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Bodipy-Labeled Nucleoside Triphosphates for Polymerase Synthesis of Fluorescent DNA

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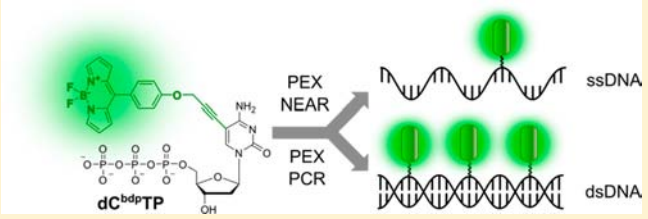
Supporting Information

ABSTRACT: New fluorescent nucleosides and nucleoside triphosphate (dNTPs) analogs bearing the F-Bodipy fluorophore linked through a short, flexible nonconjugate tether were synthesized. The Bodipy-labeled dNTPs were substrates for several DNA polymerases which incorporated them into DNA in primer extension, nicking enzyme amplification reaction, and polymerase chain reaction. The fluorescence of F-Bodipy is not quenched upon incorporation in DNA and can be detected both in solutions and on gels.



INTRODUCTION

DNA molecules labeled with fluorescent tags and reporters found broad applications in chemical, molecular, and cell biology; biomedicine; biotechnology; and materials science.[1−5] The most useful examples are molecular beacons and related probes for real-time PCR and bioimaging applications,[5−7] aptamer-based fluorescent probes developed for a wide range of analytes,[8−11] environmentally sensitive fluorescent nucleoside analogues used for probing of local structure and dynamics of nucleic acids,[1,12−17] and new DNA-based fluorescent materials.[18]

Apart from classical chemical synthesis of oligonucleotides (ONs) by the phosphoramidite method on solid support, enzymatic synthesis of base-modified ONs and DNA by polymerase incorporation of modified 2′-deoxyribonucleoside triphosphates (dNTPs) is increasingly popular for the construction of base-modified nucleic acids bearing a variety of functional and reporting groups.[19−21] Unlike the phosphoramidite chemistry method which is suitable for the synthesis of rather short base-modified oligonucleotides (<100 nt), polymerase-based methods such as primer extension (PEX) or polymerase chain reaction (PCR) can generate longer DNA conjugates, up to thousands of base pairs containing multiple modifications. The limited compatibility of some functional groups with the reagents used during the phosphoramidite protocol can also be overcome by the enzymatic incorporation, which tolerates even some highly reactive groups, azido,[22] aldehyde,[23,24] or Michael acceptors.[25] Our recent study even showed[26] that certain 7-aryl-7-deaza-dATP derivatives are better substrates for polymerases than natural dATP, which makes them promising even for in vivo synthesis of modified DNA.

Many dNTPs bearing fluorophores have been reported so far and some of them are even commercially available.[27] They have found applications in cell and molecular biology, e.g., in classical[28,29] and next-generation[30] sequencing methods. Cyanine-labeled DNA probes synthesized by PCR have been used for fluorescence in situ hybridization (FISH).[31] Fluorescent dNTPs have also been utilized for labeling of intracellular DNA in living cells and study of chromatin dynamics via fluorescence microscopy.[32−36] The majority of known fluorescent dNTPs are based on cyanine and fluorescein-type dyes, which suffer from low photostability, moderate brightness, and effect of charge of the fluorophore on physical and biological properties of labeled ONs or DNA. Therefore, there is still a need for new fluorescent dNTPs with bright photostable electroneutral fluorophores. Dyes based on a difluoro-boraindacene core (4,4-difluoro-4-borata-3*a*-azonia-4*a*-aza-*s*-indacene, F-Bodipy, Chart 1) are a class of fluorescent molecules with attractive properties for use as fluorescent tags. Major advantages of these dyes are high photostability and high brightness in combination with low sensitivity to environmental polarity and pH.[37,38] At the same time, they possess some disadvantages, such as small Stokes shift. F-Bodipy dyes have been widely used for labeling of biomolecules and their constituents, but labeling of nucleic acids has only been explored poorly. F-Bodipy-containing phosphoramidite reagents for labeling of the 5′-terminus of synthetic oligonucleotides are known.[39] A set of F-Bodipy-based electrophoretically uniform dyes of different colors for primer labeling in automated DNA sequencing was reported.[40]

Fluorescent deoxyuridine analogues based on the Bodipy fluorophore and their incorporation into DNA via solid-phase phosphoramidite synthesis have been reported (Chart 1).[41−43]

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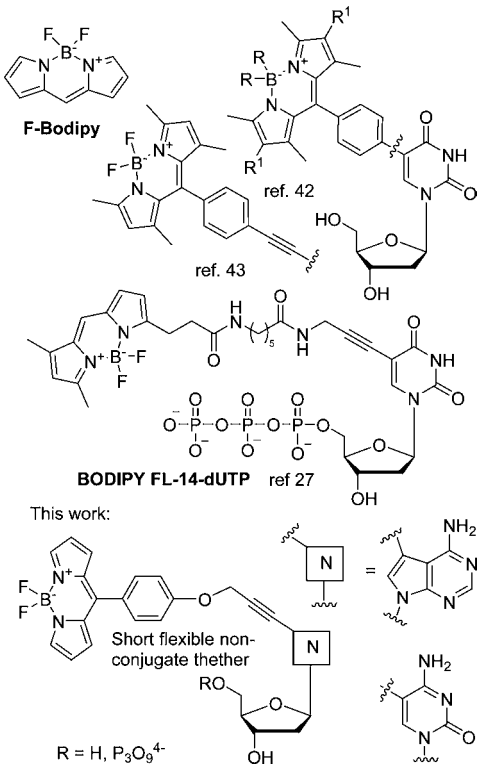
Chart 1. Chemical Structures of F-Bodipy Core, Some Known Bodipy-Labeled Nucleosides, and the Structure of New Short Tether for Attachment of Bodipy Described in This Work



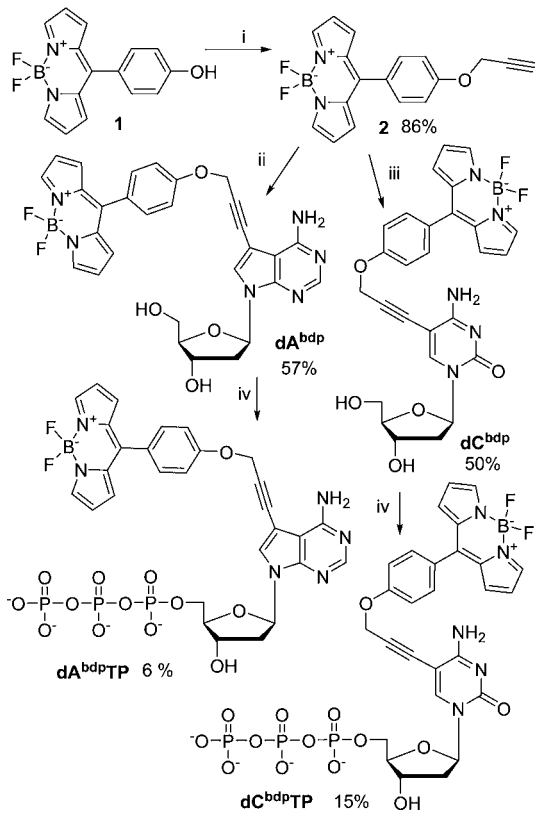
It has also been shown that some DNA polymerase can bypass F-Bodipy-labeled dU nucleoside in the template strand, correctly inserting dATP opposite to it.[44] Deoxyuridine derivatives with Bodipy fluorophore tethered via long flexible linker (as, for example, BODIPY-FL-14-dUTP shown in Chart 1) are commercially available[27] and were used for in cellulo fluorescence labeling of DNA and for detection of apoptotic cells.[36,45−47] In this work we describe synthesis of two new F-Bodipy containing dNTPs featuring a short hydroxyalkynyl linker between fluorophore and nucleobase parts, their photophysical properties, and insertion into DNA using primer extension, nicking enzyme amplification reaction, and polymerase chain reaction.

RESULTS AND DISCUSSION

Our design of new Bodipy-nucleoside analogues was based on the assumption that a flexible nonconjugate tether would electronically separate the fluorophore from the $\pi-\pi$ stacked aromatic nucleobases, and thus the brightness of fluorescence should not be influenced by any changes in secondary structure, hybridization, or interactions with other (bio)molecules. Moreover, dNTPs containing rather the bulky Bodipy moiety linked through a flexible tether should be better substrates for polymerases than the bulky rigid constructs described previously.[41−43] Another related work showed that fluorescein-dNTP constructs bearing short rigid linkers were also bad substrates for polymerases.[48] Considering synthetic accessibility also, our choice was to connect Bodipy through a propargyloxy-phenyl linker (Chart 1). We selected a nonsubstituted F-Bodipy fluorophore to reduce the possible steric clash and/or hydrophobic interaction (although it is known[37,38] that unsubsituted 8-phenyl Bodipy derivatives exhibit rather low fluorescence quantum yields). We decided to synthesize 2′-deoxycytidine and 2′-deoxy-7-deazaadenosine derivatives as one example of pyrimidine and one example of purine analogue (please note that 7-substituted 7-deazapurine dNTPs are generally used instead of purine derivatives due to their excellent substrate activity with DNA polymerases).[19−21,26]

The synthesis of Bodipy-linked dNTPs was accomplished according to a straightforward strategy as shown in Scheme 1.

Scheme 1. Synthesis of Bodipy-Linked dNTPs[a]



[a]Reagents and conditions: (i) propargyl bromide, K_2CO_3, CH_3CN, 70 °C; (ii) 7-iodo-7-deaza-2′-deoxyadenosine, $PdCl_2(PPh_3)_2$, CuI, NEt_3, DMF, 80 °C; (iii) 5-iodo-2′-deoxycytidine, $PdCl_2(PPh_3)_2$, CuI, NEt_3, DMF, 80 °C; (iv) 1: $POCl_3$, $PO(OCH_3)_3$, 0 °C, 3−4 h; 2: $(n\text{-}Bu_3NH)_2H_2P_2O_7$, NBu_3, DMF, 0 °C, 1 h; 3:1 M TEAB, 5 min; 4: HPLC; 5: Dowex (Na^+).

We started from known Bodipy-phenol **1**[49] which was readily alkylated by propargyl bromide to give Bodipy-substituted terminal acetylene **2**. The Sonogashira cross-coupling of iodinated deoxycytidine (**dC^I**) and 7-deaza-deoxyadenosine (**dA^I**) with **2** under standard conditions gave Bodipy-labeled nucleosides **dA^{bdp}** and **dC^{bdp}** in acceptable yields of 50% and 57%, respectively. The synthesis was completed by 5′-O phosphorylation[50] of the BODIPY-nucleosides yielding desired triphosphates **dA^{bdp}TP** and **dC^{bdp}TP**.

Fluorescence properties of Bodipy-labeled dNTPs are summarized in Table 1 and compared to non-nucleosidic Bodipy-intermediates **1** and **2**. A narrow absorption band is centered at 495−496 nm for all four compounds (Figure 1). Importantly, the absorbance of the fluorophore was high at 488 nm (77% of the maximal absorbance). This makes these Bodipy-linked nucleosides and nucleotides suitable for excitation with the 488 nm argon-ion laser. Emission spectra

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Table 1. Fluorescence Characteristics of the Bodipy-Derivatives

compound	solvent	λ_{abs} [a]	λ_{em} [b]	Φ [c]
1 [d]	MeOH	493	508	0.015
2	EtOH	496	512	0.03
dA^{bdp}TP	buffer [e]	496	512	0.02
dC^{bdp}TP	buffer [e]	495	512	0.03

[a]Position in nm of the absorption maximum. [b]Position in nm of the emission maximum. [c]Fluorescence quantum yield measured using fluorescein in 0.1 M NaOH (Φ = 0.92)[70] as a reference. [d]Values taken from ref 49. [e]10 mM sodium phosphate buffer, pH = 7.4.

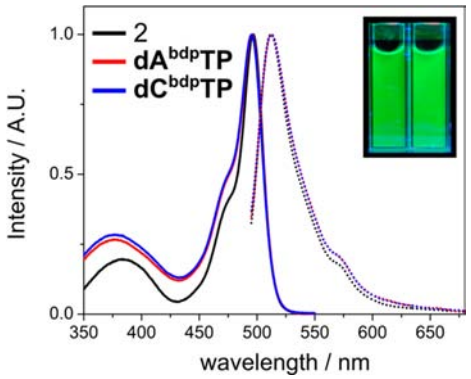

Figure 1. Normalized absorption and emission spectra of intermediate **2** (black), **dA^{bdp}TP** (red), and **dC^{bdp}TP** (blue); excitation wavelength for fluorescence was 488 nm. Photography of 15 μM solutions of **dA^{bdp}TP** (left) and **dC^{bdp}TP** (right) in 10 mM sodium phosphate buffer pH = 7.4 taken under a "blacklight blue" lamp (365 nm).

followed the mirror-image rule centered at 512 nm and shared the same shape for all compounds. The small Stokes shift observed (16−17 nm) is typical for Bodipy dyes. On the contrary, the observed low quantum yields of luminescence (2−3%) are rather uncommon for Bodipy dyes.[37,38] This is probably due to the lack of substituents in the pyrrole ring, which leads to the free rotation of the phenyl group attached at the *meso* position of the Bodipy core.[37] The low quantum yields are partially compensated by high absorption coefficient of the fluorophore. For instance, the absorption coefficient of compound **2** measured in ethanol was (6.17 ± 0.16) × 10^4 M^{-1} cm^{-1}. This corresponds to the brightness of Bodipy-labeled nucleosides of about 2000 M^{-1} cm^{-1}, which is compatible with brightness of other dyes used in chemical biology.[51]

Quenching of fluorophores by nucleobase constituents is one of the problems in fluorescence labeling of nucleic acids. Guanine is one of the most efficient quenchers of organic dyes by the photoinduced electron transfer mechanism. Several Bodipy dyes have been reported to be efficiently quenched by guanine either in free form or in DNA.[52] This phenomenon was exploited for the design of hybridization probes based on BODIPY FL fluorophore.[53,54] These literature data indicate that in principle the fluorescence of Bodipy-labeled dNTPs can be totally quenched after incorporation into DNA. To preliminarily evaluate the possible effect of quenching by guanine nucleobase, we studied the influence of increasing concentrations of 2′-deoxyguanosine 5′-monophosphate sodium salt (dGMP) on the fluorescence of **dC^{bdp}TP** in phosphate buffer solutions (steady-state Stern−Volmer experiment, Supporting Information Figure S1). In this experiment dGMP showed only a moderate quenching potency (K_{SV} = 0.013 ± 0.006 mM^{-1}). For comparison, the Stern−Volmer constant for the quenching of other nucleoside analogues by dGMP may be as high as 0.3 mM^{-1}.[55] These results mean that the fluorescence of our Bodipy-conjugates is unlikely to be significantly quenched by guanine residues after incorporation into DNA, and therefore we proceeded to the synthesis of the labeled DNA.

Both **dA^{bdp}TP** and **dC^{bdp}TP** were tested as substrates for DNA polymerases in primer extension (PEX) reaction. Oligonucleotides **P1X** (16-mer) and **T1X** (31-mer) were used as primer and template, respectively (see Table 2 for sequences). The sequence of the template was designed for incorporation of four modified nucleosides of each kind of nucleobase tested. The PEX experiments with each modified **dN^{bdp}TP** were performed in the absence of the natural counterpart but in the presence of the other three natural dNTPs. The PAGE analysis of PEX products obtained using KOD XL and Bst DNA polymerases (Figure 2) showed that enzymes were able to incorporate **dC^{bdp}TP** smoothly to form the full-length 31-mer sequence bearing four Bodipy modifications (lanes 5, 10). At the same time **dA^{bdp}TP** was incorporated by KOD XL polymerase, but less efficiently by Bst polymerase, which gave several shorter products (lanes 4, 9). We also tested five other DNA polymerases (*Vent(exo-)*, 9°N_m, DyNAzyme II, Phusion, Pwo) for their ability to incorporate **dN^{bdp}TP**. PAGE analysis (Supporting Information Figures S2−

Table 2. Deoxyoligonucleotides Used As Primers and Templates in the Enzymatic Reactions

name	used in [a]	Sequence (5′→ 3′) [b]
		Primers
P1X	PEX	CAT GGG CGG CAT GGG
P2X	PEX	TCA AGA GAC ATG CCT
P1N	NEAR	CCG ATC TAG TGA GTC CTC G
P1R	PCR	CAA GGA CAA AAT ACC TGT ATT CCT T
P2R	PCR	GAC ATC ATG AGA GAC ATC GC
		Templates
T1X	PEX	CTA GCA TGA GCT CAG TCC CAT GCC GCC CAT G
T1Xb	PEX	btn - CTA GCA TGA GCT CAG TCC CAT GCC GCC CAT G
T2X	PEX	CTA CCA TCA GCT CAC TCC CAT GCC GCC CAT G
T3Xb	PEX	btn - ATA ATA GAC ATG TCT AGG CAT GTC TCT TGA
T4X	PEX	GCT CGC CAC GCT CCC CAT GCC GCC CAT G
T1N	NEAR	TCA ACT CAT GAC CGA GGA CTC ACT AGA TCG G
T2N	NEAR	TCA GCG TAG ACT CGA GGA CTC ACT AGA TCG G
T3N	NEAR	TAC ACC TAT TTT GTC CTT CGA GGA CTC ACT AGA TCG G
T4N	NEAR	CAT CTC TCT CAT GAT CTC CGA GGA CTC ACT AGA TCG G
T1R	PCR	GAC ATC ATG AGA GAC ATC GCC TCT GGG CTA ATA GGA CTA CTT CTA ATC TGT AAG AGC AGA TCC CTG GAC AGG CAA GGA ATA CAG GTA TTT TGT CCT TG
T2R	PCR	GAG ATC ATG AGA GAG ATG GCC TCT GGG CTA ATA GGA CTA CTT CTA ATC TGT AAG AGC AGA TCC CTG GAC AGG CAA GGA ATA CAC CTA TTT TGT CCT T

[a]PEX − primer extension; NEAR − nicking enzyme amplification reaction; PCR − polymerase chain reaction. [b]btn = 5′-biotin.

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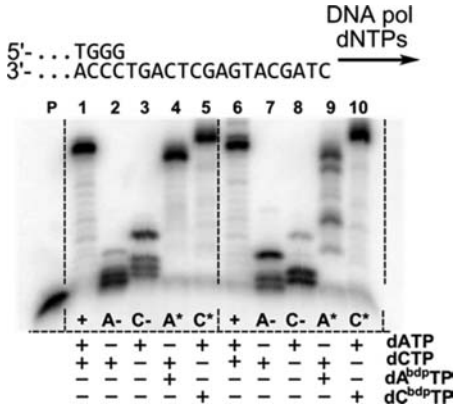

Figure 2. PAGE analysis of the products of primer extension by KOD XL DNA polymerase (lanes 1−5) and Bst DNA polymerase Large Fragment (lanes 6−10). Positive control: all four natural dNTPs are present (lanes 1, 6). Negative controls; A−: dTTP, dCTP, dGTP (lanes 2, 7); C−: dATP, dGTP, dTTP (lanes 3, 8). Modified dNTPs; A*: **dA^{bdp}TP**, dTTP, dCTP, dGTP (lanes 4, 9); C*: **dC^{bdp}TP**, dATP, dGTP, dTTP (lanes 5, 10).

S3) showed that none of them exhibited higher efficiency than KOD XL for **dA^{bdp}TP** and KOD XL with Bst LF for **dC^{bdp}TP**. The proper incorporation of four modified nucleotides into primer by KOD XL polymerase was also confirmed by primer extension with a biotinylated template (**T1Xb**) followed by magnetic separation using streptavidin-coated magnetic beads. MALDI-TOF analysis of ssDNA product obtained in this way (**ON1** and **ON2**, Table 3) revealed peaks which correspond to 31-mer ssDNA bearing four grafted Bodipy fluorophores. In both cases the peak of the product was accompanied by the peak of the template partially released from the magnetic beads during the denaturation step.

A combination of two modified dNTPs in one PEX reaction gives a way for multiple labeling of oligonucleotides at different positions. We examined the possibility to use **dC^{bdp}TP** in combination either with bulk **dA^{bdp}TP** or with 7-ethynyl-7-deaza-deoxyadenosine 5′-*O*-triphosphate (**dA^{E}TP**, Supporting Information Chart S1) bearing small ethynyl group. We performed PEX by KOD XL polymerase using primer **P1X** and template **T4X** (Table 2) which assumes incorporation of three modified deoxycytidine and two modified deoxyadenosine units within a 13-nt-long region. PAGE analysis of the products (Supporting Information Figure S4) shows that the combination of **dC^{bdp}TP** and **dA^{E}TP** with the two remaining natural dNTPs works as well as **dC^{bdp}TP** with three natural dNTPs, giving the full-length product (lanes 1 and 3). At the same time, the polymerase failed to incorporate both Bodipy-modified triphosphates in one reaction (lane 4).

In the next step, we examined the compatibility of the Bodipy-modified dNTPs with the silica-gel membrane DNA purification method. This method based on the adsorption of DNA on a silica-gel membrane in the presence of a high concentration of chaotropic salts is widely used for DNA purification, particularly in DNA manipulation kits and in microfluidics.[56] A primer−template pair (**P1X···T1X**) was incubated with **dC^{bdp}TP** and three remaining dNTPs either with or without KOD XL DNA polymerase. The resulting dsDNA samples were purified using one of the commercially available silica-gel membrane technology-based DNA purification kits, and UV−vis spectra of the DNA samples were measured. The absorption band of Bodipy-fluorophore was observed only in the spectrum of the sample incubated with DNA polymerase; the sample incubated without DNA polymerase was neat in the visible region (Figure S5 in Supporting Information). These results show that Bodipy-labeled dNTPs do not interact unspecifically with DNA or silica-gel membrane, and therefore are compatible with this DNA purification method.

As PEX is not suitable for synthesis of short ssONs (for use as primers), we tested the nicking enzyme amplification reaction (NEAR), which was recently adopted by our group for the synthesis of short base-modified ONs.[57,58] NEAR is a two-step isothermal linear amplification process performed by two enzymes: a DNA polymerase and a nicking endonuclease (Figure 3A). In the first step, a primer−template pair is elongated by DNA polymerase in the presence of one base-modified and three remaining dNTPs. Then, nickase makes a single strand break in the position defined by the sequence of template and primer. The short oligonucleotide formed dissociates at the temperature of reaction (55 °C) regenerating the primer−template pair, which is now ready to follow the cycle again. The previously reported study[58] of the scope and limitations of the NEAR for the synthesis of various base-modified oligonucleotides showed that deoxycytidine triphosphate analogues are the best modified substrates for NEAR. Therefore, we decided to test **dC^{bdp}TP** as a substrate for NEAR. Two templates from previous work were chosen (**T1N** and **T2N**, see Table 2 for sequences), assuming synthesis of 12-mer ssON products bearing one (**ON4**) and three (**ON5**) modified **dC^{bdp}** residues, respectively (sequences in Table 3).

Table 3. Single Strand Bodipy-Modified DNA

name	synth/prim/temp[a]	L/M[b]	sequence (5′ → 3′)[c]	mass calc.[d]	mass obs.[e]
ON1	PEX/P1X/T1Xb	31/4	CAT GGG CGG CAT GGG A<u>C</u>T GAG <u>C</u>T<u>C</u> ATG <u>C</u>TA G	10897	10899[f]
ON2	PEX/P1X/T1Xb	31/4	CAT GGG CGG CAT GGG <u>A</u>CT G<u>A</u>G CTC <u>A</u>TG CT<u>A</u> G	10893	10897[f]
ON3	PEX/P2X/T3Xb	30/2	TCA AGA GAC ATG CCT AGA <u>C</u>AT GT<u>C</u> TAT TAT	9829	9830[f]
ON4	NEAR/P1N/T1N	12/1	P - GT<u>C</u> ATG AGT TGA	4100	4101
ON5	NEAR/P1N/T2N	12/3	P - AGT <u>C</u>TA <u>C</u>GC TGA	4684	4688
ON6	NEAR/P1N/T3N	18/1	P - AAG GA<u>C</u> AAA ATA GGT GTA	6004	6005
ON7	NEAR/P1N/T4N	18/1	P - GAG AT<u>C</u> ATG AGA GAG ATG	6036	6038[g]

[a]Enzymatic reaction which was used for the synthesis of the sequence/primer used/template used. [b]Length of oligonucleotide (nt)/Number of modified nucleosides. [c]<u>Underline</u> = Bodipy-modified nucleoside unit (<u>A</u> = dA^{bdp} and <u>C</u> = dC^{bdp}, respectively); P = 5′-phosphate group. [d]Calculated using ChemBioDraw13 and OligoCalc.[69] [e]Results of MALDI-TOF analysis; the *m*/*z* value of major peak only is given; for copies of MALDI spectra see Supporting Information . [f]Signal of biotinylated template was also observed. [g]Signal of oligonucleotide longer by one dA was also observed.

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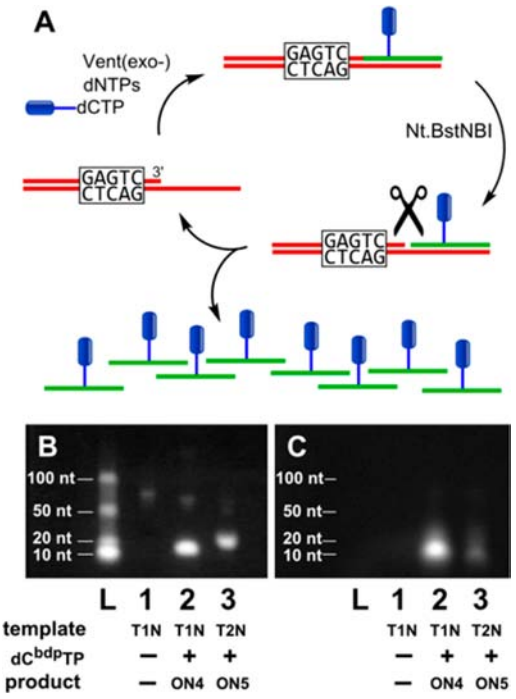

Figure 3. (A) Schematic representation of nicking enzyme amplification reaction (NEAR); the sequence of the *Nt.BstNBI* recognition site is given in the rectangle. (B) Agarose gel (4%) showing the results of NEAR in the presence of **dC^{bdp}TP** visualized using GelRed dye. (C) The same gel imaged using the 488 nm laser for excitation. L = single-stranded DNA ladder; lane 1 = negative control; lane 2 = amplification of a product incorporating one fluorescently modified dC; lane 3 = amplification of the product incorporating three modified dC.

The NEAR was performed by *Vent(exo−)* DNA polymerase and nickase *Nt.BstNBI* under previously optimized reaction conditions.[58] The products analyzed by 4% agarose gel electrophoresis are shown in Figure 3B. These results indicate that formation of the NEAR product was much more efficient in the case of template **T1N** assuming incorporation of only one modified **dC^{bdp}**; whereas the yield was lower in the case of template **T2N** resulting in ON bearing three Bodipy residues. Interestingly, Bodipy-containing ONs can be visualized in two ways, either by the use of intercalating fluorescent dyes (Figure 3B) or by the use of intrinsic fluorescence of Bodipy fluorophore (Figure 3C). As was noticed above, the fluorophore suits perfectly for the excitation by the 488 nm argon-ion laser. Because of that we were able to image gels containing labeled DNA using fluorescence readout. The possibility to image the gels containing Bodipy-labeled DNA supports our suggestion for the Bodipy-labeled dNTPs to be used as fluorescent tags for DNA. The formation of correct NEAR products **ON4** and **ON5** was also confirmed by MALDI-TOF mass spectrometry (results are summarized in Table 3).

We also tested Bodipy-modified dNTPs in polymerase chain reaction (PCR). PCR with chemically modified deoxynucleoside triphosphates is a useful method for the synthesis of long base-modified DNA functionalized at multiple positions. In was also used for the synthesis of fluorescently labeled DNA using different fluorescent dNTPs.[31,59−61] At first we tested the capability of KOD XL DNA polymerase to perform PCR amplification of 98-base template (**T1R**) in the presence of modified **dA^{bdp}TP** and/or **dC^{bdp}TP** and remaining natural dNTPs. We found that only in the case of **dC^{bdp}TP** in combination with the three remaining natural dNTPs, the polymerase was able to amplify the target sequence, although with a decreased efficiency comparing to PCR with all natural dNTPs (Figure 4A). In the 98-bp-long PCR product obtained

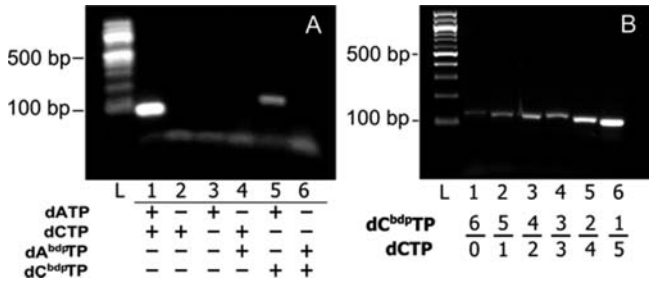

Figure 4. Polymerase chain reaction (PCR) with Bodipy-modified dNTPs. (A) Agarose gel (2%) stained with GelRed showing the results of amplification of 98-bp template **T1R** by KOD XL DNA polymerase in the presence or absence of dATP, dCTP, **dA^{bdp}TP**, and **dC^{bdp}TP**. (B) Agarose gel (2%) stained with GelRed showing the products of PCR amplification of 98bp template **T1R** in the presence of dGTP, dATP, dTTP, and a mixture of dCTP and **dC^{bdp}TP**. The content of dCTP increases from 0% (lane 1) to 83% (lane 6).

in the presence of **dC^{bdp}TP** (Figure 4A, lane 5) virtually all dC residues are substituted by **dC^{bdp}** (except those in primers). This result indicates that **dC^{bdp}** is tolerated by DNA polymerase both as incoming substrate and as modification in the template strand. We also performed mixed PCR using a mixture of **dCTP** and **dC^{bdp}TP** in various proportions. PCR amplification of the 98-nt template showed (Figure 4B) that the yield is larger at higher concentration of the natural triphosphate, in line with observations made by Sawai et al.[59] The increase of the yield is accomplished by an increase in electrophoretic mobility of DNA, which indicates a lower degree of incorporation of modified dNTPs.

The PCR with modified **dN^{X}TP**s generally produces dsDNA containing modifications everywhere along the strand except the primer regions, which remain unlabeled. This situation can be inverted by the use of a tandem of NEAR and PCR to produce DNA containing labels only in the primer regions.[58] We used this approach to synthesize 97-bp-long dsDNA containing Bodipy labels in the primer regions only. At first we designed a 97-nt template for PCR (**T2R**, Table 2) containing only one dC within each of the 18-mer primer regions. Corresponding primers (**ON6** and **ON7**) containing one Bodipy-labeled deoxycytidine each were synthesized by NEAR using templates **T3N** and **T4N**, respectively. The formation of the products **ON6** and **ON7** was observed by gel electrophoresis (Supporting Information Figure S6). Semipreparative NEAR (500 μL scale) followed by RP HPLC purification gave us ca. 1 nmol of each Bodipy-labeled primer. PCR was performed using template **T2R**, primers **ON6** and **ON7** in the presence of four natural dNTPs. We also performed PCR with 7-ethynyl-7-deaza-deoxyadenosine 5′-*O*-triphosphate (**dA^{E}TP**) and three natural dNTPs to generate the DNA bearing multiple ethynyl groups along the strand in addition to the Bodipy labels in the primer regions (Figure 5A). Both PCR worked well producing double-stranded DNA of desired lengths as was evidenced by the electrophoresis in agarose gel stained with intercalating dye (Figure 5B). The presence of Bodipy-

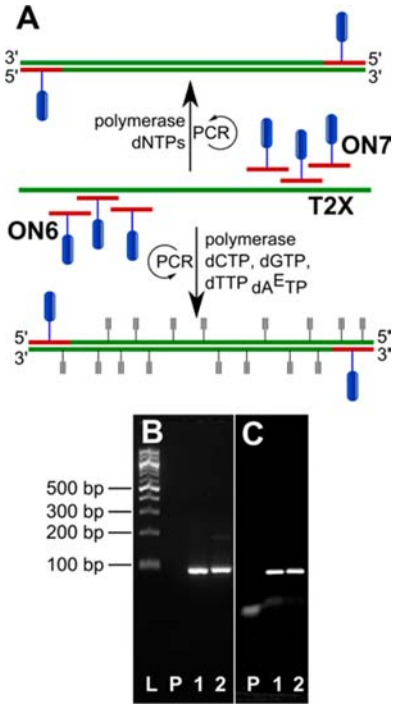

Figure 5. (A) Scheme of PCR reactions with fluorescently labeled primers **ON6** and **ON7**; the primers were obtained by NEAR. (B) Agarose gel (2%, stained with GelRed) showing the PCR producs; L = DNA ladder; P = mixture of primers (**ON6**+**ON7**); lane 1 = PCR with four natural dNTPs; lane 2 = PCR with **dA^{E}TP**, **dTTP**, **dGTP**, and **dCTP**. (C) The PCR product visualized by intrinsic fluorescence of Bodipy fluorophore (ex = 488 nm).

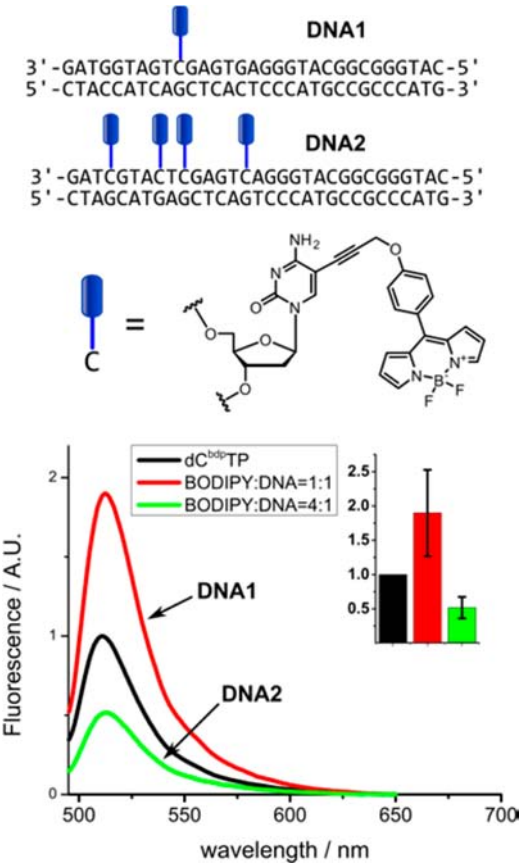

Figure 6. Sequences of dsDNA samples labeled by Bodipy fluorophores in proportion of 1:1 (**DNA1**) and 1:4 (**DNA2**). Uncorrected emission spectra of **DNA1** (red) and **DNA2** (green) in comparison with the spectrum of **dC^{bdp}TP**; absorbance of the samples was identical (0.08) at the excitation wavelength (480 nm). The inset shows quantum yields of **DNA1** and **DNA2** normalized to the quantum yield of **dC^{bdp}TP**; error bars show SD of the mean for n = 3.

fluorophores within the primers and products was confirmed by fluorescence readout of the gel using 488 nm laser for excitation (Figure 5C).

Like other organic dyes featuring a small Stokes shift, Bodipy dyes are known to suffer from homo-FRET self-quenching when multiple fluorophores are attached to one biomolecule.[62−64] In view of this fact we addressed the possible self-quenching of Bodipy within DNA via the homo-FRET mechanism. We prepared dsDNA samples by PEX bearing either one (**DNA1**) or four (**DNA2**) Bodipy-modified dC analogs **dC^{bdp}**. **DNA1** and **DNA2** were prepared by using primer **P1X** and templates **T2X** and **T1X** (Table 2), respectively. Fluorescence spectra as well as quantum yields of these samples were compared with those of **dC^{bdp}TP** (Figure 6). We found that the shapes of the spectra are the same for all three substances, whereas variations in quantum yields were observed. No quenching comparing with the triphosphate was observed for monolabeled **DNA1**; at the same time a 2-fold decrease in quantum yield was observed for multiply labeled **DNA2**. The latter sequence contains four Bodipy fluorophores located at positions +2, +7, +9, and +13 of the elongated primer. Since typical B-DNA conformation contains 10.5 nucleobases per turn[65] (36 Å), the distance between edge fluorophores is approximately 38−40 Å. This distance is smaller than the homotransfer Förster radius for Bodipy dyes which is approximately 57 Å.[64] It makes self-quenching for **DNA2** probable. The observed efficiency of quenching for **DNA2** (~50%) is in reasonable agreement with Bodipy-homotransfer quenching observed on proteins, as, for example, for Bodipy-labeled avidin (70% quenching, ~30 Å between fluorophores).[64] One can speculate that for lower density of DNA labeling (<1 **dC^{bdp}** per 15−20 bp) the homo-FRET quenching will be lower due to its strong dependence on the distance ($\sim r^{-6}$). Altogether, these results obtained for densely labeled **DNA2** indicate that the homo-FRET self-quenching of the Bodipy fluorophores does not seem to be a problem if **dC^{bdp}** is used for labeling of either short or long DNA.

CONCLUSIONS

To conclude, we have synthesized and characterized two new deoxynucleoside triphosphate analogues bearing Bodipy-fluorophore attached via short flexible linker. The modified **dC^{bdp}TP** is well accepted by KOD XL and Bst DNA polymerases in primer extension experiments, by *Vent(exo−)* polymerase in nicking enzyme amplification reaction, and by KOD XL polymerase in polymerase chain reaction. On the other hand, **dA^{bdp}TP** is a worse substrate for polymerases, as it is accepted only by KOD XL polymerase in primer extension (which is surprising when comparing to other 7-substituted 7-deazaadenine dNTPs which were found[26] to be superior substrates even in the presence of natural dATP). The fluorescence of Bodipy dyes is not significantly quenched upon incorporation into DNA, which makes them prospective for labeling of DNA in molecular and cell biology.

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EXPERIMENTAL SECTION

General Remarks. Reagents and solvents were purchased from Sigma-Aldrich and Alfa Aesar and were used as supplied. 5-Iodo-2′-deoxycytidine was purchased from Berry & Associates. Known compounds were prepared according to literature procedures: 4,4-difluoro-8-(4-hydroxyphenyl)-4-bora-3*a*,4*a*-diaza-*s*-indacene (**1**),[49,66] 7-iodo-7-deaza-2′-deoxyadenosine (**dA^{I}**),[67] 2′-deoxy-7-ethynyl-7-deazaadenosine-5′-*O*-triphosphate (**dA^{E}TP**).[68] The reactions were monitored by thin-layer chromatography using Merck silica gel 60 F254 plates and visualized by UV (254 and 356 nm). Column chromatography purification was performed using silica gel (40−63 μm). NMR spectra were measured on a Bruker AVANCE 500 (^{1}H at 500.0 MHz, ^{13}C at 125.7 MHz, and ^{31}P at 202.3 MHz) NMR spectrometer in $CDCl_3$, DMSO-d_6, or D_2O solutions. Chemical shifts (in ppm, δ scale) were referenced to the residual solvent signal in ^{1}H spectra ($\delta(CHCl_3)$ = 7.26 ppm, $\delta(CHD_2SOCD_3)$ = 2.5 ppm) or to the solvent signal in ^{13}C spectra ($\delta(CDCl_3)$ = 77.0 ppm, $\delta((CD_3)_2SO)$ = 39.7 ppm). 1,4-Dioxane was used as an internal standard for D_2O solutions (3.75 ppm for ^{1}H and 69.3 ppm for ^{13}C). ^{31}P NMR spectra were referenced to phosphate buffer signal (2.35 ppm). Coupling constants (*J*) are given in Hz. The complete assignment of ^{1}H and ^{13}C signals was performed by an analysis of the correlated homonuclear H,H-COSY, and heteronuclear H,C-HSQC, and H,C-HMBC spectra. High resolution mass spectra were measured on a LTQ Orbitrap XL instrument using ESI technique.

Synthetic oligonucleotides (primers and templates for PEX, NEAR, and PCR, Table 2) were purchased from Generi Biotech (Czech Republic) and Sigma-Aldrich. Double-stranded 100bp DNA ladder was purchased from New England Biolabs. Single-stranded DNA ladder (10, 20, 50, 100 bases) was prepared as described previously.[58] *Vent(exo−)* DNA polymerase (2U/μL), *Bst* DNA polymerase Large Fragment (8U/μL), 9°N$_m$ DNA polymerase (2U/μL), nicking endonuclease *Nt.BstNBI* (10U/μL), corresponding reaction buffers, as well as natural nucleoside triphosphates (**dATP**, **dCTP**, **dGTP**, **dTTP**) were purchased from New England Biolabs. KOD XL DNA polymerase (2.5 U/μL) and corresponding reaction buffer were obtained from Merck Millipore. DyNAzyme II DNA Polymerase (2 U/μL) and Phusion High-Fidelity DNA Polymerase (2U/μL) were from Finnzymes. *Pwo* DNA polymerase (1 U/μL) was purchased from Peqlab. Streptavidin magnetic particles were obtained from Roche. All solutions for biochemical reactions were prepared using Milli-Q water. Primers for analytical primer extension experiments were labeled using T4 polynucleotide kinase (New England Biolabs) and [γ^{32}P]-ATP (Institute of Isotopes Co., Ltd.; Hungary) according to standard techniques. Radioactive gels were visualized by phosphorimaging using Storage Phosphor Screens (GE Healthcare) and Typhoon 9410 imager (Amersham Biosciences). Properties of ODNs (ε_{260}, M_w) were estimated using the OligoCalc.[69] Mass spectra of oligonucleotides were measured by MALDI-TOF, on UltrafleXtreme mass spectrometer (Bruker Daltonics, Germany), with 1 kHz smartbeam II laser.

Chemicals and spectroscopy grade solvents for fluorescence measurements were purchased from Sigma-Aldrich and Alfa-Aesar. Stock solutoin of **2** was prepared in DMSO; stock solutions of **dGMP**, **dA^{bdp}TP**, and **dC^{bdp}TP** were prepared in water. UV−visible spectra of individual compounds were measured on a Cary 100 UV−vis spectrometer (Agilent Technologies). UV−vis spectra of oligonucleotides were measured on a Nanodrop 1000 spectrophotometer (Thermo Scientific). Fluorescence spectra were measured on a Jasco 6600 spectrofluorimeter.

4,4-Difluoro-8-[4-(propargyloxy)phenyl]-4-borata-3*a*-azonia-4*a*-aza-*s*-indacene (2). Potassium carbonate (861 mg, 3 equiv) and propargyl bromide (80 wt % toluene solution, 0.3 mL, 1.6 equiv) were added to a stirred solution of **1** (591 mg, 2.08 mmol) in dry acetonitrile (16 mL). The reaction mixture was stirred at 70 °C until the complete consumption of the starting material was evidenced by TLC. Then the reaction mixture was cooled down to room temperature and portioned between water and dichloromethane; the organic layer was separated, and the water layer was extracted with dichloromethane (2×). The combined organic layers were dried over $MgSO_4$, filtered, and concentrated on a rotavapor. The crude product was purified by silica gel column chromatography eluted by ethyl acetate in hexane (0−20%) to give the product as orange solid (575 mg, 86%). ^{1}H NMR (500.0 MHz, $CDCl_3$): 2.60 (t, 1H, ^4J = 2.4, HC≡C−); 4.80 (d, 2H, ^4J = 2.4, CH_2O); 6.55 (ddd, 2H, $J_{4,3}$ = 4.2, $J_{4,5}$ = 1.9, $J_{H,F}$ = 0.8, H-4-pyrr); 6.97 (bd, 2H, $J_{3,4}$ = 4.2, H-3-pyrr); 7.14 (m, 2H, H-*o*-phenylene); 7.56 (m, 2H, H-*m*-phenylene); 7.93 (bs, 2H, H-5-pyrr). ^{13}C NMR (125.7 MHz, $CDCl_3$): 55.94 (CH_2O); 76.26 (HC≡C−); 77.82 (HC≡**C**−); 114.89 (CH-*o*-phenylene); 118.35 (CH-4-pyrr); 127.11 (C-*p*-phenylene); 131.39 (CH-3-pyrr); 132.29 (CH-*m*-phenylene); 134.82 (C-2-pyrr); 143.60 (CH-5-pyrr); 147.09 (C-pyrr); 159.88 (C-*i*-phenylene). HRMS: calculated for $C_{18}H_{14}BN_2OF_2$ $[M + H]^+$: 323.1167; found: 323.1156.

7-{[4-(4,4-Difluoro-4-borata-3*a*-azonia-4*a*-aza-*s*-indacen-8-yl)phenoxy]ethynyl}-7-deaza-2′-deoxyadenosine (dA^{bdp}). Dry DMF (2.5 mL) was added to a flask containing 7-iodo-7-deaza-2′-deoxyadenosine (110 mg, 0.29 mmol), Bodipy-acetylene **2** (0.35 mmol), $PdCl_2(PPh_3)_2$ (10 mg, 5% mol), CuI (3 mg, 5% mol), and the mixture was purged-and-refilled with argon for 3−4 times. Triethylamine (0.1 mL, 0.725 mmol) was added via syringe and the mixture was stirred at 80 °C until complete consumption of the starting nucleoside as monitored by TLC (ca. 1 h). Then the mixture was concentrated on a rotavap and purified by silica gel column chromatography eluted with methanol in dichloromethane (0−10%) to give the desired nucleoside as orange solid (95 mg, 57%). ^{1}H NMR (500.0 MHz, DMSO-d_6): 2.14 (ddd, 1H, J_{gem} = 13.1, $J_{2'b,1'}$ = 6.0, $J_{2'b,3'}$ = 2.9, H-2′b); 2.46 (ddd, 1H, J_{gem} = 13.1, $J_{2'a,1'}$ = 8.0, $J_{2'a,3'}$ = 5.7, H-2′a); 3.51 (ddd, 1H, J_{gem} = 11.8, $J_{5'b,OH}$ = 5.8, $J_{5'b,4'}$ = 4.3, H-5′b); 3.57 (ddd, 1H, J_{gem} = 11.8, $J_{5'a,OH}$ = 5.3, $J_{5'a,4'}$ = 4.3, H-5′a); 3.82 (td, 1H, $J_{4',5'}$ = 4.3, $J_{4',3'}$ = 2.6, H-4′); 4.33 (m, 1H, H-3′); 5.06 (dd, 1H, $J_{OH,5'}$ = 5.8, 5.3, OH-5′); 5.25 (s, 2H, CH_2O); 5.26 (d, 1H, $J_{OH,3'}$ = 4.1, OH-3′); 6.47 (dd, 1H, $J_{1',2'}$ = 8.0, 6.0, H-1′); 6.68 (dd, 2H, $J_{4,3}$ = 4.2, $J_{4,5}$ = 1.9, H-4-pyrr); 7.06 (dd, 2H, $J_{3,4}$ = 4.2, $J_{3,5}$ = 0.9, H-3-pyrr); 7.31 (m, 2H, H-*o*-phenylene); 7.69 (m, 2H, H-*m*-phenylene); 7.83 (s, 1H, H-6); 8.10 (bs, 2H, H-5-pyrr); 8.12 (s, 1H, H-2). ^{13}C NMR (125.7 MHz, DMSO-d_6): 40.04 (CH_2−2′); 57.05 (CH_2O); 61.99 (CH_2−5′); 71.07 (CH-3′); 80.72 (−**C**≡C−CH_2−); 83.44 (CH-1′); 86.97 (−C≡**C**−CH_2−); 87.74 (CH-4′); 93.94 (C-5); 102.31 (C-4a); 115.38 (CH-*o*-phenylene); 119.24 (CH-4-pyrr); 126.19 (C-*p*-phenylene); 127.49 (CH-6); 131.81 (CH-3-pyrr); 132.84 (CH-*m*-phenylene); 134.21 (C-2-pyrr); 144.21 (CH-5-pyrr); 147.05 (C-pyrr); 149.49 (C-7a); 152.98 (CH-2); 157.59 (C-4); 160.25 (C-*i*-phenylene). HRMS:

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calculated for $C_{29}H_{25}O_4N_6BF_2Na$ $[M + Na]^+$: 593.1890; found: 593.1894.

5-{[4-(4,4-Difluoro-4-borata-3*a*-azonia-4*a*-aza-*s*-indacen-8-yl)phenoxy]ethynyl}-2′-deoxycytidine (dC^{bdp}). Dry DMF (6 mL) was added to a flask containing 5-iodo-2′-deoxycytidine (274 mg, 0.77 mmol), Bodipy-acetylene **2** (300 mg, 0.93 mmol), $PdCl_2(PPh_3)_2$ (27 mg, 5% mol), CuI (7 mg, 5% mol), and the mixture was purged-and-refilled with argon for 3−4 times. Triethylamine (0.22 mL, 1.6 mmol) was added via syringe and the mixture was stirred at 80 °C until the complete consumption of the starting nucleoside as monitored by TLC (ca. 1 h). Then the reaction mixture was concentrated on a rotavap and purified by silica gel column chromatography eluted with methanol in dichloromethane (0−15%) to give the desired nucleoside as an orange solid (212 mg, 50%). ^{1}H NMR (500.0 MHz, DMSO-d_6): 1.99 (ddd, 1H, J_{gem} = 13.1, $J_{2'b,1'}$ = 6.8, $J_{2'b,3'}$ = 6.1, H-2′b); 2.15 (ddd, 1H, J_{gem} = 13.1, $J_{2'a,1'}$ = 6.1, $J_{2'a,3'}$ = 3.8, H-2′a); 3.55, 3.62 (2 × ddd, 2 × 1H, J_{gem} = 11.9, $J_{5',OH}$ = 5.2, $J_{5',4'}$ = 3.5, H-5′); 3.79 (q, 1H, $J_{4',3'} = J_{4',5'}$ = 3.5, H-4′); 4.20 (m, 1H, H-3′); 5.09 (t, 1H, $J_{OH,5'}$ = 5.2, OH-5′); 5.14 (s, 2H, CH_2O); 5.20 (d, 1H, $J_{OH,3'}$ = 4.3, OH-3′); 6.09 (dd, 1H, $J_{1',2'}$ = 6.8, 6.1, H-1′); 6.69 (dd, 2H, $J_{4,3}$ = 4.2, $J_{4,5}$ = 2.0, H-4-pyrr); 7.04 (bs, 1H, $NH_a\mathbf{H}_b$); 7.07 (dd, 2H, $J_{3,4}$ = 4.2, $J_{3,5}$ = 1.1, H-3-pyrr); 7.31 (m, 2H, H-*o*-phenylene); 7.68 (m, 2H, H-*m*-phenylene); 7.80 (bs, 1H, $N\mathbf{H}_aH_b$); 8.11 (bs, 2H, H-5-pyrr); 8.27 (s, 1H, H-6). ^{13}C NMR (125.7 MHz, DMSO-d_6): 41.04 (CH_2−2′); 56.97 (CH_2O); 61.04 (CH_2−5′); 70.08 (CH−3′); 79.65 (−**C**≡C−CH_2−); 85.63 (CH-1′); 87.64 (CH-4′); 88.65 (C-5); 90.04 (−C≡**C**−CH_2−); 115.32 (CH-*o*-phenylene); 119.22 (CH-4-pyrr); 126.14 (C-*p*-phenylene); 131.76 (CH-3-pyrr); 132.74 (CH-*m*-phenylene); 134.19 (C-2-pyrr); 144.19 (CH-5-pyrr); 145.63 (CH-6); 147.01 (C-pyrr); 153.49 (C-2); 160.19 (C-*i*-phenylene); 164.50 (C-4). HRMS: calculated for $C_{27}H_{24}O_5N_5BF_2Na$ $[M + Na]^+$: 570.1731; found: 570.1733.

7-{[4-(4,4-Difluoro-4-borata-3*a*-azonia-4*a*-aza-*s*-indacen-8-yl)phenoxy]ethynyl}-7-deaza-2′-deoxyadenosine 5′-*O*-triphosphate (dA^{bdp}TP). Dry trimethyl phosphate (1.5 mL) was added to argon purged flask containing nucleoside **dA^{bdp}** (89 mg, 156 μmol). The resulting solution was cooled down to 0 °C and solution of $POCl_3$ (21 μL, 218 μmol) in dry trimethyl phosphate (0.5 mL) was added dropwise. After 4 h 30 min stirring at 0 °C, a solution of $(Bu_3NH)_2H_2P_2O_7$ (342 mg, 624 μmol) and Bu_3N (150 μL, 264 μmol) in dry DMF (1.5 mL) was added. The solution was stirred for another 60 min at 0 °C and then quenched by the addition of 1 M TEAB (2 mL). The mixture was concentrated on a rotavap; the residue was coevaporated with distilled water three times. After that the crude mixture was diluted with water to a total volume of ca. 10 mL and filtered from unreacted nucleoside. The crude aqueous solution was purified by semipreparative HPLC using a linear gradient of methanol (5−90%) in 0.1 M TEAB buffer. The appropriate fractions were combined and evaporated on a rotavap. The viscous orange oil was coevaporated with distilled water three times. The product was converted to sodium salt on an ion-exchange column (Dowex 50WX8 in Na^+ cycle) and freeze-dried. The product was obtained as orange solid (8 mg, 6%). ^{1}H NMR (500.0 MHz, D_2O, pD = 7.1, phosphate buffer): 2.20, 2.29 (2 × bm, 2 × 1H, H-2′); 4.04 (bm, 2H, H-5′); 4.15 (bm, 1H, H-4′); 4.51 (bm, 1H, H-3′); 4.86 (bs, 2H, CH_2O); 6.14 (bs, 2H, H-4-pyrr); 6.18 (bt, 1H, $J_{1',2'}$ = 6.6, H-1′); 6.30 (bs, 2H, H-3-pyrr); 6.79 (bm, 2H, H-*o*-phenylene); 6.91 (bm, 2H, H-*m*-phenylene); 7.28 (bs, 1H, H-6); 7.60 (bs, 2H, H-5-pyrr); 7.90 (s, 1H, H-2). ^{13}C NMR (125.7 MHz, D_2O, pD = 7.1, phosphate buffer): 40.60 (CH_2−2′); 59.20 (CH_2O); 68.43 (d, $J_{C,P}$ = 5.6, CH_2−5′); 73.49 (CH-3′); 82.68 (−**C**≡C−CH_2−); 85.39 (CH-1′); 87.50 (d, $J_{C,P}$ = 7.4, CH-4′); 89.63 (−C≡**C**−CH_2−); 98.03 (C-5); 105.36 (C-4a); 117.37 (CH-*o*-phenylene); 121.37 (CH-4-pyrr); 128.77 (C-*p*-phenylene); 129.38 (CH-6); 134.06 (CH-3-pyrr); 135.21 (CH-*m*-phenylene); 136.50 (C-2-pyrr); 145.93 (CH-5-pyrr); 148.72 (C-pyrr); 150.84 (C-7a); 154.42 (CH-2); 159.30 (C-4); 162.30 (C-*i*-phenylene). ^{31}P{^{1}H} NMR (202.3 MHz, D_2O, pD = 7.1, phosphate buffer): −20.60 (t, J = 18.8, P_β); −9.83 (d, J = 18.8, P_α); −6.68 (d, J = 18.8, P_γ). HRMS: calculated for $C_{29}H_{24}O_{13}N_6BF_2P_3Na_3$ $[M-Na]^-$: 875.0374; found: 875.0364.

5-{[4-(4,4-Difluoro-4-borata-3a-azonia-4a-aza-*s*-indacen-8-yl)phenoxy]ethynyl}-2′-deoxycytidine 5′-*O*-triphosphate (dC^{bdp}TP). Dry trimethyl phosphate (1 mL) was added to an argon-purged flask containing nucleoside **dC^{bdp}** (37 mg, 67.6 μmol). The resulting solution was cooled down to 0 °C and a solution of $POCl_3$ (32 μL, 107 μmol) in dry trimethyl phosphate (0.5 mL) was added dropwise. After 3 h stirring at 0 °C, a solution of $(Bu_3NH)_2H_2P_2O_7$ (185 mg, 337 μmol) and *n*-Bu_3N (72 μL, 303 μmol) in dry DMF (0.9 mL) was added. The solution was stirred for another 60 min at 0 °C and then quenched by the addition of 1 M TEAB (2 mL). The mixture was concentrated on a rotavap; the residue was coevaporated with distilled water three times. After that the crude mixture was diluted with water to a total volume of ca. 10 mL and filtered from unreacted nucleoside. The crude aqueous solution was purified by semipreparative HPLC using a linear gradient of methanol (5−90%) in 0.1 M TEAB buffer. The appropriate fractions were combined and evaporated on a rotavap. The viscous orange oil was coevaporated with distilled water three times. The product was converted to sodium salt on an ion-exchange column (Dowex 50WX8 in Na^+ cycle) and freeze-dried. The product was obtained as orange solid (8.4 mg, 15%). ^{1}H NMR (500.0 MHz, D_2O, pD = 7.1, phosphate buffer, ref(dioxane) = 3.75 ppm): 2.17 (dt, 1H, J_{gem} = 14.3, $J_{2'b,1'} = J_{2'b,3'}$ = 6.5, H-2′b); 2.36 (ddd, 1H, J_{gem} = 14.3, $J_{2'a,1'}$ = 6.5, $J_{2'a,3'}$ = 4.7, H-2′a); 4.09−4.22 (m, 3 × 1H, H-4′,5′); 4.53 (m, 1H, H-3′); 5.14 (bs, 2H, CH_2O); 6.10 (t, 1H, $J_{1',2'}$ = 6.5, H-1′); 6.58 (bm, 2H, H-4-pyrr); 6.90 (bd, 2H, $J_{3,4}$ = 3.5, H-3-pyrr); 7.25 (m, 2H, H-*o*-phenylene); 7.54 (m, 2H, H-*m*-phenylene); 7.94 (bs, 2H, H-5-pyrr); 8.09 (s, 1H, H-6). ^{13}C NMR (125.7 MHz, D_2O, ref(dioxane) = 69.3 ppm): 10.94 ($\mathbf{C}H_3CH_2N$); 41.86 (CH_2−2′); 49.33 ($CH_3\mathbf{C}H_2N$); 59.31 (CH_2O); 67.56 (d, $J_{C,P}$ = 5.3, CH_2−5′); 72.46 (CH-3′); 80.85 (cyt-**C**≡C−CH_2−); 88.20 (d, $J_{C,P}$ = 8.9, CH-4′); 88.94 (CH-1′); 93.36 (cyt-C≡**C**−CH_2−); 94.00 (C-5); 117.98 (CH-*o*-phenylene); 121.78 (CH-4-pyrr); 129.30 (C-*p*-phenylene); 134.85 (CH-3-pyrr); 135.52 (CH-*m*-phenylene); 136.99 (C-2-pyrr); 146.29 (CH-5-pyrr); 148.12 (CH-6); 149.64 (C-pyrr); 158.37 (C-2); 162.32 (C-*i*-phenylene); 167.47 (C-4). ^{31}P NMR (202.4 MHz, D_2O, pD = 7.1, phosphate buffer, ref(phosphate buffer) = 2.35 ppm): −21.38 (bm, P_β); −10.46 (bd, J = 18.7, P_α); −6.78 (bd, J = 14.8, P_γ). HRMS: calculated for $C_{27}H_{26}O_{14}N_5BF_2P_3$ $[M-4Na+3H]^-$: 786.0756; found: 786.0757.

PEX on Analytical Scale. The reaction mixture (20 μL) contained 5′-^{32}P-labeled primer (3 μM, 1 μL), unlabeled primer (100 μM, 0.77 μL), template (100 μM, 0.8 μL), corresponding DNA polymerase (0.23 U of KOD XL; 0.72 U of *Bst LF*; 0.19 U of Vent(exo-), 9°N_m, DyNAzyme II, Phusion, Pwo), either natural or modified dNTPs (4 mM each, 0.4 μL), and 10× concentrated reaction buffer (2 μL) supplied by the manufacturer with the enzyme. The reaction mixture was

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incubated at 60 °C for 40 min. The reaction was stopped by the addition of PAGE stop solution (40 μL; 80% [v/v] formamide, 20 mM EDTA, 0.025% [w/v] bromophenol blue, 0.025% [w/v] xylene cyanol), and denatured at 95 °C for 5 min. Aliquots (3 μL) were subjected to vertical electrophoresis in 12.5% denaturing polyacrylamide gel containing 1× TBE buffer (pH 8.0) and 7 M urea at 42 mA for 50 min. The gel was dried in vacuo (80 °C, 70 min) and visualized by phosphor imaging autoradiography.

PEX on Semipreparative Scale. The reaction mixture (250 μL) contained either KOD XL DNA polymerase (2.5 U/μL, 1.14 μL) or Bst DNA polymerase Large Fragment (8 U/μL, 1 μL), 10× concentrate of corresponding reaction buffer provided by the manufacturer (25 μL), three natural and one modified dNTPs (4 mM each, 5 μL), primer (100 μM, 10 μL), and template (100 μM, 10 μL). The reaction mixture was incubated for 40 min at 60 °C in a thermal mixer. The reaction was stopped by cooling to 4 °C. The modified dsDNA was purified using QIAquick Nucleotide Removal Kit (QIAGEN); reaction mixture was divided to two columns; the product was eluted from each column by adding 100 μL of elution buffer EB (10 mM Tris·HCl, pH 8.5). Concentration of the resulting DNA solutions was determined on a NanoDrop.

PEX on Semipreparative Scale with Magnetic Separation. The reaction mixture (100 μL) containing KOD XL DNA polymerase (2.5 U/μL, 0.45 μL), 10× concentrate of the reaction buffer provided by the manufacturer of the enzyme (10 μL), biotinylated template (100 μM, 4 μL), primer (100 μM, 4 μL), and dNTPs (dGTP, dTTP, dATP, **dC^{bdp}TP**; 4 mM each, 5 μL) was incubated at 60 °C for 40 min. The reaction was stopped by cooling to 4 °C.

Streptavidine magnetic particles (120 μL) were washed with binding buffer (3 × 300 μL, 10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.5). The PEX solution (100 μL) and binding buffer (100 μL) were added. The mixture was incubated in a thermal mixer for 35 min at 15 °C and 1200 rpm. Then the magnetic beads were washed with wash buffer (3 × 300 μL, 10 mM Tris, 1 mM EDTA, 500 mM NaCl, pH 7.5) and water (4 × 300 μL). Then water (100 μL) was added and the sample was denatured for 2 min at 40 °C and 900 rpm. The beads were collected on a magnet and the solution containing desired ssDNA was transferred into a clean vial. The product was analyzed by MALDI-TOF mass spectrometry.

NEAR on Analytical Scale. The reaction mixture (50 μL) contained the template (0.125 μM), primer (0.125 μM), modified **dC^{bdp}TP** (187.5 μM), natural dNTPs (dGTP, dTTP, dATP; 125 μM each), 1× ThermoPol buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl/pH 8.8/, 0.1% Triton-X-100, and 2 mM $MgSO_4$), and 0.5× NEBuffer 3 (50 mM NaCl, 25 mM Tris-HCl/pH 7.9/, 5 mM $MgCl_2$, and 0.5 mM DTT), *Vent(exo−)* DNA polymerase (7.5 U), and nickase *Nt.BstNBI* (30 U). The reaction mixture was incubated in a thermal mixer at 55 °C, 400 rpm for 3 h. The reaction was stopped by cooling to 4 °C. The products were analyzed by agarose gel electrophoresis using 4% agarose gels stained with GelRed (Biotium). Samples for electrophoresis were prepared by mixing 1.6 μL of 6× DNA loading dye (Thermo Scientific) and 8 μL of the reaction mixture or ssDNA ladder. The gel was run for 60 min at 120 V. Gels were imaged using electronic dual wave transilluminator equipped with UltraCam 8gD digital imaging system (Ultra-Lum). Bodipy fluorophore was imaged on a Typhoon 9410 imager (Amersham Biosciences) using 488 nm laser for excitation. For MALDI-TOF mass spectrometry analysis the reaction mixtures were purified on Illustra MicroSpin G-25 columns (GE Healthcare) to remove the unincorporated nucleotides and buffer salts.

PCR with Modified dNTPs. The reaction mixture (10 μL) contained KOD XL DNA polymerase (2.5 U/μL, 0.1 μL), KOD XL reaction buffer supplied by the manufacturer (10×, 1 μL), primers **P1R** and **P2R** (10 μM; 1 μL of each), 98-mer template **T1R** (1 μM, 0.25 μL). The amount of dNTPs depends on whether only natural dNTPs or Bodipy-modified dNTP(s) together with the remaining natural dNTPs were used. The following amounts were used: positive control, four natural dNTPs (2 mM of each, 0.15 μL); negative control, three natural dNTPs (10 mM of each, 0.6 μL); Bodipy-modified dNTPs, **dA^{bdp}TP** or **dC^{bdp}TP** with remaining natural dNTPs (10 mM of each, 0.6 μL). After the initial denaturation for 3 min at 94 °C, 40 PCR cycles were run under the following conditions: denaturation for 1 min at 94 °C, annealing for 1 min at 51 °C, extension for 2 min 30 s at 72 °C. These PCR processes were terminated with a final extension step for 5 min at 72 °C. The reaction was stopped by cooling to 4 °C. The products were analyzed by agarose gel electrophoresis using 2% agarose gel stained with GelRed (Biotium). Samples for electrophoresis were prepared by mixing 1 μL of 6× DNA Loading Dye (Thermo Scientific) and 5 μL of the reaction mixture. The gel was run for 70 min at 120 V and imaged using electronic dual wave transilluminator equipped with UltraCam 8gD digital imaging system (Ultra-Lum).

NEAR on Semipreparative Scale. The reaction mixture (500 μL) contained the template (0.125 μM), primer (0.125 μM), **dC^{bdp}TP** (187.5 μM), natural dNTPs (dGTP, dTTP, dATP; 125 μM each), 1× ThermoPol buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl/pH 8.8/, 0.1% Triton-X-100, and 2 mM $MgSO_4$), and 0.5× NEBuffer 3 (50 mM NaCl, 25 mM Tris-HCl/pH 7.9/, 5 mM $MgCl_2$, and 0.5 mM DTT), *Vent(exo−)* DNA polymerase (50 U), and nickase *Nt.BstNBI* (280 U). The reaction mixture was incubated at 55 °C, 400 rpm for 3 h. After that the reaction was stopped by cooling to 4 °C, the solution was concentrated on a vacuum concentrator to approximately 100 μL. The viscous concentrate was subjected to HPLC purification on a XBridge OST C18 Column (Waters; 2.5 μm particle size, 4.6 mm × 50 mm). A linear gradient of triethylammonium acetate buffer (TEAA, pH = 7.0) and acetonitrile with flow rate 1 mL/min was used. Mobile phase A corresponds to 0.1 M TEAA in HPLC-grade water, mobile phase B to 0.1 M TEAA in HPLC-grade water/acetonitrile 80/20 (v/v). The gradient started with 50% mobile phase B, going linearly to 80% mobile phase B in 60 min. The fractions containing product were combined and evaporated on a vacuum concentrator. The residue was dissolved in water (40 μL) and UV−vis absorption spectra were measured on a Nanodrop. The products were analyzed by MALDI-TOF mass spectrometry.

PCR with Bodipy-Labeled Primers. Reaction mixture (10 μL) contained KOD XL DNA polymerase (2.5 U/μL, 0.1 μL), 10× concentrated reaction buffer provided by the manufacturer of the polymerase (1 μL), template **T2R** (1 μM, 0.25 μL), primers **ON6** and **ON7** (10 μM each, 1 μL), and dNTPs (1 mM each, 0.4 μL). After the initial denaturation for 2 min at 94 °C, 30 PCR cycles were run under the following conditions: denaturation for 30 s at 94 °C, annealing for 1 min at 40 °C, extension for 1 min at 65 °C. These PCR processes were terminated with a final extension step for 2 min at 65 °C. The PCR products were analyzed by 2% agarose gel electrophoresis.

The samples for electrophoresis were prepared by mixing an aliquot of reaction mixture (5 μM) and 1 μL of 6× DNA Loading Dye (Thermo Scientific). The gel was run for 70 min at 100 V. The gel stained with GelRed was imaged using electronic dual wave transilluminator equipped with UltraCam 8gD digital imaging system (Ultra-Lum). The Bodipy fluorophore was imaged in unstained gel by Typhoon 9410 imager (Amersham Biosciences) using 488 nm laser for excitation.

Measurement of the Quantum Yields. Quantum yields (Φ) of fluorescence were determined by comparison with a standard. We used fluorescein sodium salt in 0.1 M NaOH as a standart (Φ = 0.92 ± 0.03).[70] Excitation wavelenth was 488 nm. Integration range was 490−640 nm. Notice that the quantum yields of this standard reported by different authors are slightly different.[71] Quantum yields were calculated using eq 1.

$$\Phi = \Phi_R \frac{I}{I_R} \frac{\mathrm{abs}_R}{\mathrm{abs}} \frac{n^2}{n_R^2} \quad (1)$$

Here Φ_R is the quantum yield of fluorescein in 0.1 M NaOH solution; I is the integrated fluorescence intensity of the sample; I_R is the integrated fluorescence intensity of the reference; abs and abs_R are the absorbance at 488 nm of the sample and the reference, respectively; n and n_R are the values of the refractive index for the solvents used for the sample and reference, respectively. The optical densities of sample and reference solutions were kept below 0.1 to avoid inner filter effects.

Quenching Studies and Stern−Volmer Plot. A series of solutions containing **dC^{bdp}TP** (1 μM) and **dGMP** (0, 2, 4, 6, or 8 mM) in 10 mM sodium phosphate buffer (pH = 7.4) was prepared. Concentration of **dGMP** stock solution was calculated from UV spectra (ε_{253} = 13 700 M^{-1} cm^{-1}). The samples were excited at 488 nm. Intensity of fluorescence (F) was recorded at the emission maximum. Stern−Volmer plot was obtained by plotting F_0/F vs concentration of the quencher. The data was fit into eq 2.

$$\frac{F_0}{F} = 1 + K_{SV}[\mathrm{dGMP}] \quad (2)$$

Here F_0 and F are the intensity of fluorescence in the absence and presence of the quencher, respectively; K_{SV} is the Stern−Volmer constant; [dGMP] is concentration of **dGMP**.

Fluorescence of Labeled dsDNA. Double-stranded DNA incorporating either one (**DNA1**) or four (**DNA2**) modified **dC^{bdp}** residues was prepared by semipreparative PEX from primer **P1X** and templates **T2X** and **T1X**, respectively (Table 2). Fluorescence of **DNA1** and **DNA2** was compared with **dC^{bdp}TP**. Fluorescence measurements were performed in phosphate buffered saline (10 mM phosphate, pH = 7.4, 100 mM NaCl) using 100 μL quartz cuvettes. Excitation wavelength was 480 nm. Absorbance of the samples at 480 nm was equalized to 0.08 according to UV−vis spectra measured on a Nanodrop.

■ ASSOCIATED CONTENT

Supporting Information

Additional figures; copies of NMR and MALDI spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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