Clear Distinction of Purine Bases on the Complementary Strand by a Fluorescence Change of a Novel Fluorescent Nucleoside

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Many fluorescent nucleosides have been used as site-specific probes for studying structures and dynamics of nucleic acids.[1] Nucleosides possessing various fluorophores have been explored, including fluorescent nucleoside analogues and fluorophore-linked base conjugates, as exemplified by 2-aminopurine,[2] 1,N-etheno-adenine,[3] ethynyl-extended pyrimidines and deazapurines,[4] and nucleoside analogues replaced by flat aromatic fluorophores.[5] Such fluorescent nucleosides are valuable as sensitive reporter probes for detecting the change in the microenvironment of DNA, such as that which occurs in hybridization and the conformational change. However, there is no effective method that provides a clear distinction of the type of counterbase by using the fluorescence change caused by the change in DNA microenvironment.

We herein report on the clear distinction of purine bases on the complementary strand using the fluorescence from a novel nucleoside, benzopyridopyrimidine (BPP). BPP has a base-pairing degeneracy, which strongly contributes to the clear distinction of purine bases on the complementary strand by a sharp fluorescence change. The BPP-containing oligodeoxynucleotide (ODN) is applicable as a very effective reporter probe for typing of the A/G single-nucleotide polymorphism (SNP).

The synthesis of BPP is outlined in Scheme 1. The hydroxy groups of 5-iodo-2′-deoxycytidine **1** were protected, and then two benzoyl groups were incorporated into the amino group to give **2**. Photoirradiation with a high-pressure Hg lamp efficiently afforded the cyclized product **3**. The deprotection of **3** gave BPP 2′-deoxynucleoside **4**, which was converted to phosphoramidite **5** for use in a DNA synthesizer. The ODNs synthesized are summarized in Table 1.

Scheme 1 [a]
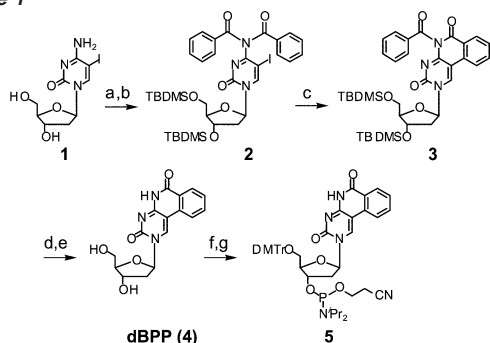
[a] Reagents and conditions: (a) TBDMSCl, imidazole, DMF, room temperature, 5 h; (b) benzoyl chloride (2.5 equiv), pyridine, room temperature, 14 h, 82% (two steps); (c) hν (high-pressure Hg lamp), benzene, 10 min, 57%; (d) concentrated ammonia−methanol (1:1), 50 °C, 1.5 h, 82%; (e) TBAF, THF, room temperature, 2 h, 72%; (f) 4,4′-dimethoxytrityl chloride, pyridine, room temperature, 6 h, 51%; (g) (iPr₂N)₂PO(CH₂)₂CN, 1H-tetrazole, acetonitrile, room temperature, 30 min, quant.

We measured the absorption and fluorescence spectra of **ODN-(BPP)/ODN(A)** and **ODN(BPP)/ODN(G)** duplexes (Figure 1).

Table 1. The Oligodeoxynucleotides (ODNs) Used in This Study

|	sequences
ODN(BPP)	5′-d(CGCAAT[**BPP**]TAACGC)-3′
ODN(BPP*)[a]	5′-d(CGCAAT[**BPP***]TAACGC)-3′
ODN(A)	5′-d(GCGTTA**A**ATTGCG)-3′
ODN(G)	5′-d(GCGTTA**G**ATTGCG)-3′
ODN(ᵐG)[b]	5′-d(GCGTTA**ᵐG**ATTGCG)-3′
ODN_IFNG(BPP)	5′-d(CCACAT[**BPP**]TTATGA)-3′
ODN_IFNG(A)	5′-d(TCATAA**A**ATGTGG)-3′
ODN_IFNG(G)	5′-d(TCATAA**G**ATGTGG)-3′

[a] BPP* = [3-¹⁵N]- or [4-¹⁵N]-BPP. [b] ᵐG = N¹-methylguanine.
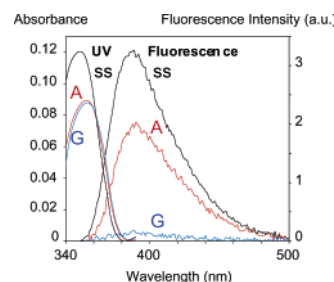
Figure 1. Absorption and fluorescence spectra of 2.5 μM **ODN(BPP)** hybridized with 2.5 μM **ODN(G)** and **ODN(A)** (50 mM sodium phosphate, 0.1 M sodium chloride, pH = 7.0, room temperature). λ_{ex} = 347 nm.

ODN(BPP) exhibited an absorption band at 347 nm arising from BPP, and the absorption maxima shifted slightly to longer wavelengths by duplex formation with **ODN(A)** and **ODN(G)**. In contrast, the fluorescence behavior of **ODN(BPP)** was strongly dependent on the purine bases opposite BPP. The fluorescence spectra of **ODN(BPP)/ODN(A)** had a strong fluorescence peak at 390 nm, whereas the fluorescence of the **ODN(BPP)/ODN(G)** duplex was almost completely quenched. The fluorescence quantum yield of **ODN(BPP)/ODN(G)** (Φ = 0.0018) was approximately 20 times less than that observed for **ODN(BPP)/ODN(A)** (Φ = 0.035).[6]

The fluorescent nucleobase BPP can form stable base pairs with both A and G. In melting temperature (T_m) measurements of the duplex,[7] very high duplex stabilities were observed for both **ODN-(BPP)/ODN(A)** and **ODN(BPP)/ODN(G)** duplexes (T_m = 54.7 and 57.1 °C, respectively), which were comparable to those of the natural base pairs T/A (T_m = 52.5 °C) and C/G (T_m = 56.0 °C). Next, hydrogen bonds between BPP and purines have been investigated by the ¹⁵N NMR of ODNs containing ¹⁵N-enriched BPP. [3-¹⁵N]- and [4-¹⁵N]-enriched BPP (>98% ¹⁵N) were prepared from [3-¹⁵N]- and [4-¹⁵N]-2′-deoxycytidine,[8] respectively, and incorporated into ODN (**ODN(BPP*)**). As is apparent in chemical shifts shown in Figure 2, BPP in **ODN(BPP*)** exists in the N3-imino and N4-amide forms, regardless of the type of purine opposite the BPP. The change in chemical shift on hybridization indicated that the hydrogen bond at N3 was tight for the BPP/G base pair,
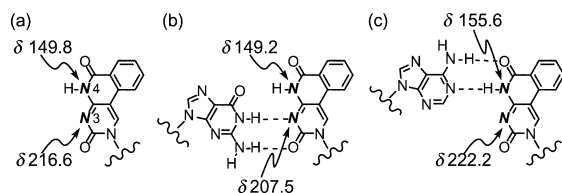
Figure 2. ^{15}N NMR chemical shifts of ^{15}N-enriched BPP in **ODN(BPP*)** and the base-pairing mode of BPP and purines. (a) BPP in **ODN(BPP*)**, (b) BPP/G base pair in **ODN(BPP*)/ODN(G)**, (c) BPP/A base pair in **ODN-(BPP*)/ODN(A)**. The ^{15}N-enriched nitrogen atoms are shown in bold.

Table 2. Fluorescence Lifetime Analysis for **ODN(BPP)**^{a}

| strands hybridized with ODN(BPP)	Φ (10^{-2})	τ_1 (ps) (α_1)	τ_2 (ns) (α_2)	τ_3 (ns) (α_3)	χ_r^2
single strand	4.09	158 (48)	0.91 (31)	2.40 (21)	1.00
ODN(A)	3.52	113 (54)	0.44 (43)	2.43 (3)	1.13
ODN(G)	0.18	37^b (81)	1.03 (12)	3.28 (7)	1.07
ODN(^{m}G)^c	1.26	122 (73)	0.91 (16)	2.67 (11)	1.01

^{a} **ODN(BPP)** hybridized with **ODN(G)** and **ODN(A)** was monitored (2.5 μM in 50 mM sodium phosphate, 0.1 M sodium chloride, pH = 7.0, room temperature). λ_{ex} = 295 nm, λ_{em} = 380 nm. The fluorescence lifetime profile is shown in the Supporting Information. ^{b} This value is shorter than the measurement time scale (41.2 ps/channel). ^{c} ^{m}G = N^1-methylguanine.

whereas N4 was preferentially used for the BPP/A base pair hydrogen bond. Thus, BPP is an effective degenerate base, forming stable base pairs in the Watson−Crick pairing mode for BPP/G and in the wobble mode for BPP/A.

The base-pairing degeneracy of BPP plays an important role in the sharp fluorescence change, in particular, the quenching in BPP/G. Accordingly, we measured the quenching of the fluorescence of BPP by N^1-methylguanine (^{m}G),[9] which does not form a stable base pair with BPP,[10] to investigate the effect of the stable BPP/G base pair on the quenching of BPP. The fluorescence of **ODN-(BPP)** obtained by hybridization with **ODN(^{m}G)** was considerably quenched (Φ = 0.0126), but the extent of the quenching was not as large as that with **ODN(BPP)/ODN(G)**. This result suggests that the formation of a stable BPP/G base pair is a key factor for the effective quenching of the BPP fluorescence.

To further analyze the character of the fluorescence of BPP in the duplex in detail, we examined the fluorescence lifetime of duplexes containing **ODN(BPP)**. The fluorescent decay profiles of these duplexes were fitted to triexponential functions (Table 2). The fluorescence decay was strongly influenced by the type of purine base opposite BPP. An extremely rapid quenching process that contributed to a strong quenching was only found in **ODN-(BPP)/ODN(G)**, as judged by the shortest lifetime and the preexponential factor, α_1. This observation strongly suggests that tight hydrogen bonds between BPP as a fluorophore and G as a quencher form a complex that undergoes an extremely rapid quenching and result in an effective quenching of the BPP fluorescence.

This clear change in fluorescence that depends on the type of purine base opposite BPP will be very useful for SNP typing of genes. We tested the distinction of purine bases by BPP hybridization using the A/G SNP sequence of the human interferon-γ gene (IFNG).[11] As a result, a strong visible emission was obtained with the **ODN$_{IFNG}$(BPP)/ODN$_{IFNG}$(A)** duplex, whereas the emission from the **ODN$_{IFNG}$(BPP)/ODN$_{IFNG}$(G)** duplex was negligible
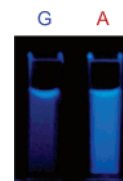
Figure 3. A/G SNP typing using the change of BPP fluorescence. The fluorescence from a solution containing 5 μM **ODN$_{IFNG}$(BPP)/ODN$_{IFNG}$(G)** (left) or **ODN$_{IFNG}$(BPP)/ODN$_{IFNG}$(A)** (right) in 50 mM sodium phosphate, 0.1 M sodium chloride, pH = 7.0, was measured using a transilluminator at 366 nm.

(Figure 3). The hybridization of the BPP-containing ODN with a target DNA facilitates the judgment with the naked eye of the type of purine located at a specific site on the target DNA, although the general utility of our method is limited by the flanking base pair of BPP.[12]

In summary, we have devised a new fluorescent nucleoside, BPP, which can sharply distinguish between A and G bases opposite BPP. The base-pairing degeneracy of BPP may play a key role in the fluorescence emission and quenching of BPP. The hybridization of an ODN probe containing BPP with a target DNA facilitates judgment with the naked eye of the type of purine base located at a specific site on the target DNA. BPP-containing ODN is a very effective probe for A/G SNP typing.

Supporting Information Available: Detailed experimental data of BPP and the related ODNs (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(6) The fluorescence quantum yields (Φ) of **ODN(BPP)/ODN(T)** and **ODN-(BPP)/ODN(C)** were 0.026 and 0.025, respectively.
(7) All T_m's of the duplexes (2.5 μM) were measured in 50 mM sodium phosphate and 100 mM sodium chloride, pH = 7.0. The absorbance of the duplexes was monitored at 260 nm from 10 to 80 °C using a heating rate of 1 °C/min.
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(10) The T_m of **ODN(BPP)/ODN(^{m}G)** was 44.0 °C, indicating that the base pair between BPP and N^1-methylguanine is very loose.
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(12) When the flanking base pair is a G/C base pair, the fluorescence of BPP is considerably suppressed. Thus, the SNP typing method would be inaccurate for the sequence containing a G/C base pair flanking to SNP site.

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