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Synthesis and properties of purine-type base-discriminating fluorescent (BDF) nucleosides: distinction of thymine by fluorescence-labeled deoxyadenosine derivatives

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Abstract—Novel base-discriminating fluorescent (BDF) nucleoside, 8-fluorescence-labeled adenosine derivative (^{8Py}**A**), was developed for the detection of thymine base on a target DNA. The BDF nucleoside was incorporated into oligodeoxynucleotides by post-synthetic modification. BDF probes containing ^{8Py}**A** selectively emit fluorescence only when the base opposite BDF nucleoside is thymine and act as effective reporter probes for homogeneous SNP typing.

Single nucleotide polymorphisms (SNPs) are the most abundant genetic variations in the human genome and appear on average once every 1.0–1.9 kb.[1] They are of medical and pharmacological interests in studies of disease susceptibility and drug response. Various techniques for SNP typing have been developed, such as 5′-exonuclease assay using TaqMan,[2] molecular beacon,[3] the Invader assay,[4] and the DNA microarray method.[5] However, the single base discrimination in nearly all reported methods is directly or indirectly based on the different hybridization efficiency between matched and mismatched duplexes. Such DNA probes have inherent limitations of selectivity because the differences in the hybridization efficiency vary with the sequence context and are often very small for the detection of a single base mismatch in a long target DNA. Particularly, to attain enough signal to noise (S/N) ratio when using the DNA microarray method, the hybridization and washing conditions must be carefully selected to minimize undesirable responses from the mismatched hybridization probes. Considering these limitations, alternative probes that do not rely on hybridization events are highly desired.

Recently, we reported a new homogeneous assay method to discriminate a single base alteration by base-discriminating fluorescent (BDF) probes.[6] Particularly, we reported novel pyrenecarboxamide-labeled pyrimidine-type BDF nucleosides ^{Py}**U** (**1**) and ^{Py}**C** (**2**) and indicated that these BDF probes selectively emit fluorescence only when the complementary bases are perfectly matched.[7] The use of ^{Py}**U**- and ^{Py}**C**-containing BDF made it possible to discriminate adenine and guanine at specific sites on the target DNA by simple mixing. However, discrimination of pyrimidine bases by purine-type BDF nucleosides has not yet been achieved. In this paper, we report novel adenosine-type BDF probes for the discrimination of thymine base, the first thymine selective fluorophore (Fig. 1).

The synthesis of ODN containing an aminopropyl linker (**ODN**(^{NH2}**A**), 5′-d(CGCAAT^{NH2}ATAACGC)-3′), is outlined in Scheme 1. 8-Bromo-2′-deoxyadenosine **4** was coupled with a pyrene substituted propargylamine under Sonogashira conditions by using $Pd(PPh_3)_4$ to afford **6**, which was then hydrogenated on 10% Pd/C in MeOH to give **7**. Protection of the amino group with DMF acetal and the 5′-hydroxyl group of deoxyribose with the 4,4′-dimethoxytrityl group resulted in formation of **9**. After conversion to phosphoramidite, **10** was incorporated into oligonucleotides by an automated DNA synthesizer.[8]

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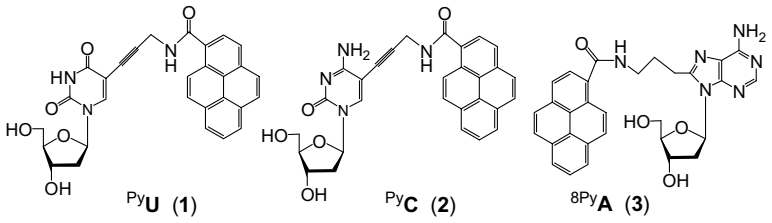
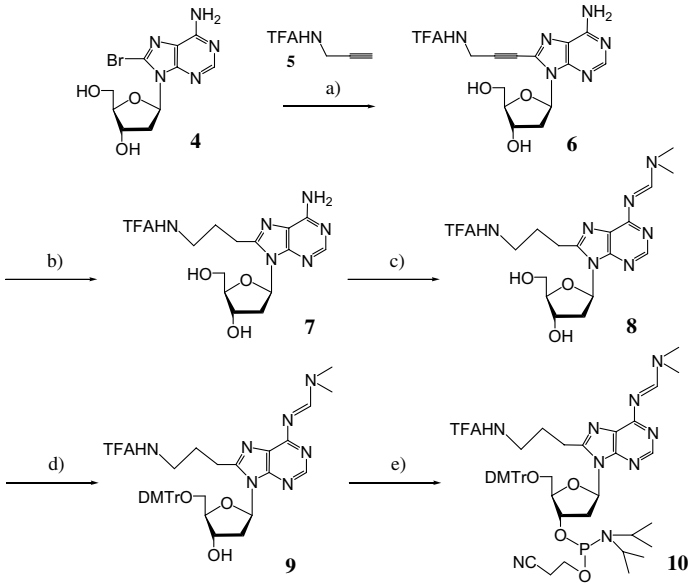
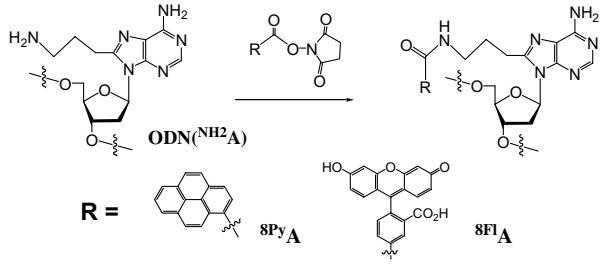


Figure 1. Structure of pyrenecarboxamide-labeled BDF nucleosicles.



Scheme 1. Reagents and conditions: (a) **5**, $Pd(PPh_3)_4$, CuI, Et_3N, DMF, rt, 13 h, 35%; (b) 10% Pd/C, MeOH, rt, 5 h, 88%; (c) DMF acetal, DMF, 50 °C, 2 h, 90%; (d) DMTrCl, pyridine, rt, 8 h, 75%; (e) $(i\text{-}Pr_2N)_2PO(CH_2)_2CN$, 1*H*-tetrazole, CH_3CN, rt, 1.5 h.



Scheme 2. Reagents and conditions: succinimidyl ester, 1 M $NaHCO_3$, aq DMF.

Table 1. Oliaonucleotides (ODNs) used in this study

	Sequences
ODN(^{8Py}**A**)	5′-d(CGCAAT^{8py}**A**TAACGC)-3′
ODN(^{8Fl}**A**)	5′-d(CGCAAT^{8Fl}**A**TAACGC)-3′
ODN(**N**)	3′-d(GCGTTA**N**ATTGCG)-5′, **N** = **T**, **C**, **G**, **A**
ODN_{BRCA1}(^{8Py}**A**)	5′-d(GGTACCA^{8Py}**A**TGAAATA)-3′
ODN_{BRCA1}(**T**)	3′-d(CCATGGT**T**ACTTTAT)-5′
ODN_{BRCA1}(**C**)	3′-d(CCATGGT**C**ACTTTAT)-5′

The synthesis of BDF probes ODN(^{8Py}**A**) and ODN(^{Fl}**A**) has been accomplished by using ODN(^{NH2}**A**). As shown in Scheme 2, each fluorophore was post-synthetically incorporated into ODN(^{NH2}**A**). A DMF solution of the succinimidyl ester of each fluorophore was added to ODN(^{NH2}**A**) in 1 M $NaHCO_3$, and this was incubated at 37 °C for 17 h. The resulting suspended mixture was filtered off and evaporated to dry. ODNs were purified by reverse phase HPLC. The newly synthesized ODNs are summarized in Table 1.

Initially, the fluorescence of ^{8Py}**A**-containing ODN was examined. The fluorescence spectra of the mismatched duplexes (ODN(^{8Py}**A**)/ODN(**N**); **N** = C, G, A) and single-stranded ODN (ODN(^{8Py}**A**)) showed weak fluorescence emission ($\Phi_F = 0.065$, 0.017, 0.051, and 0.040, respectively).[9] In contrast, the fluorescence of the perfectly matched duplexes (ODN(^{8Py}**A**)/ODN(**T**)) showed strong emission at 406 nm ($\Phi_F = 0.167$) (Fig. 2). In melting temperature (T_m) measurements of the duplex, a high duplex stability was observed for ^{8Py}**A**-containing duplex (ODN(^{8Py}**A**)/ODN(**T**), $T_m = 64.7$ °C, Table 2), which was more stable than a natural A/T base pair ($T_m = 51.2$ °C).

It was reported that bulky substituents on purine nucleosides at the C-8 position favor the *syn* conformation around the *N*-glycosidic bond in solution.[10] When

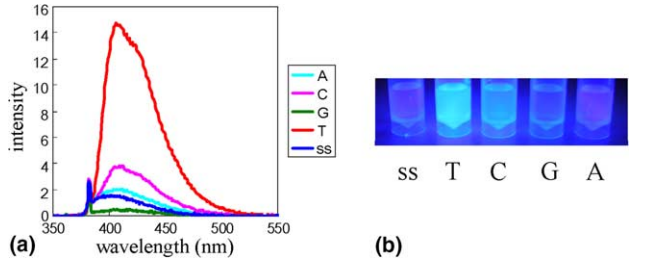


Figure 2. (a) Fluorescence spectra of 2.5 μM **ODN** (**^{8py}A**) hybridized with 2.5 μM **ODN**(**A**), **ODN** (**G**), **ODN** (**C**), or **ODN** (**T**), and that of single-stranded **ODN**(**^{8py} A**) (50 mM sodium phosphate, 0.1 M sodium chloride, pH 7.0. room temperature). Excitation wavelength was at 381 nm. (b) Comparison of the fluorescence for the bases opposite **^{8Py}A** (50 mM sodium phosphate, 0.1 M sodium chloride, pH 7.0, room temperature), "ss" denotes a single-stranded BDF probe. The sample solutions were illuminated with a 365 nm transilluminator.

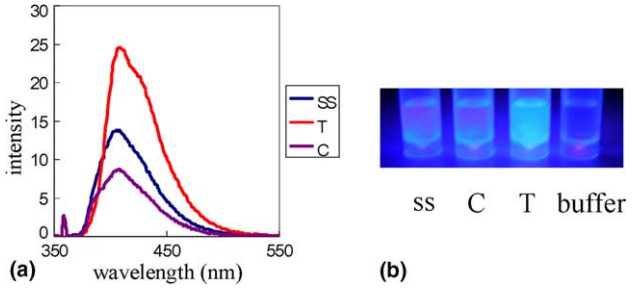


Figure 3. (a) Fluorescence spectra of 2.5 μM **ODN_{BRCA1}**(**^{8Py}A**) hybridized with 2.5 μM **ODN_{BRCA1}**(**T**) or **ODN_{BRCA1}**(**C**), and that of single-standard **ODN_{BRAC1}**(**^{8Py}A**) (50 mM sodium phosphate, 0.1 M sodium chloride, pH 7.0, room temperature). Excitation wavelength was at 358 nm. (b) Comparison of the fluorescene for the bases opposite **^{8Py}A** (50 mM sodium phosphate, 0.1 M sodium chloride, pH 7.0, room temperature). "SS" denotes a single-standard BDF probe. The sample solutions were illuminated with a 365 nm transilluminator.

Table 2. Melting temperature (T_m)

5'-d(CGCAAT **^{8Py}A**TAACGC)-3'

3'-d(GCGTTA **N** ATTGCG)-5'

Sample (N)	T_m (°C)
T	64.7
C	55.9
G	59.5
A	56.4

pyrenecarboxamide fluorophore is attached to adenine at the C-8 position, the polarity-sensitive fluorophore is expected to be extruded to the outside of the groove, a highly polar aqueous phase, due to base-pairing with thymine (*anti*-conformation), and a strong fluorescence emission should be observed. In contrast, the mismatched base pair containing **^{8Py}A** shows a weak fluorescence emission probably because conformational change from *anti* to *syn* occurs due to the lack of base-pairing. Thus, the fluorophore relocates to a low-polarity hydrophobic site within the groove, which weakens the fluorescence emission. Therefore, **^{8Py}A**-containing BDF probe is thought to emit a highly T-selective fluorescence.

Although **^{8Py}A**-containing ODN showed a clear T-selective fluorescence emission, completely no selectivity was observed in the fluorescein-labeled BDF probe (**ODN** (**^{8Fl}A**)), possibly because fluorescein does not interact with DNA duplex nor has a polarity-sensitive fluorescence property.

Since **^{8Py}A**-containing ODN shows a clear change in fluorescence that depend upon the type of base on the complementary strand, we examined the SNP detection of the T/C (wild type/mutant) SNP sequence of human breast cancer 1 gene (BRCA1)[11] by using **^{8Py}A**-containing BDF probe. We added BDF probe **ODN_{BRCA1}** (**^{8Py}A**) to the solution of the target sequences, **ODN_{BRCA1}**(**T**) (wild type) and **ODN_{BRCA1}**(**C**) (mutant), and incubated these solutions for one minute at ambient temperature. The sample solutions were then illuminated at 365 nm, and the fluorescence images were taken by a digital camera. A strong fluorescence emission was observed with the **ODN_{BRCA1}**(**^{8Py}A**)/**ODN_{BRCA1}**(**T**) duplex, whereas the mismatched duplex (**ODN_{BRCA1}** (**^{8Py}A**)/**ODN_{BRCA1}**(**C**)) and single-stranded **ODN_{BRCA1}**-(**^{8Py}A**) showed a weak emission (Fig. 3). The fluorescence quantum yield of the matched duplex (**^{8Py}A/T** base pair, $\Phi_F = 0.280$) was at least 2.3 times larger than that observed for the mismatched duplexes.

The **^{8Py}A**-containing BDF probe facilitates the distinction of thymine on a target DNA by the drastic fluorescence change. These fluorescence properties of **^{8Py}A** are favorable as an SNP typing probe. However, when BDF probes containing a –C**^{8Py}A**C– sequence were used, the fluorescence emission was strongly quenched by the flanking C/G base pairs. This indicates that there are some sequence limitations for the use of **^{8Py}A**-containing BDF probe.

In conclusion, we have developed a novel purine-type BDF probe, which can clearly distinguish thymine base on the complementary strand. The clear fluorescence change observed here is very useful for SNP typing. SNP typing using BDF probes serves as a powerful alternative to current SNP typing methods.

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8. Spectroscopic data of compound **9**: ^{1}H NMR (CD_3OD, 400 MHz) δ 2.01–2.11 (m, 2H), 2.23 (ddd, 1H, $J = 4.8$, 6.4, 12.0 Hz), 2.98 (t, 2H, $J = 7.6$ Hz), 3.11 (s, 3H), 3.12 (s, 3H), 3.20 (m, 1H), 3.25 (s, 2H), 3.31 (t, 2H, $J = 6.8$ Hz), 3.61 (s, 3H), 3.64 (s, 3H), 4.02 (ddd, 1H, $J = 4.4$, 4.8, 5.2 Hz), 4.74 (m, 1H), 6.29 (t, 1H, $J = 6.4$ Hz), 6.54–6.64 (m, 4H), 6.99–7.10 (m, 7H), 7.20–7.23 (m, 2H), 8.09 (s, 1H), 8.72 (s, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 26.3, 27.7, 35.1, 37.9, 40.2, 41.4, 55.7, 55.7, 65.1, 73.1, 85.9, 87.2, 87.7, 113.9 (×2), 117.6 ($J = 285.0$ Hz), 113.9 (×2), 126.0, 127.7, 128.6 (×2), 129.3 (×2), 131.1 (×2), 131.3 (×2), 137.2, 137.4, 146.5 (×2), 152.5, 153.6, 156.3, 159.0 ($J = 37.0$ Hz), 159.9 (×2), 160.0; HRMS: calcd for $C_{39}H_{43}F_3N_7O_6$ [$(M+H)^+$] 762.3227, found 762.3228.
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