An exceptional fluorescence turn-on nucleoside: Lighting up single-stranded DNA with constant brightness regardless neighboring bases

Jinsi Li [a,b], Lei He [a], Junlin Wen [a], Chenghe Xiong [a], Jingwei Zhou [c], Chenguang Lou [d], Xiaoluo Huang [a], Hui Mei [a] *, Xin Ming [b] *

[a] Shenzhen Key Laboratory of Synthetic Genomics, Guangdong Provincial Key Laboratory of Synthetic Genomics, Key Laboratory of Quantitative Synthetic Biology, Shenzhen Institute of Synthetic Biology, Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences, Shenzhen 518055, China

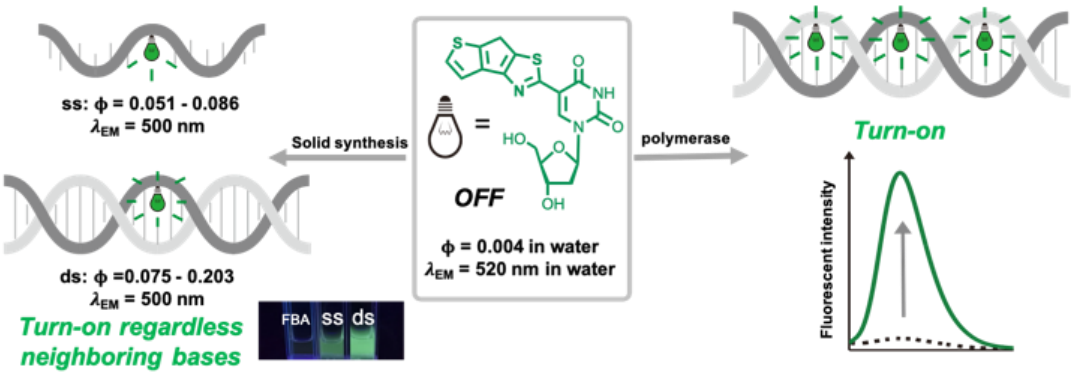
[b] Study on the Structure-Specific Small MolecularDrug in Sichuan Province College Key Laboratory, School of Pharmacy, Chengdu Medical College, Chengdu, Sichuan 610500, P. R. China

[c] Department of Quantum Chemistry, Wecomput Technology Company, Ltd. (Guangzhou), Guangzhou, 100085, P. R. China

[d] Biomolecular Nanoscale Engineering Center, Department of Physics, Chemistry and Pharmacy, University of Southern Denmark, Campusvej 55, 5230 Odense M, Denmark

Corresponding to:
Prof. Xin Ming, Email: mingxin@cmc.edu.cn
Prof. Hui Mei, E-mail: hui.mei@siat.ac.cn

Abstract



Fluorescent nucleobase analogs (FBAs) have proven valuable for studying nucleic acid structure and dynamics. Regrettably, most FBAs exhibit reduced quantum yields when incorporated into DNA, particularly when neighboring residues are present. In this study, we introduce a turn-on nucleoside (**thieno cyclopenta -dU, 3b**) that increases the brightness of single-stranded oligonucleotides by approximately 10-fold compared to the free nucleoside, regardless of neighboring bases. Furthermore, an up to 50-fold increase in brightness is observed during duplex formation. To the best of our knowledge, compound **3b** is the only turn-on type fluorescent nucleoside known to maintain a stable quantum yield after incorporation, and it can be well-accepted by DNA polymerases. These findings highlight the potential of turn-on FBAs for fluorescence sensing applications in enzymatic DNA synthesis and in vivo strand hybridization.

Fluorescent nucleobase analogs (FBAs) have emerged as powerful tools for studying the structure, dynamics, and biomolecular interactions of nucleic acids while maintaining the canonical Watson-Crick base pairing.[1-3]However, despite the development of numerous types of fluorescent base analogs (FBAs) (Figure 1), most of them exhibit only moderate quantum yields in water, which decrease significantly when integrated into DNA and quenched by neighboring residues.[4, 5] For instance, fluorescent pyrimidine nucleoside analogs are prominently quenched when flanked by a G residue in both single-stranded (ss) and double-stranded (ds) DNA due to a photoinduced electron transfer (PET) process.[6] Moreover, monomers in water with high brightness produce a substantial level of background fluorescence, thereby limiting their utility as reporters in living cells.[7] Consequently, the emergence of turn-on FBAs, which remain nonfluorescent as free nucleosides or triphosphates but become much brighter when base-stacked, holds great promise for fluorescence sensing studies in enzymatic DNA synthesis and in vivo strand hybridization. However, only a few turn-on fluorescent nucleosides, such as 5-methoxybenzofuran uridine[8], ^{UDB}T nucleoside[9], ^{DMA}T nucleoside[10] and 8-DEA-tC nucleoside[11], have been identified so far. Herein, our aim is to introduce a new turn-on fluorescence nucleoside that enhances the brightness of single-stranded oligonucleotides by around 10-fold compared to the free nucleoside, independent of neighboring bases. The turn-on mechanism of this new fluorescent nucleoside **3b** is primarily attributed to solvent quenching and excited-state proton transfer, as supported by the theoretical calculations and experimental measurements. The successful incorporation of **3b** triphosphate by DNA polymerases in primer extension experiments and polymerase chain reaction (PCR) emphasizes its potential for applications in fluorescence sensing and in vivo DNA labelling studies. Furthermore, it exhibits the ability to replace specific site or even all natural counterparts in DNA without causing fluorescence quenching.

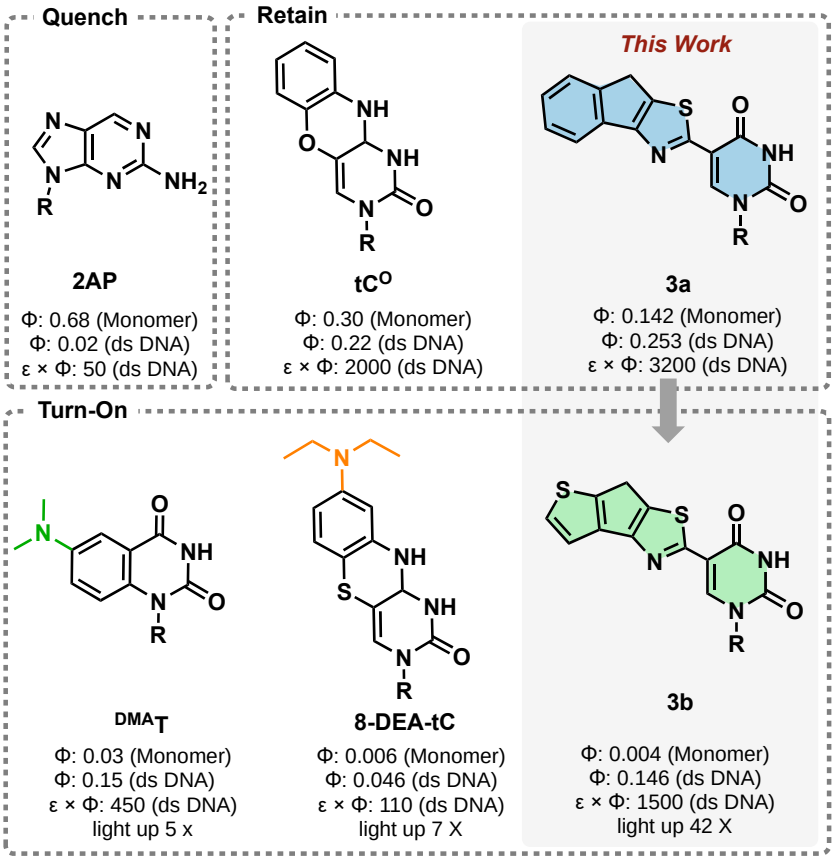
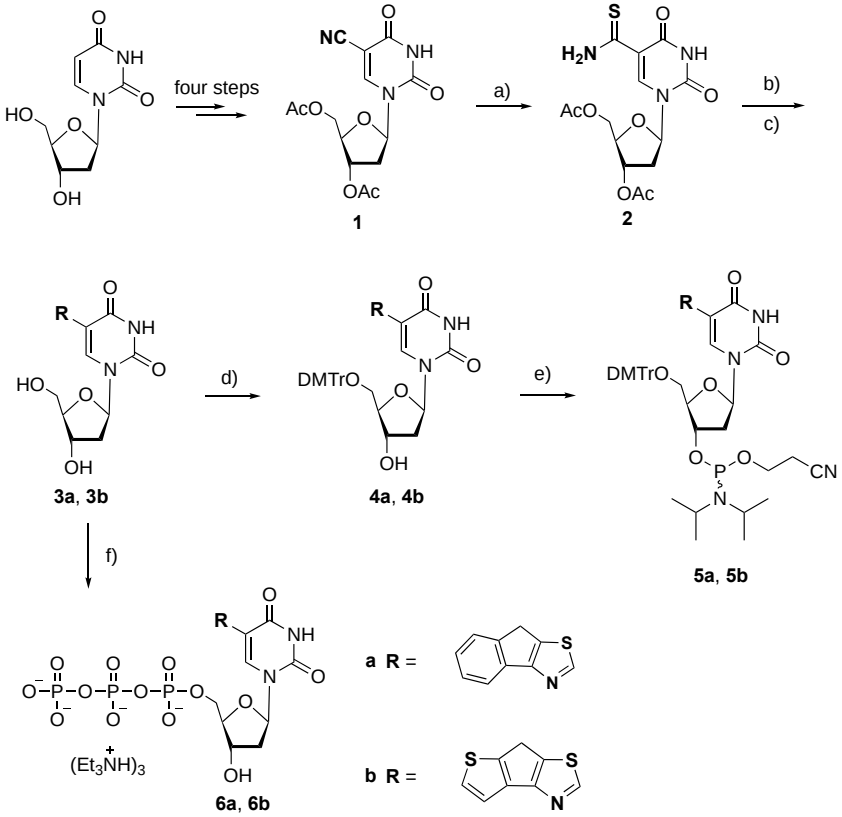


Figure 1. Structure of three types of fluorescent nucleosides and our work. The average brightness and quantum yield of FBAs over various DNA sequence were listed. [4, 10, 12]

Recently, our laboratory has published findings on a novel series of tricyclic thiazolyl-conjugated uridine analogs with exceptional environmental sensitivity.[13] The current study aims to comprehensively investigate the photophysical properties of these FBAs when incorporated into oligonucleotide DNAs. To commence, we synthesized the pivotal intermediate - protected 5-cyano-2'-deoxyuridine 1 from commercially available 2'-deoxyuridine on a multigram scale, yielding 96% over four steps[14, 15] (Supporting Information). Subsequently, compound **2** was synthesized using an enhanced method, resulting in a 77% yield with NaHS treatment. Fluorescent deoxyribonucleosides **3a-b** were then prepared via a Hantzsch reaction using compound **2** and aromatic α-bromocarbonyl compounds. The overall yield for **3a** was 55% (52% for **3b**) achieved in seven steps without the need for chromatographic purifications. The nucleosides were converted into phosphonamidites and triphosphates via conventional methods, as illustrated in Scheme 1. Subsequently, these compounds were utilized for solid-phase oligonucleotide synthesis and enzymatic incorporation in subsequent experiments.

Scheme 1. Synthesis of fluorescent nucleosides, the corresponding phosphoramidites and triphosphates.



Reagents and conditions: (a) NaSH (3 equiv), $Et_2N{\cdot}HCl$ (4.5 equiv), pyridine (6.0 equiv), H_2O (40.0 equiv), DMF, N_2, 80 °C, 5 h, 77%; (b) aromatic α-bromocarbonyl compounds (1.4 equiv), DMF, 105 °C, 0.5 h; (c) NaOH (2.0 equiv), H_2O, EtOH, 25 °C, 0.5 h, 82% (78%); (d) DMTrCl (1.3 equiv), Py, 25 °C, 18 h, 66% (69%) ; (e) 2-cyano ethyl N, N-diisopropyl chlorophosphoroamidite (1.3 equiv), DIPEA (3.0 equiv), CH_2Cl_2, N_2, 25 °C, 20 min, 85% (88%); (f) $POCl_3$ (1.6 equiv), trimethylphosphate, N_2, 0°C, 2h; 0.5 M $(Bu_3NH)_2H_2P_2O_7$ solution in DMF (7.0 equiv), Bu_3N (6.0 equiv) 25°C, 20 min; 2 M triethylammonium bicarbonate buffer (TEAB), 0°C, 1min, 43% (64%).

With fluorescent nucleosides at our disposal, we initially measured the photophysical characteristics of **3a** and **3b** in various solvents with differing polarity, pH levels, and viscosity. Both **3a** and **3b** are solvent and pH dependent fluorescent nucleosides, nearly identical to their ribonucleoside analogues.[13] Analogous to most microenvironment-sensitive fluorescent nucleoside analogues,[4, 5, 16-19] **3a** exhibited negligible differences in quantum yields across diverse solvents (Φem = 0.14–0.25). Significantly, nucleoside **3b** demonstrated a more pronounced fluorescence solvatochromism than **3a**, as evidenced by our findings. In water, **3b** exhibited a substantial reduction in fluorescence (Φem = 0.004), while in organic solvents (DMSO, EtOH, and MeOH), the quantum yield increased dramatically by over 30-fold (Φem = 0.14–0.19). In MeOH, the emission maximum wavelength of **3b** red shifted by 30 nm compared to nucleoside **3a** (λmax, em = 455nm) (Figure 2B). A correction plot of the Stokes Shift in solvents of varying polarity versus E_T^{30} (Reichardt's microscopic solvent polarity parameter)[20] produced a nearly linear correlation for **3a** and **3b**, hinting at their potential intramolecular charge transfer (ICT) character.[18, 21]

To gain insight into the intramolecular electronic transitions and transition probabilities related to the observed polarity-dependent emission spectra, we conducted Density Functional Theory (DFT) calculations (Supporting Information).In the ground-state structure, the HOMO of **3a** or **3b** was found to be delocalized throughout the tricyclic thiazole, while the lowest unoccupied molecular orbital (LUMO) was localized to the pyrimidine in the excited-state structure, as demonstrated in Figure 2. This observation indicated a push-pull character, showing charge transfer from the benzene group (**3a**) or thiophene group (**3b**) to pyrimidine upon transition from HOMO to LUMO. The expanded thiazolyl-2'-deoxyuridine family displayed a notable push-pull core, with the benzene group (**3a**) and thiophene group (**3b**) serving as electron donors, and pyrimidine as an electron acceptor. Compounds featuring a donor structure with strong electron-donating ability and an acceptor structure with strong electron-accepting ability are anticipated to undergo significant quenching.[22] The significantly low initial quantum yields of **3b** in water were greatly influenced by the intramolecular charge transfer (ICT).

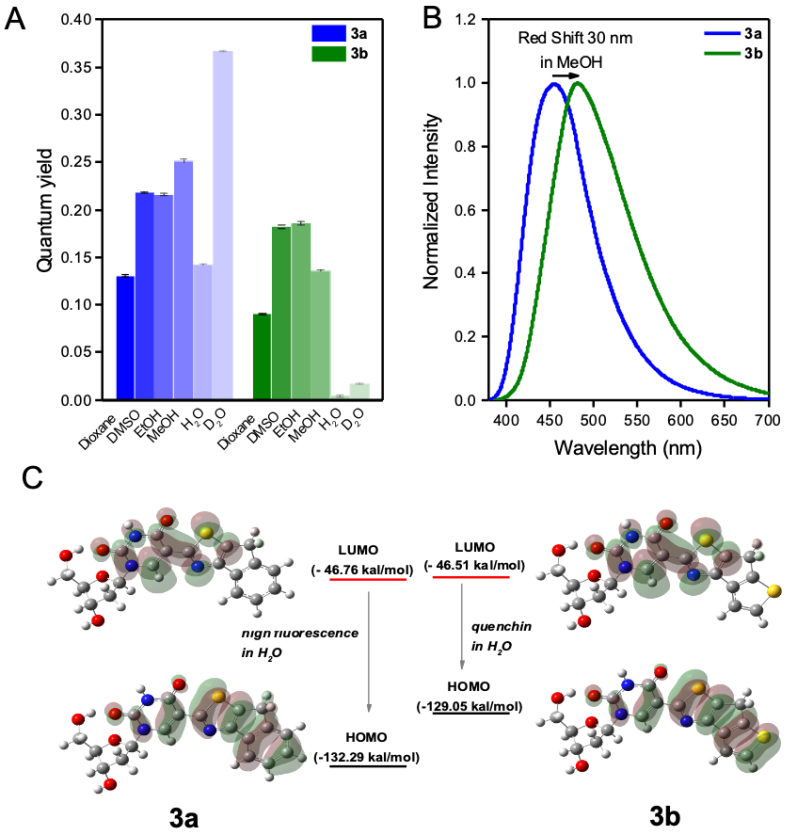


Figure 2. (A) Quantum yields of fluorescent nucleosides in different solvents. (B) Normalized fluorescence spectra of two fluorescent nucleosides in MeOH (C)HOMO and LUMO of the representative fluorophores **3a** and **3b**.

Inspired by the distinctive fluorescence properties of nucleoside monomers, we incorporated them into oligonucleotides for a study on duplex stability, geometry, and fluorescence. To accomplish this, we incorporated

FBAs **3a** and **3b** into the central position of the oligonucleotide sequence (ACT CAX **3** YGC CGT) via standard solid-phase supported synthesis (XY were neighboring bases). We measured the impact of these FBAs on the DNA structure by quantifying the circular dichroism (CD) and melting temperature (Tm) of the modified sequences when annealed to their complements. The results indicated that all the modified sequences maintained similar canonical B-form structures (Supporting Information). Furthermore, the Tm of the modified DNAs showed a slight decrease of -3.0 (for **3a**) and -2.4 (for **3b**) on average. These CD and thermal denaturation results suggest that the 5-position thiazolyl modification aligns well with the major groove and does not significantly disrupt the secondary structure of DNA, consistent with findings on other 5-position modified nucleosides.[23-25]

Table 1. Properties of Single-Stranded (Double-Stranded)DNA[a]

Sequence Name[b]	λ_{ex} (nm) ss/ds	Ɛ (cm^{-1} M^{-1}) ss(ds)	λ_{em} (nm) ss (ds)	Φ[c] ss (ds)	Ɛ×Φ (cm^{-1} M^{-1}) ss (ds)	T_m (°C)/ ΔT_m(°C)
AA-**3b**	375 (375)	10400 (11300)	500 (500)	0.073 (0.104)	759 (1175)	52.7/-2.8
AT-**3b**	376 (376)	11800 (12300)	501 (500)	0.068 (0.174)	802 (2140)	53.7/-3.7
TT -**3b**	373 (375)	12000 (11600)	497 (500)	0.067 (0.203)	800 (2360)	54.4/-2.8
TG-**3b**	371 (372)	11300 (10300)	498 (500)	0.069 (0.165)	780 (1700)	58.8/-2.4
TC-**3b**	373 (374)	11100 (11200)	498 (499)	0.051 (0.143)	566 (1602)	57.0/-3.6
GG-**3b**	373 (372)	11600 (10600)	500 (498)	0.053 (0.115)	615 (1219)	59.8/-2.7
GA-**3b**	373 (373)	11000 (11200)	500 (500)	0.063 (0.075)	693 (840)	55.9/-2.1
GC-**3b**	375 (374)	11100 (10000)	500 (499)	0.063 (0.105)	699 (1050)	60.5/-3.2
CC-**3b**	374 (373)	10700 (11200)	498 (497)	0.064 (0.146)	685 (1635)	62.5/-1.4
CA-**3b**	375 (372)	10300 (11200)	499 (499)	0.086 (0.097)	886 (1086)	57.1/-2.6
ACC-**3b**[d]	376 (373)	10400 (10400)	500 (500)	0.066 (0.130)	686 (1352)	56.2/+0.7
3b	365	9100	520	0.004	36	

[a] More detail for photophysical measurements were given in the Supporting Information. All measurements were performed at 25 °C in in 0.1m NaCl, 10 mm $MgCl_2$, 10mm Na-cacodylate, pH 7.0 with 3+3 μM single strand concentration. [b]Sequences named for neighboring bases. Sequence: 5'-d (ACT CAX **3** YGC CGT)-3**'** (sequence name AA-**3b**: X = Y = A, **3** = **3b**). [c]Standard deviations for quantum yield (Φ)≤0.002. Errors based on two independent T_m measurements were less than 0.4 °C. [d]Sequence: 5'-d (AC**3b** CAX TYG CCGT)-3**'.**

Next, the quantum yields of fluorescent oligonucleotides were measured in the presence of different neighboring bases in both single-stranded and double helix states. The quantum yields of single-stranded oligonucleotides containing **3a** ranged from Φ_{em} = 0.04 to 0.44, depending on its sequence context. Electron transfer from G and its high reduction potential resulted in **3a** being quenched 3.4 times in a single strand and 5.7 times in a double strand when flanked by a G residue. However, the overall brightness of **3a** showed an average of 1821 M^{-1} cm^{-1} in single-stranded DNA and 3289 M^{-1} cm^{-1} in double-stranded DNA. Exceptionally high brightness (~6000 M^{-1} cm^{-1}) was obtained when AA, TT, and AC were the nearest neighbors. Therefore, **3a** could potentially exhibit one of the brightest fluorescent base analogs (FBAs) in DNA, like ABN[26], ^{ts}T[27], and 2CNqA[28].

More remarkably, an unconventional turn-on fluorescent base analog (FBA) **3b** was obtained by replacing the phenyl group of **3a** with thiophene. Specifically, this substitution leads to an approximately 10-fold increase in green fluorescence in single-stranded (ss) DNA, and a noteworthy 10 to 54-fold increase in double-stranded (ds) DNA as compared to the nucleoside monomer **3b** in water. Due to the electron-rich nature of thiophene, **3b** displayed lower fluorescence quenching in ssDNA or dsDNA (ss: 5.1-8.6%, ds: 7.5-20.3%) compared to the nucleoside **3a** (ss: 4.0-44.2%, ds: 2.5-47.5%). Surprisingly, unlike **3a** and many other fluorescent nucleobase analogs, the fluorescence of **3b** incorporated into DNA oligonucleotides was not quenched when flanked by a G residue (**3a**: 0.025, **3b**: 0.115). Notably, the quantum yields of single-stranded oligonucleotides containing **3b** were almost independent of its sequence context, ranging from Φem = 0.05 to 0.08 (Figure 3 and Table 1). This is attributed to the fluorescence quenching of FBAs by neighboring residues through photoinduced electron transfer (PET), a phenomenon that can be circumvented by having higher HOMO energy levels than that of guanine and lower LUMO energy levels than that of thymidine., It was found that the HOMO energy of nucleosides **3a** and **3b** was higher than that of guanine, particularly for **3b** (refer to Figure S8 in the Supporting Information), resulting in partial quenching of **3a** and no quenching of **3b.**

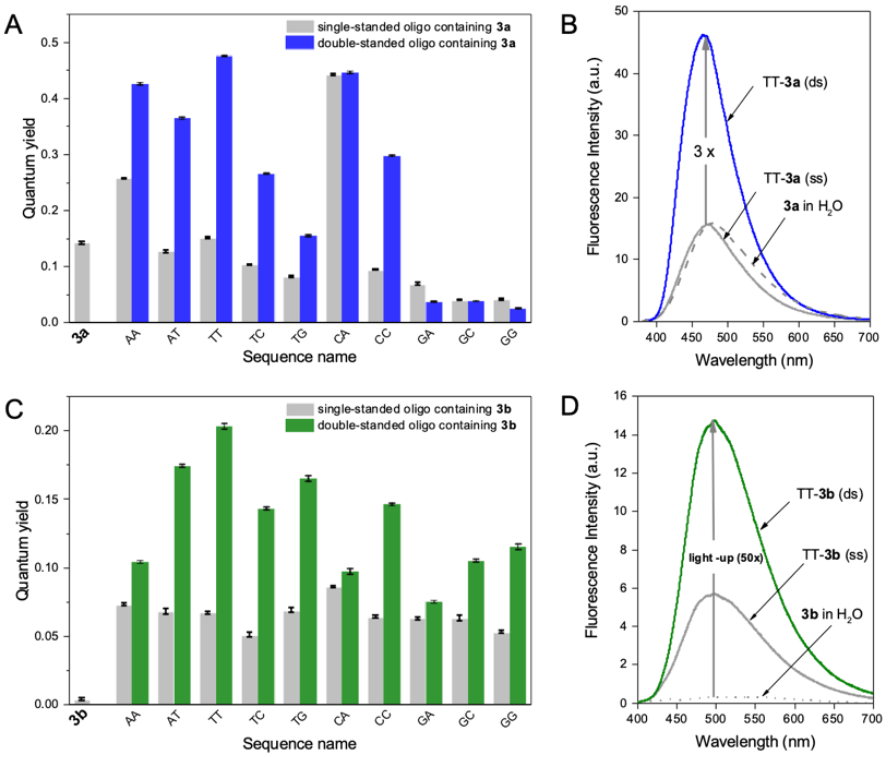


Figure 3. (A)(C) Quantum yields of single-stranded and double-stranded DNA containing single nucleosides (**3a**).and (**3b)**. (B) (D) Fluorescence spectra of **3a** and **3b**, single-stranded (ss) and double-stranded (ds) DNA at 3 μM concentration flanked by T residue.

To further investigate the unusual fluorescence enhancement of **3b** within oligonucleotides, various potential mechanisms were explored, including solvent quenching, excited-state proton transfer, chloride quenching, and a molecular rotor effect. Contrary to enhancement, most nucleosides experienced fluorescence quenching within oligonucleotides due to these factors. (1) Specifically, the sensitivity of **3b** to quenching by water was found to be about 30 times higher than that in methanol, which could explain the observed 10-fold increase in fluorescence in single-stranded DNA (ssDNA). (2) Furthermore, the influence of the duplex on excited-state proton transfer was investigated by measuring the emission quantum yield (Φ_{em}) in deuterated buffer, which showed a kinetic isotope effect that slowed proton transfer. In deuterated buffer, nucleoside **3a** exhibited 2.6 times higher brightness (Φ_{em} = 0.367), while nucleoside **3b** showed 4.3 times higher brightness (Φ_{em} = 0.017) (Table 2). The two- to four-fold increase in quantum yields for these compounds in D_2O suggested that proton-transfer reactions with the bulk solvent could account for the rapid non-emissive decay of nucleoside derivatives **3** in water. The hydrophobic interactions of bases reduced the excited-state proton transfer between nucleosides **3** and water when they were incorporated into DNA oligonucleotides [29] (Table 2, Φ_{D_2O}/Φ_{H_2O} < two-fold). The nearly unchanged quantum yield for the duplex formation further confirmed this effect.

Table 2. Fluorescence Quantum Yield (Φ) of Double-Stranded DNA or monomers in H_2O and D_2O

Name	Φ_{H_2O} (ds)	Φ_{D_2O} (ds)	Φ_{D_2O}/Φ_{H_2O} (ds)
3a	0.142	0.367	2.6
TT-**3a**	0.151(0.475)	0.197 (0.667)	1.3 (1.4)
AT-**3a**	0.121 (0.364)	0.217 (0.606)	1.8 (1.7)
GA-**3a**	0.069 (0.037)	0.081 (0.035)	1.2 (0.95)
3b	0.004	0.017	4.3
TT-**3b**	0.067 (0.203)	0.125 (0.303)	1.9 (1.5)
AT-**3b**	0.068 (0.174)	0.076 (0.201)	1.1 (1.2)
GA-**3b**	0.063 (0.075)	0.081 (0.133)	1.3 (1.8)

(3) The influence of ions, particularly chloride[11,30], on excited-state proton transfer was investigated with oligonucleotides **3a** and **3b** in aqueous NaCl solutions. To analyze this, a Stern−Volmer analysis was conducted using nucleoside **3b** and the GA duplex oligonucleotide (Supporting Information). It was observed that chloride exhibited a modest quenching effect on **3b** when present in the matched GA duplex, thereby ruling out the possibility of protection against chloride quenching contributing to the fluorescence turn-on effect.

(4) A reported characteristic of 5-position modified fluorescence uridine is its potential to act as a molecular rotor, which may impact fluorescence in diverse microenvironments through a twisted excited-state mechanism.[31-33] To

study its influence, the fluorescence emission of nucleosides **3a** and **3b** was measured in various methanol and glycerol mixtures. Surprisingly, it was found that unlike other 5-position modified fluorescence uridines, **3a** and **3b** exhibited low fluorescence intensity in response to viscosity (Supporting Information), indicating that they did not adopt a twisted excited state upon excitation.

Overall, the results suggest that the observed fluorescence turn-on effect of **3b** in the oligonucleotide is mainly attributed to escaping solvent quenching and excited-state proton transfer, even within a single-stranded oligonucleotide. Additionally, this effect is further enhanced in the double-helical state.

Many fluorescence-sensing applications of oligonucleotide probes rely on enzymatic DNA synthesis and amplification.[34, 35] Therefore, the enzymatic incorporation of **3a** and **3b** nucleoside triphosphates was further investigated. The study involved testing the incorporation of single and multiple nucleotides in primer extension reactions (figure 5A).[36, 37] It was observed that the intrinsic fluorescence of **3a** and **3b** could serve as turn-on probes to monitor their incorporation in the polymerization reaction, as evidenced by the fluorescence of the extended product (Figure 5). In these experiments, deep vent DNA polymerase was used and the results revealed an expected increase in fluorescence for **3b** even after a single incorporation of FBA in full extended double-stranded DNA, demonstrating a significant 25-fold increase in fluorescence compared to the free triphosphate (Figure 5C and 5D). Further study also tested the incorporation of multiple fluorophores. The results indicated that the KOD XL DNA polymerase effectively incorporated four modifications into the extended primer, resulting in the formation of a full-length 43-mer double-stranded DNA product (figure 5E), while the Deep Vent DNA polymerase could not achieve this (Figure S16). Moreover, the oligonucleotides containing **3b** showed a substantial 18-fold increase in fluorescence brightness, as shown in Figure 5F and G.

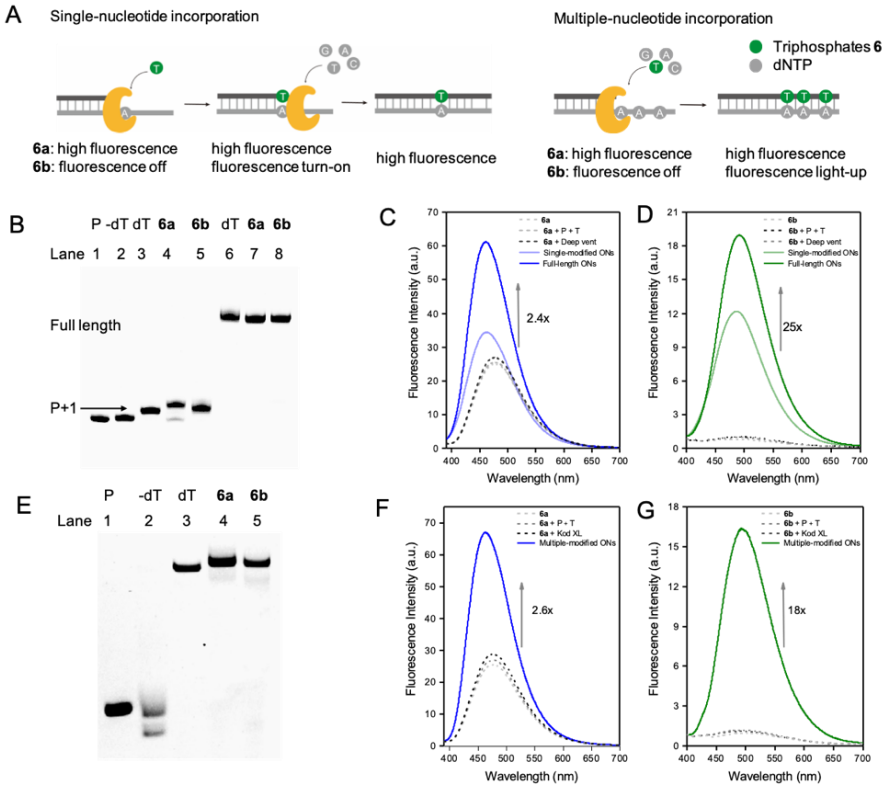


Figure 5. (A) scheme of single-nucleotide and multiple-nucleotide incorporation of FBAs to dsDNA. (B) In the presence of dTTP (lane 3) or modified nucleotides (lane 3−5), Deep vent DNA polymerase produced only singly-modified ONs. dT or fluorescent nucleosides **3a-b** labeled primer P was efficiently extended by deep vent DNA polymerase to give the full-length site-specifically functionalized ON product (lane 6-8). (Lane 1: primer, lane 2: negative control (PEX in absence of dTTP)). (C, D) Fluorescence spectroscopy of single-nucleotide incorporation **6a** (C) or **6b** (D) to DNA, followed by PEX after the addition of natural dNTPs. (E) Incorporation of multiple nucleotides of **3a** (lane 4) and **3b** (lane 5) by KOD XL DNA polymerase using **Primer2** and **Temp2PEX**. All reactions were conducted in the presence of dATP, dGTP, and dCTP. (Lane 1: primer, lane 2: negative control (PEX in absence of dTTP)). (E, F) Fluorescence spectroscopy of multiple -nucleotide incorporation **6a** or **6b** to DNA.

Next, we investigated the efficiency of modified dTTP **6b** in the polymerase chain reaction (PCR). PCR that employs chemically modified deoxynucleotide triphosphates presents a valuable method for synthesizing long base-modified DNA functionalized at multiple positions. Initially, we assessed the capability of KOD XL DNA polymerase to conduct PCR amplification using 98-mer and 321-mer templates in the presence of modified **6b** and natural dNTPs, with a primer/template ratio of 50:1(figure S17-20). The fully replacement of dTTP with **6b** resulted in a complete PCR failure (figure S). These findings suggest that fluorescent **6b** could potentially act as a substrate for certain DNA polymerases, potentially being at least partially incorporated in the presence of natural dTTP. The fluorescence of the dsDNA (98 nt) synthesized by PCR was also significantly enhanced compared to the triphosphate itself (figure S21).

In summary, we have successfully synthesized two new thymidine analogs on a large scale with a 53% yield over 7 steps, eliminating the need for column chromatography purification. Nucleoside **3b** demonstrates a significant increase in fluorescence upon incorporated into oligonucleotide, regardless of sequence context, while is nearly nonfluorescent in water. This makes it the first universal and environment-sensitive turn-on green FBA reporter, to the best of our knowledge. Additionally, the modified triphosphate of **3b** shows promise in primer extension experiments with DeepVent and KOD XL, as well as in the polymerase chain reaction with KOD XL polymerase. These findings provide valuable insights for fluorescence sensing in enzymatic DNA synthesis and potential in vivo applications. In vivo applications of these FBAs are currently under further exploration in our laboratory.

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