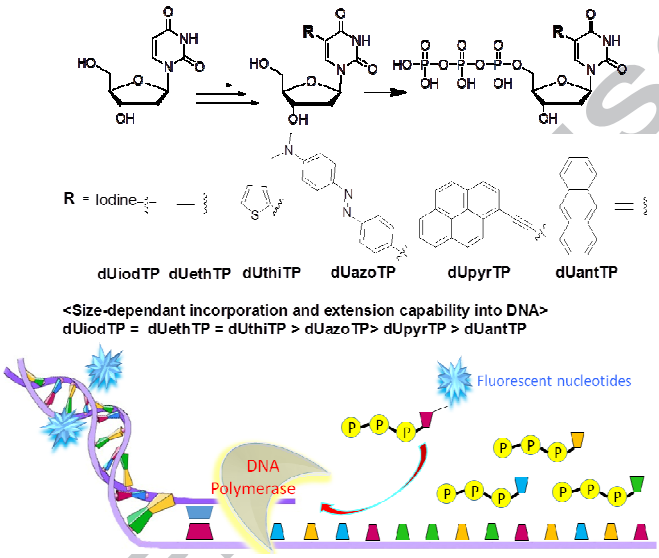
Binh Huy Le[1a], Ja Choon Koo[2a], Han Na Joo[3], Young Jun Seo[1,3,*]



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Diverse size approach to incorporate and extend highly fluorescent unnatural nucleotides into DNA

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ABSTRACT

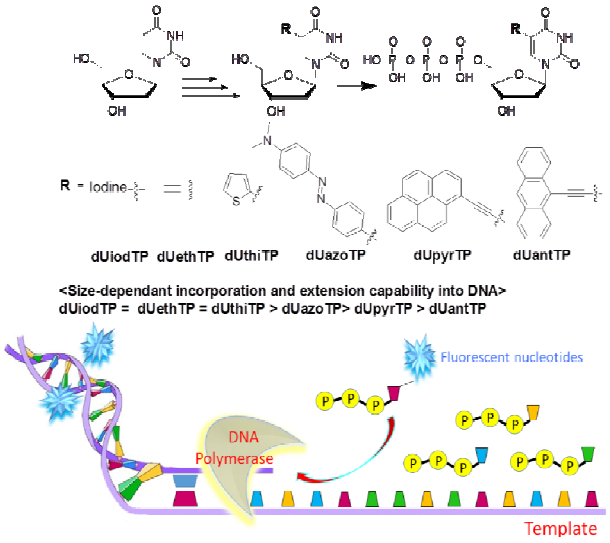
We have prepared a series of size-diverse unnatural nucleotides containing fluorescent (**dApyrTP**, **dUpyrTP**, **dUantTP**, **dUthiTP**) and quencher (**dUazoTP**) units, as well as nucleotides presenting small functional groups (**dAethTP**, **dAoctTP**, **dUethTP**, **dUiodTP**), all based on deoxyadenosine and deoxyuridine, and examined their suitability for use in enzymatic incorporation and extension into DNA. We observed a size-dependence of the incorporation and extension capability (following the order **dUiodTP** = **dUethTP** = **dUthiTP** > **dUazoTP** > **dUpyrTP** > **dUantTP**) during primer extension. This result was supported by circular dichroism (CD) spectra, which revealed a trend in the different B-form DNA structures depending on the size of the unit at the 5-position of the deoxyuridine (**dUiodTP** > **dUethTP** > **dUthiTP** > **dUpyrTP**), obtained from the PCR products. Interestingly, **dUthiTP** could be incorporated and extended into long DNA strands during primer extension and even PCR amplification, with CD spectroscopy confirming a stable secondary B-form duplex DNA structure. We observed full-length extension products even when combining **dUthiTP** with a template containing 24 continuous dA units during the primer extension. Thus, we believe that **dUthiTP** is a promising fluorescent nucleotide for a diverse range of biological applications requiring multiple incorporation and extension directly without disruption of B-form DNA structures.

1. Introduction

Nucleic acids have been used in a diverse range of applications, including enzymatic replication,[1] nucleic acid–based sensors,[2] catalysts,[3] diagnostics,[4] and therapeutics.[5] Enzymatic incorporation and extension of functionalized nucleotide triphosphates can be applied to introduce additional functionality into DNA and, thereby, expand the scope of the applicability of nucleic acids.[6–14]

Chemical modification of DNA to introduce novel photophysical properties can facilitate biophysical studies of the structural changes of DNA in vitro and in vivo.[15–17] Accordingly, direct incorporation of fluorescent materials into DNA would be very valuable for a diverse array of nucleic acid–based applications. One way to incorporate fluorescent nucleotides is through chemical solid-phase synthesis, which can be used to obtain oligonucleotides having less than 100 nucleotides.[18] This approach is limited, however, when attempting to prepare long-chain DNA because of the relatively low yields of chemical synthesis. Enzymatic synthesis is potentially an alternative method for incorporating fluorescent nucleotides into long DNA chains.[19–22] The development of fluorescent nucleotides that can be incorporated into DNA directly is, however, challenging because the active site of DNA polymerase is very tight (i.e., the fluorescent nucleotide might not be recognized).[23–26] Accordingly, many researchers have focused on the incorporation and extension of small functional nucleotides (e.g., those functionalized with azido, amino, or ethynyl groups) into DNA and then applying post-synthetic labeling with fluorophores.[27] Such post-synthetic methods are not always efficient because of steric factors (a bulky DNA secondary structure may disrupt the reaction), sequence-dependent reactivity, or the need for extra steps. Another approach is to attach the fluorophore at the 5-position of deoxyuridine or the 7-position of deoxyadenosine through a long linker to avoid steric effects in the enzyme active site. Indeed, the syntheses of dNTPs bearing fluorophores at the base and their polymerase-mediated incorporation into DNA has been reported many times.[28–31] This approach might be limited, however, when incorporating multiple fluorophores, which can also induce unstable B-form DNA structures.

Thus, we were interested in enzymatic methods for the direct incorporation and extension of fluorophores into long DNA strands—approaches that would be much more convenient and efficient than post-synthetic methods.[32] Our goal was to screen several fluorescent nucleotides that would be recognized by DNA polymerase and, thereby, discover highly efficient fluorescent nucleotides that could be incorporated and extended into DNA.



Scheme 1. Synthesis of fluorophore-attached deoxyuridine triphosphates and their use in enzymatic DNA synthesis mediated by DNA polymerase.

2. Synthesis

Toward this goal we synthesized several novel nucleotides presenting fluorophores of various sizes: pyrene-attached nucleotides (**dApyrTP**, **dUpyrTP**), an anthracene-attached nucleotide (**dUantTP**), and a thiophene-attached nucleotide (**dUthiTP**),[33] all of which have been prepared previously as phosphoramidite-type compounds for solid phase chemical synthesis. In addition, we synthesized two nucleotides presenting small functional groups (ethynyl and iodo) as reference compounds (**dUethTP** and **dUiodTP**, respectively) which is commercial available, because such functional nucleotides are well established for incorporation into DNA. Pyrene, anthracene, and thiophene are fluorescent materials that have been used in a diverse array of fluorescence imaging applications, each with unique photophysical properties: a high quantum yield and excimer-forming properties for pyrene,[34–41] light-induced dimer formation for anthracene,[42] and thiophene as a basic monomer unit for fluorescent polymers.[43] Their fluorescent nucleotides may be useful for diverse bioimaging and diagnostic applications targeting DNA and RNA; for example, sensing single nucleotide polymorphisms,[44–46] probing structural changes,[47] and other diverse biological applications.[48]

Table 1. Oligonucleotides employed in this study

Name	Sequence
Primer **E1**	5′-TAA TAC GAC TCA CTA TAG GGA GC
Template **E1**	3′-ATT ATG CTG AGT GAT ATC CCT CGT CTA GGT TAC GGC AGG ATC GC
Template **E2**	3′-ATT ATG CTG AGT GAT ATC CCT CGA CTA GGT TAC GGC AGG ATC GC
Template **H1**	3′-ATT ATG CTG AGT GAT ATC CCT CGA AAA AAA AAA AAA AAA AAA AAA AAA
Primer **P1**	5′-dCAC ACA GGA AAC AGC TAT GAC
Primer **P2**	5′-dGAA ATT AAT ACG ACT CAC TAT AGG
Template **T1**	5′-dGAA ATT AAT ACG ACT CAC TAT AGG GTT AAC TTT AAG AAG GAG ATA TAC CAT GGG CTC CAA GAA GCC GGT CCC CAT CAC AGT GCA ACC GC
Template **T2**	5′-dTTT CAC ACA GGA AAC AGC TAT GAC CCG GGT TAT TAC ATG CGC TGG CAC TTG CCC GTA CGG CGG TTG CAC TGT GAT GGG G

We attached these fluorescent groups and the quencher at the 8-position of deoxyadenosine and the 5-position of deoxyuridine. To synthesize the deoxyadenosine-based fluorescent nucleotides, we employed 8-brominated deoxyadenosine as a starting material and attached pyrene, ethynyl, and octyl groups at the 8-position. To form the fluorescent deoxyuridine-based nucleotides, we reacted 5-iododeoxyuridine through Sonogashira coupling (pyrene, anthracene, and ethynyl), Stille coupling (thiophene), and Suzuki coupling (benz-azo).[49,50] Next, we used the Yoshikawa method[51] to prepare their triphosphate derivatives (see Supporting Information). For primer extension and the polymerase chain reaction (PCR), we synthesized several oligonucleotides using the methods of solid phase DNA synthesis.[52]

We examined the basic photophysical properties of the fluorophore-labeled nucleotides **dApyrTP**, **dUpyrTP**, **dUantTP**, and **dUthiTP**. Each exhibited a quantum yield (QY; 0.700 for **dApyrTP**; 0.921 for **dUpyrTP**; 0.078 for **dUantTP**; 0.017 for **dUthiTP**) sufficiently high for in vitro and in vivo fluorescence imaging applications (Figure S1).

3. Primer extension

We prepared Primer **E1**, Template **E1**, and Template **E2** to monitor the template-dependent incorporation and extension of the fluorescent nucleotides. Template **H1**, which has a homogeneous dA sequence, was synthesized to examine the sequential incorporation and extension of the functional nucleotides. Primers **P1** and **P2** and Templates **T1** and **T2** were synthesized to examine the PCR (**Table 1**). To check the primer extension, we labeled the ^{32}P radiotracer at the 5′-end of each primer. We also applied three different types of DNA polymerase—Vent(exo-), nPfu special, and Kf(exo-) enzymes—as template-dependent polymerases for the primer extension. We also employed terminal deoxynucleotidyl transferase (TdT) to study the DNA template–independent incorporation and extension of the functional nucleotides.

First we examined the incorporation and extension ability of the functionalized deoxyadenosines **dApyTP**, **dAethTP**, and **dAoctTP**, as alternatives to dATP, on the context of Primer **E1**:Template **E1** using the three different polymerases. Gel electrophoresis data revealed, however, no incorporation and extension, even when using octyl- and ethynyl-attached deoxyadenosines, from any of the polymerases. Figure 1 exhibits the gel data for Vent(exo-). This result is consistent with previous reports from other groups; namely, that 8-substituted dATP derivatives cannot be incorporated using polymerases.[53] We conclude that the 8-positionof deoxyadenosine cannot be modified if we wish to perform a successful enzymatic synthesis.

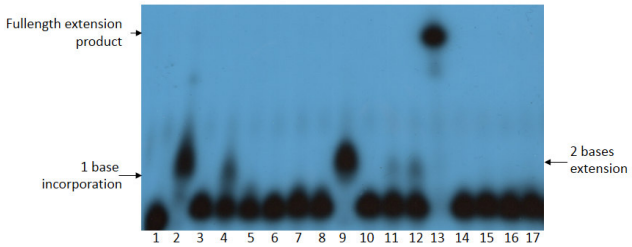


Figure 1. Primer extension of functionalized deoxyadenosines using Primer **E1**:Template **E1**. Lane 1: dATP; lane 2: dCTP; lane 3: dGTP; lane 4: dTTP; lane 5: **dApyrTP**; lane 6: **dAoctTP**; lane 7: **dAethTP**; lane 8: dATP, dGTP; lane 9: **dApyrTP**, dGTP; lane 10: **dAotcTP**, dGTP; lane 11: **dAethTP**, dGTP; lane 12: dATP, dTTP, dCTP, dGTP; lane 13: **dApyrTP**, dTTP, dCTP, dGTP; lane 14: **dAoctTP**, dTTP, dCTP, dGTP; lane 15: **dAethTP**, dTTP, dCTP, dGTP; lane 16: only Primer **E1**. All primers were labeled by 5′-[^{32}P]. Vent(exo-) polymerase catalyzed the reactions. All

samples were obtained using polyacrylamide gel 20%. Labeled DNA Primer **E1** (5´-labeled with ^{32}P; 0.5 μM) and Template **E1** (1 μM) were annealed in buffer by heating to 95 °C and then slowly cooling to room temperature. DNA polymerase (2 U) was then added. This sample was added into the samples containing 1 mM dNTP without dATP (5 μL) and then the mixtures were incubated at 37 °C for 3 h. The polymerase extension was stopped by adding twice the amount of stop buffer [10 mM EDTA, 10 mM NaOH, 0.1% xylene cyanol, and 0.1% bromophenol blue in formamide], and then the solution was loaded onto a 20% denaturing polyacrylamide gel.

We used the PyMOL program to examine the structure of the duplex context of Primer **E1**:Template **E1**. It revealed that the 8-position of dATP was blocked by the phosphoric diester backbone in the minor grove (data not shown). Thus, we examined the suitability of incorporating dUTP derivatives within the DNA structure. Interestingly, the large space at the 5-position of dUTP was open on the minor groove. Accordingly, we examined the incorporation and extension of fluorophore-attached deoxyuridine derivatives (**dUpyrTP**, **dUantTP**, **dUthiTP**, **dUazoTP**, **dUethTP**, **dUiodTP**) using the Primer **E1**:Template **E2** duplex context. Interestingly, **dUpyrTP**, **dUthiTP**, **dUazoTP**, **dUethTP**, and **dUiodTP** (but not **dUantTP**) were incorporated well against deoxyadenosine on the Primer **E1**:Template **E2** duplex context (Figure 2). We suspect that anthracene may be too big to be recognized by the polymerase active site in the closer 5-position of deoxyuridine. Furthermore, we examined the extension at the next dC position, located on Template **E2**. Again, **dUpyrTP**, **dUthiTP**, **dUazoTP**, **dUethTP**, and **dUiodTP** (but not **dUantTP**) were all extended well (Figure 2).

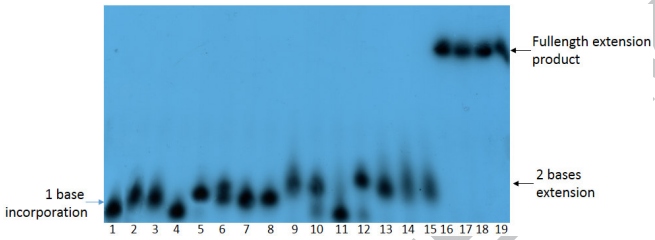


Figure 2. Primer extension of functionalized deoxyuridines using Primer **E1**:Template **E2**. Lane 1: only Primer **E1**; lane 2: dTTP lane 3: **dUpyrTP**; lane 4: **dUantTP**; lane 5: **dUazoTP**; lane 6: **dUthiTP**; lane 7: **dUethTP**; lane 8: **dUiodTP**; lane 9: dTTP, dGTP; lane 10: **dUpyrTP**, dGTP; lane 11: **dUantTP**, dGTP; lane 12: **dUazoTP**, dGTP; lane 13: **dUthiTP**, dGTP; lane 14: **dUethTP**, dGTP; lane 15: **dUiodTP**, dGTP; lane 16: **dUazoTP**, dATP, dCTP, dGTP; lane 17: **dUthioTP**, dATP, dCTP, dGTP; lane 18: **dUethTP**, dATP, dCTP, dGTP; lane 19: **dUiodP**, dATP, dCTP, dGTP. All primers were labeled by 5´-[^{32}P]. Vent(exo-) polymerase catalyzed the reactions. Labeled DNA Primer **E1** (5´-labeled with ^{32}P; 0.5 µM) and Template **E2** (1 µM) were annealed in buffer by heating to 95 °C and then slowly cooling to room temperature. DNA polymerase (2 U) was then added. This sample was added into the samples containing 1 mM dNTP without dUTP (5 µL) and then the mixtures were incubated at 37 °C for 3 h. The polymerase extension was stopped by adding twice the amount of stop buffer [10 mM EDTA, 10 mM NaOH, 0.1% xylene cyanol, and 0.1% bromophenol blue in formamide], and then the solution was loaded onto a 20% denaturing polyacrylamide gel.

Encouraged by this result, we also performed the full-length primer extension experiment. Surprisingly, **dUazoTP** and **dUthiTP** were still incorporated and extended well: **dUthiTP** provided a high yield of its full-length product, while **dUazoTP** formed its full-length product with a small amount of the early terminated product (Figure 2). Thus, the 5-position of deoxyuridine is relatively less restricted in the DNA polymerase active site than is the 8-position of deoxyadenosine, but the former's reactions remained sensitive to size. This result was promising because we had obtained directly incorporated and extended fluorophores (**dUthiTP, dUpyTP**) and a quencher (**dUazoTP**).

Next we examined the multiple continuous incorporation and extension of the fluorophore-attached deoxyuridines during primer extension using the Primer **E1**:Template **H1** duplex, with Template **H1** containing a sequence of 24 continuous dA units (Figure 3). Interestingly, even with this long continuous sequence we observed fully incorporated and extended products from the reactions of **dUthiTP**, **dUethTP**, and **dUiodTP**, while the reaction of **dUazoTP** provided some truncated products (Figure 3). The reactions of **dUpyTP** and **dUantTP**, however, did not result in any incorporation and extension products. Most notably, the small fluorescent compound **dUthiTP** still functioned in the incorporation and extension process over the 24 homogeneous continuous sequence. We conclude that the incorporation of deoxyuridine-based fluorescent nucleotides into DNA can be very efficient, even when the size of the fluorescent unit is large, but the extension process is sensitive to the size of the fluorophore at the 5-position of the deoxyuridine derivative. It appears that one aromatic unit at the 5-position can be tolerated during primer extension; indeed, **dUthiTP** exhibited a high incorporation and extension capability, similar to that of natural dTTP.

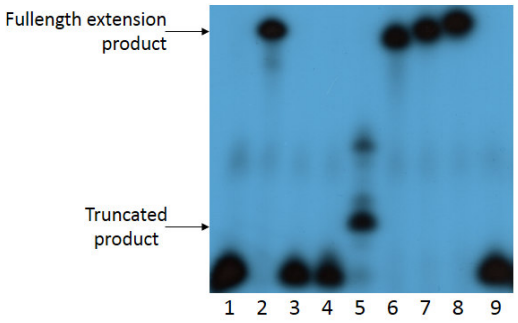


Figure 3. Primer extension of functionalized deoxyuridine using Primer **E1**:Template **H1**. Lane 1: only Primer **E1**; lane 2: dTTP; lane 3: **dUpyrTP**; lane 4: **dUantTP**; lane 5: **dUazoTP**; lane 6: **dUthioTP**; lane 7: **dUethTP**; lane 8: **dUiodTP**; lane 9: only Primer **E1**. All primers were labeled by 5´-[^{32}P]. Vent(exo-) catalyzed the reactions. All samples were obtained using polyacrylamide gel 20%. Labeled DNA Primer **E1** (5´-labeled with ^{32}P; 0.5 μM) and Template **H1** (1 μM) were annealed in buffer by heating to 95 °C and then slowly cooling to room temperature. DNA polymerase (2 U) was then added. This sample was added into the samples containing 1 mM dNTP or 5´-modified dUTP with dCTP, dGTP, with dATP (5 μL) and then the mixtures were incubated at 37 °C for 3 h. The polymerase extension was stopped by adding twice the amount of stop buffer [10 mM EDTA, 10 mM NaOH, 0.1% xylene cyanol, and 0.1% bromophenol blue in formamide], and then the solution was loaded onto a 20% denaturing polyacrylamide gel.

To further examine the incorporation and extension processes, we investigated the effects of different types of DNA polymerase (e.g., TdT, a template-independent polymerase). We used Primer **P1** as a single-strand sequence for the template-independent polymerase experiment. Unlike other template-dependent DNA polymerases, the use of TdT resulted in a low degree of incorporation and extension, even when using deoxyuridines presenting small functional groups (Figure S4). When we added dATP, dTTP, dGTP, or dCTP to the Primer **P1**, we obtained different incorporated and extended products. In contrast, most of the functionalized deoxyuridines did not form their fully extended products. For example, **dUthiTP** and **dUiodTP** provided only slightly elongated products.

4. PCR

To explore the potential applications of the functionalized deoxyuridines in replication, we examined their use in 30 cycles of PCR. We used Primers **P1** and **P2** and Templates **T1** and **T2** to form 160-mers of full-length DNA. We used dNTP as a positive control and dNTP without dTTP as a negative control; we obtained the 160-mer full-length product with the positive control, but not with the negative control (Figure 4a). To check the amplification capability of our deoxyuridine-based fluorescent nucleotides, we ran the PCR with them as substitutes for dTTP. Interestingly, **dUthiTP**, **dUethTP**, and **dUiodTP** exhibited sufficient amplified signals at the same position of dNTP amplification (Figure 4a). In contrast, **dUantTP** and **dUazoTP** did not feature sufficient amplification bands. **dUpyrTP** did, however, still exhibit more amplified product than that from the negative PCR. We also observed strong fluorescence from **dUpyrTP** and **dUthiTP** after direct PCR amplification, without any extra post-synthesis processing (Figure 4b). Although the amplification efficiency of **dUpyrTP** was lower than that of **dUthiTP**, the former exhibited strong fluorescence because of the higher QY. We also screened two other polymerases; Pfu displayed almost the same behavior toward the deoxyuridine-based fluorescent nucleotides, but Kf provided less amplification than the other polymerases [this behavior was not unexpected because Kf polymerase is unstable at the high temperature of the PCR[54] (see Supporting Information)]. Thus, we conclude that the deoxyuridine-based fluorescent nucleotides, particularly **dUthiTP**, can function as incorporated substrates and also as platforms in the template for the incorporation of dATP.

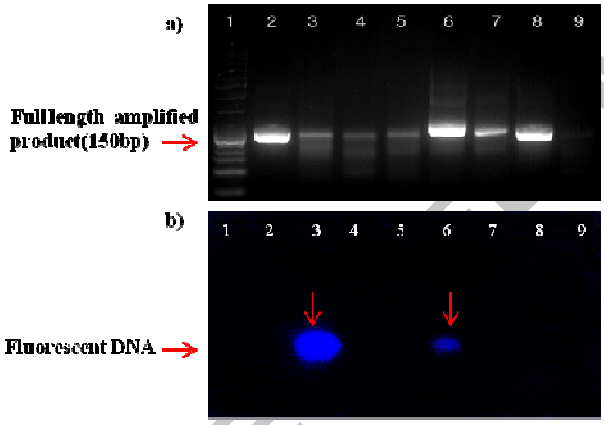


Figure 4. a. PCR data and b. photograph obtained using Primers **P1** and **P2** and Templates **T1** and **T2**. Lane 1: Marker; lane 2: dATP, dTTP, dGTP, dCTP; lane 3: **dUpyrTP**; lane 4: **dUantTP**; lane 5: **dUazoTP**; lane 6: **dUthiTP**; lane 7: **dUethTP**; lane 8: **dUiodTP**, all lanes 3 to 8 treated with dATP, dGTP, dCTP; lane 9: dATP, dGTP, dCTP. Vent(exo-) catalyzed all of the reactions. All data were obtained using 2.5% agarose gel. The reactions were performed in an overall volume of 25 μL in reaction buffer (1X) using modified nucleotides **dUpyrTP**, **dUantTP**, **dUthiTP**, **dUethTP**, and **dUiodTP** (final concentration: 200 μM) with dNTP (200 μM) without dUTP, 0.25 μM of each Primer **P1** and **P2,** 0.125 μM of each Template **T1** and **T2**, and DNA polymerase (2 U). As a control, PCRs were performed under the same conditions using 200 μM natural dNTPs instead of the modified nucleotides; and using 200 μM dATP, dGTP, and dCTP as a negative control. Amplification was performed through an initial denaturing at 95 °C for 3 min, followed by 30 cycles of denaturing at 95 °C for 2 min, primer annealing at 55 °C for 2 min, and extension at 70 °C for 5 min. We used 312nm wavelength as a excitation wavelength to obtain fluorescence photograph image using UV trans illuminator.

5. DNA structure

We recorded CD spectra to examine the stability of the DNA secondary structures incorporated and extended with the fluorophore-attached deoxyuridines (Figure 5). Interestingly, all of the deoxyuridine derivatives presenting small functional groups exhibited stable B-form duplex characteristics, with a positive band from 275 to 270 nm and a negative band from 235 to 245 nm, with slight differences depending on the size (the large substituent of **dUthiTP** provided a slightly blue-shifted band pattern relative to those provided by the small substituents of **dUiodTP** and **dUethTP**). Nevertheless, **dUpyrTP**, the deoxyuridine derivative presenting the largest fluorophore, did not result in a stable B-form DNA structure. The CD spectra clearly revealed different B-form duplex characteristics depending on the size at the 5′-position of deoxyuridine: **dTTP** ≥ **dUiodTP** > **dUethTP** > **dUthiTP** > **dUpyrTP**. This result supports the notion that the active site of DNA polymerase is very tight and sensitive to modification of the 5′-position of deoxyuridine. Impressively, **dUthiTP** exhibited particularly significant amplification capability as well as stability of its DNA secondary structure, much like that of natural dTTP. We suspect that **dUthiTP** will be a promising fluorescent nucleotide for use as a replacement for dTTP in a variety of biological applications.

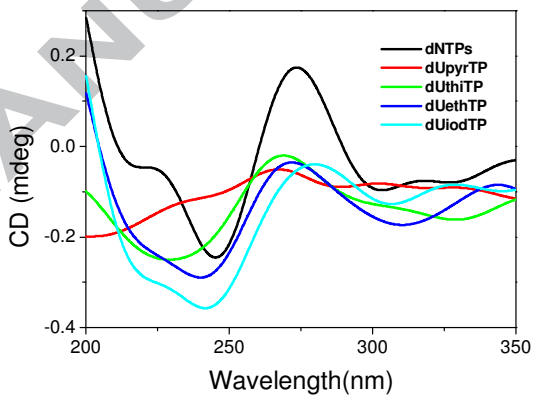


Figure 5. CD spectra of PCR products using Primers **P1** and **P2** and Templates **T1** and **T2.** Deoxyuridine based Fluorescent nucleotides (**dUpyrTP, dUthiTP**, **dUethTP, dUiodTP**) were treated with dATP, dGTP, and dCTP for PCR reaction. All samples were prepared in the condition of 30 cycles of PCR using Vent(exo-) polymerase under the same PCR conditions (See supporting information). The products were purified using a Generation Capture Column Kit and then their CD spectra were measured in the range 200–400 nm.

6. Conclusions

We designed and synthesized several functionalized deoxyadenosine triphosphates (**dApyrTP, dAethTP, dAoctTP**) and deoxyuridine triphosphates (**dUpyrTP, dUantTP, dUthiTP, dUazoTP, dUethTP, dUiodTP**) and examined their enzymatic incorporation and extension using three different types of DNA template–dependent polymerases [Vent(exo-), nPfu special, Kf(-)] and also a template-independent polymerase (terminal transferase). **dUpyrTP**, **dUthiTP**, **dUazoTP**, **dUethTP**, and **dUiodTP** were well incorporated and extended through primer extension, but **dUantTP**, **dApyrTP**, **dAethTP**, and **dAoctTP** were not. We observed a size-dependence in the incorporation and extension capability (following the order **dUiodTP = dUethTP = dUthiTP > dUazoTP > dUpyrTP > dUantTP**) during primer extension with the heterogeneous sequence. This result was supported by CD spectra, which revealed different tendencies in the B-form DNA structures depending on the size of

the unit at the 5-position of the deoxyuridine (**dUiodTP > dUethTP > dUthiTP > dUpyrTP**). Furthermore, interestingly, **dUthiTP** could be incorporated and extended well into long DNA for primer extension and even for PCR. This multiple-thiophene-incorporated PCR product exhibited a stable B-form DNA structure, as demonstrated using CD spectroscopy. **dUthiTP** also exhibited full-length extension products even when using **dUthiTP** with 24 continuous oligo dA units as a template. Thus, we believe that **dUthiTP** is a promising fluorescent nucleotide for diverse biological applications that require multiple incorporation and extension directly (i.e., without additional post-synthesis treatment) without disruption of the B-form DNA structure.

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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.bmc.

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Synthesis of a series of size-diverse fluorescent nucleotides for incorporation and extension into DNA.

Size-dependence of the incorporation and extension capability of the functional unnatural nucleotides into DNA.

The fluorescent nucleotide dUthiTP could be incorporated and extended into long DNA strands.

dUthiTP appears to be a promising fluorescent nucleotide for a diverse range of biological applications.