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Singly and doubly labeled base-discriminating fluorescent oligonucleotide probes containing oxo-pyrene chromophore

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ABSTRACT

We have developed new oxo-pyrene labeled fluorescent nucleoside, $^{Oxo\text{-}Py}$**U** which showed a strong fluorescence dependency on solvent polarity at long wavelength. The designed singly and doubly $^{Oxo\text{-}Py}$**U** labeled fluorescent oligonucleotide probes were found highly efficient for the discrimination of A and consecutive AA bases of target DNA opposite to the labeled base via generation of enhanced fluorescence signal.

Fluorescent oligonucleotide probes are powerful tools for structural studies of nucleic acids, sequencing, molecular diagnostics, and other applications relating genomics.[1] Oligonucleotide probes labeled with solvatochromic fluorophores are particularly useful for detecting the change in microenvironment around the nucleic acids and thus can be used for DNA detection and other applications like single nucleotide polymorphisms (SNPs) typing.[1] The concept of the labeled probe is based on the fluorescence change of the labeled base itself in response to the opposite bases on a complementary strand. As a part of our ongoing project in searching for long wavelength emissive and highly solvatochromic fluorescent nucleosides, we demonstrated a novel method for homogeneous fluorescence assay that allows a clear distinction of the base opposite to the labeled base by a drastic fluorescence change.[2] Such fluorescent nucleosides named as base-discriminating fluorescence (BDF) nucleosides have been used extensively for gene detection and SNP genotyping.[1c,f,2,3]

Previously reported fluorescent probes containing BDF base ^{MPy}**U**- and ^{AMPy}**U** with oxo-pyrene chromophore were found to be useful for the discrimination of perfectly matched base A opposite to the BDF base in a complementary strand.[3a] However, the preparation of nucleosides ^{MPy}**U**- and ^{AMPy}**U** needed rather complicated synthetic steps of low yield and the synthesis in large quantity was very difficult.[3a] Therefore, the assay using these BDF bases are not practical. In contrast, a new fluorescent nucleoside $^{Oxo\text{-}Py}$**U** described here was very easily synthesized from readily available γ-oxo-pyrene butyric acid and the fluorescence emission was more solvent polarity sensitive with stronger fluorescence intensity and higher quantum yield (Fig. 1). Therefore, $^{Oxo\text{-}Py}$**U** is more convenient and practically more useful for the preparation of oxo-pyrene labeled BDF probes as compared with previous ^{MPy}**U**- and ^{AMPy}**U** probes.[3a]

Aiming a practical application of the BDF base, we thought that it would be worthwhile to tether oxo-pyrene chromophore to the oligonucleotide sequence and follow up the fluorescence behavior in the presence of its complementary target sequences. We also thought that there is a possibility of detecting two consecutive opposite bases from the target strand if we could incorporate two consecutive $^{Oxo\text{-}Py}$**U** in the probe, either by an enhanced monomer emission or via excimer emission, depending on the geometrical disposition of the two pyrene rings. With this view in mind, we have synthesized oxo-pyrene labeled 2′-deoxyuridine, $^{Oxo\text{-}Py}$**U**, which was incorporated into short oligonucleotides as one and two consecutive BDF bases.

Thus, we synthesized nucleoside **1** bearing a rigid acetylene linker via Sonogashira cross-coupling[4] of 5-iodo-2′-deoxyuridine with γ-oxo-pyrene carboxamide **2** (Scheme 1). The synthesis of γ-oxo-pyrene butyric acid (**3**) was accomplished from commercially available pyrene and succinic anhydride via Friedel–Craft acylation. Nucleoside **1** was ultimately incorporated into three oligonucleotide sequences ODN **1**, **2** and **3** via standard DNA synthesis protocol and the ODNs were analyzed by MALDI-TOF mass spectrometry (Table 1). While 13-mer ODN **1** and **2** contained

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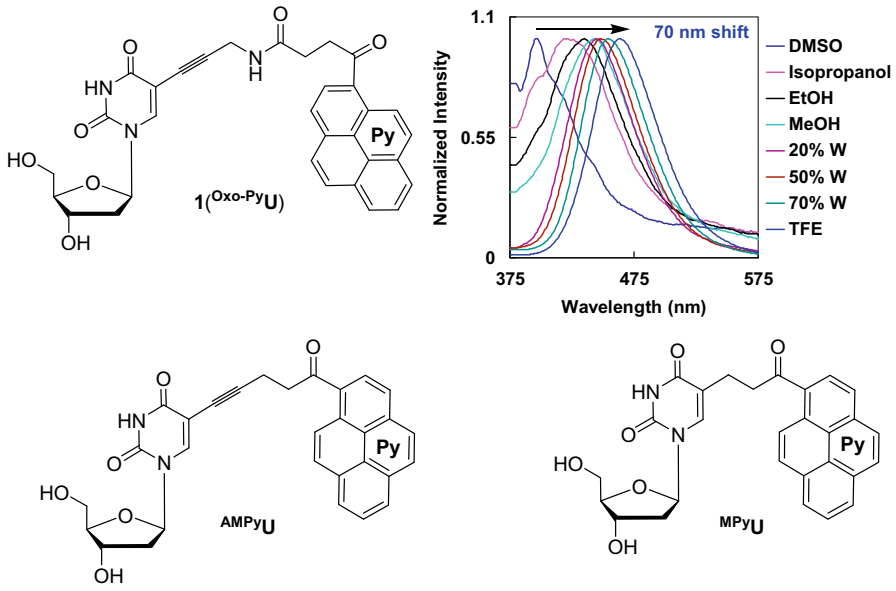
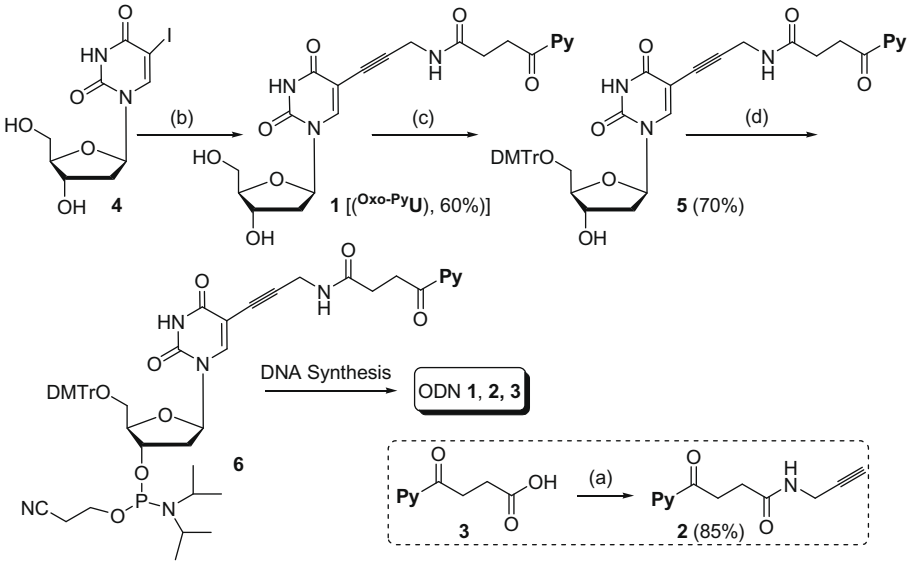


Figure 1. BDF nucleoside **1** ($^{Oxo\text{-}Py}$**U**), its solvatochromicity and the structures of ^{AMPy}**U** and ^{MPy}**U**.



Scheme 1. Synthesis of BDF nucleoside **1** and ODNs.

Table 1
Oligonucleotide sequences used in this study

ODNs	Sequences
1	5′-d(CGCAAC$^{Oxo\text{-}Py}$**U**CAACGC)-3′
2	5′-d(CGCAAT$^{Oxo\text{-}Py}$**U**TAACGC)-3′
3	5′-d(CGCAAT$^{Oxo\text{-}Py}$**U**$^{Oxo\text{-}Py}$**U**TAACGC)-3′
4	5′-d(CGCAAC **T** CAACGC)-3′
5	5′-d(CGCAAT **T** TAACGC)-3′
6	5′-d(CGCAAT **TT** TAACGC)-3′
7	5′-d(GCGTTG **N** GTTGCG)-3′ [**N** = A, G]
8	5′-d(GCGTTA **N** ATTGCG)-3′ [**N** = A, G,]
9	5′-d(GCGTTA **NN** ATTGCG)-3′ [**NN** = AA, AG, GG]

singly labeled $^{Oxo\text{-}Py}$**U** and differed only by flanking bases near the labeled nucleoside (ODN **1** contained C, while ODN **2** contained T as the flanking bases). 14-mer ODN **3** labeled with two consecutive $^{Oxo\text{-}Py}$**U** was also prepared.

Photophysical properties of nucleoside **1** were then examined. We observed that with increasing solvent polarity a decreased and a red shifted patterns were observed in the UV absorption spectra of **1** (λ_{max} = 345 in DMSO; λ_{max} = 355 nm on TFE). Most interestingly, both the fluorescence intensity and the wavelength were markedly affected by solvent polarity (Fig. 1). In DMSO nucleoside **1** displayed a very weak emission with a maximum at 396 nm (Φ = 0.015), while in TFE, the most polar organic solvent tested, **1** showed a strong emission (Φ = 0.22) at around 467 nm and decayed deeply into the visible region (>565 nm). We have also calculated fluorescence brightness factor (F.B. = $\varepsilon\Phi$) which showed an 11-fold increase in TFE in comparison with DMSO (Supplementary data, Fig. S1, Table S1). The fluorescence titration of a solution of **1** in EtOH with water revealed a similar observation. In pure EtOH, the emission was around 438 nm (Φ = 0.023) which was shifted to 452 nm and decayed deeply into visible region, 580–600 nm, when the ratio of water and ethanol was 70:30 (Φ = 0.12). As expected, the fluorescence of oxo-pyrene-labeled nucleoside

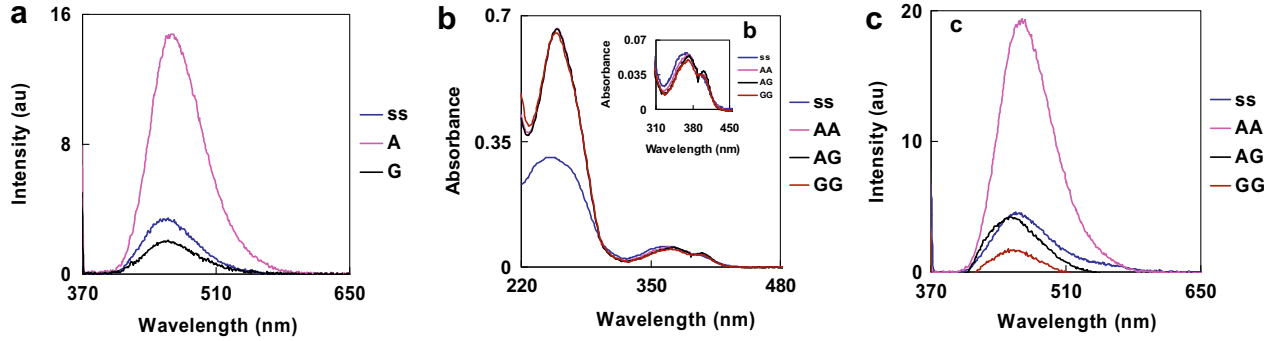


Figure 2. (a) Fluorescence of single strand ODN **2**, (b) UV–vis and (c) fluorescence spectra of ODN **3** (2.5 μM) and the different duplexes formed by hybridization with ODN **8** [**N** = A, G] and **9** [**NN** = AA, AG, GG], (2.5 μM, 50 mM sodium phosphate, 0.1 M sodium chloride, pH 7.0, rt; λ_{ex} = 369 nm.).

showed a high sensitivity to the solvent polarity. It is noteworthy that the emission wavelength in 30% water is about 55 nm longer than that reported for previous pyrenecarboxamide (^{Py}**U**)-labeled probes (397 nm in water)[2c] and the solvatochromicity (Δ70 nm) was 20 nm larger than those of ^{MPy}**U** and ^{AMPy}**U** (Δ50 nm).[3a]

Thus, the solvent polarity dependent fluorescence property of $^{Oxo\text{-}Py}$**U** would be usable for monitoring the change in DNA microenvironment. Therefore, we examined the photophysical properties of single stranded ODN **1** (5′-CGCAAC $^{Oxo\text{-}Py}$**U** CAACGC-3′) and **2** (5′-CGCAAT $^{Oxo\text{-}Py}$**U** TAACGC-3′) in the presence and absence of their complementary strands. As revealed from the fluorescence spectra (Supplementary data, Fig. S2), it was observed that the fluorescence of ODN **1** with flanking GC base pair was completely quenched when hybridized to perfectly matched or single base mismatched complementary sequences ODN **7** [**N** = A, G]. This is so obvious and a positive result of our expectation that the flanking GC base pair is responsible for the quenching of $^{Oxo\text{-}Py}$**U** fluorescence. Thus, $^{Oxo\text{-}Py}$**U** could potentially be useful in designing G-quenched molecular beacon as well.[5]

On the other hand, ODN **2** (5′-CGCAAT $^{Oxo\text{-}Py}$**U** TAACGC-3′) upon hybridization with perfectly matched sequence ODN **8** [**N** = A] showed a very strong emission at 465 nm (Fig. 2a). A very weak fluorescence was observed in case of the duplex ODN **2**/**8** [**N** = G] where mismatched base G is opposite to the labeled nucleoside in the complementary strand. This observation again indicated that the fluorescence of $^{Oxo\text{-}Py}$**U** is tremendously quenched by neighboring G-base and can allow us to detect matched base A with an intense fluorescence signal.[3,6]

The strong fluorescence emission for perfectly matched duplex ODN **2**/**8** [**N** = A] can be explained if we consider the more hydrophilic microenvironment faced by oxo-pyrene chromophore outside the groove. As the opposite base is matched base A, the fluorophore has to extrude outside the groove and thus faced to more aqueous microenvironment, resulting in an enhancement of fluorescence (Fig. 2a). The thermal melting behaviors of the duplex ODNs also support our explanation. The thermal melting behavior showed that the matched duplex ODN **2**/**8** [**N** = A] is more stable (T_m = 64.1 °C) than unmodified duplex ODN **5**/**8** [**N** = A] (T_m = 56.1 °C). Thus, the fluorophore resides along the major groove and involved in stacking type interaction and hence bringing more stability. Molecular modeling study using AMBER* force field in water employing MacroModel vs. 9.0 also supported this explanation.

Recently, Yoshida et al., have reported a doubly labeled probe for efficient SNP typing using ^{Py}**U** and ^{AMPy}**U**.[3b] Since ODN **2** showed a highly A-selective fluorescence emission, we thought that there could be a possibility of detecting opposite two consecutive AA sequence from the target strand if we incorporate two consecutive $^{Oxo\text{-}Py}$**U** in the probe either by an enhanced monomer emission or via an excimer emission depending on the geometrical disposition of the two oxo-pyrene rings. Therefore, we prepared a doubly $^{Oxo\text{-}Py}$**U**-labeled probe, ODN **3** [5′-d(CGCAAT $^{Oxo\text{-}Py}$**U** $^{Oxo\text{-}Py}$**U** TAACGC)-3′] and measured the fluorescence of the duplexes formed via hybridization with complementary strands ODN **9** [{5′-d(GCGTTA **NN** ATTGCG)-3′}; **NN** = AA, AG, GG]. Unfortunately, in any case we could not observe an excimer fluorescence. However, the detection of –AA– bases was perfectly accomplished by a strong monomer emission at 467 nm (Φ = 0.15) with a long tail in the visible region (>567 nm) together with a high signal to noise ratio and a large fluorescence brightness factor (F.B. = 3284). In contrast, the emission of the single stranded ODN **3** and the duplexes ODN **3**/**9** [**NN** = AG] and ODN **3**/**9** [**NN** = GG] was strongly suppressed (Fig. 2b and Table 2). Therefore, ODN **3** can act as a useful probe which emits strong fluorescence with a high AA selectivity. To understand the fact of no excimer emission by two consecutive $^{Oxo\text{-}Py}$**U** we have examined the molecular modeling study. From the optimized structure it was found that the pyrene rings are far apart and positioned parallel and in cofacial configuration to each other, thus inhibiting an excimer formation (Supplementary data, Fig. S5).[8]

In conclusion, we have investigated the fluorescence emission properties of fluorescent DNA probes containing singly and doubly labeled $^{Oxo\text{-}Py}$**U**[9] in the presence of complementary strands. We showed that the fluorescence of $^{Oxo\text{-}Py}$**U** is strongly quenched by neighboring G-base. We have also shown that the probes are highly specific for the detection of matched base A. The doubly labeled ODN which exhibit unique fluorescence properties depending on the number of adenines on the complementary strands would be also useful for the detection of consecutive A sequence.

Table 2
Thermal melting temperatures and photophysical properties of ODNs

Duplexes	T_m (°C)	ε_{max} (M^{-1} cm^{-1})	Φ[7]	F.B.
ODN **1**	–	11,200	0.054	605
ODN **1**/**7** [**N** = A]	70.9	11,200	0.002	22
ODN **1**/**7** [**N** = G]	60.7	10,800	0.001	11
ODN **4**/**7** [**N** = A]	59.8	–	–	–
ODN **2**	–	11,600	0.046	543
ODN **2**/**8** [**N** = A]	64.1	12,000	0.216	2640
ODN **2**/**8** [**N** = G]	52.5	11,200	0.030	336
ODN **5**/**8** [**N** = A]	56.1	–	–	–
ODN **3**	–	22,648	0.036	815
ODN **3**/**9** [**NN** = AA]	64.1	21,896	0.150	3284
ODN **3**/**9** [**NN** = AG]	58.4	21,492	0.025	550
ODN **3**/**9** [**NN** = GG]	53.2	19,696	0.007	153
ODN **6**/**9** [**NN** = AA]	61.1	–	–	–

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.04.063.

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9. *Spectroscopic data for compound* **1**: ^{1}H NMR (DMSO-d_6, 400 MHz) δ = 11.68 (d, 1H, J = 8.0 Hz), 8.76 (d, 1H, J = 9.4 Hz), 8.58 (d, 1H, J = 8.1 Hz), 8.41–8.27 (m, 5H), 8.25–8.15 (m, 2H), 8.14 (s, 1H), 6.13 (t, 1H, J = 8.2 Hz), 5.26 (br, 1H), 5.16 (br, 1H), 4.23 (br, 1H,), 4.14 (br, 1H), 3.80 (br, 1H), 3.6 (m, 2H), 3.49 (m, 1H), 3.36 (br, 2H), 2.69 (broad t, 1H), 2.50 (t, 2H, J = 1.8 Hz), 2.12 (br m, 2H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ = ^{13}C NMR (DMSO-d_6, 100 MHz) δ = 203.5, 170.9, 161.6, 149.4, 143.6, 132.9, 132.7, 130.6, 129.9, 129.2, 129.0, 128.0, 127.1, 126.7, 126.4, 126.3, 125.9, 124.5, 124.4, 123.9, 123.4, 98.1, 89.6, 87.5, 84.6, 74.3, 70.2, 60.9, 40.0, 37.0, 29.5, 28.7; FABMS, m/z 566 ([M+H]$^+$), HRMS calcd for $C_{32}H_{27}N_3O_7$ ([M+H]$^+$) 566.1927, found 566.1929.