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Synthesis and properties of a novel fluorescent nucleobase, naphthopyridopyrimidine

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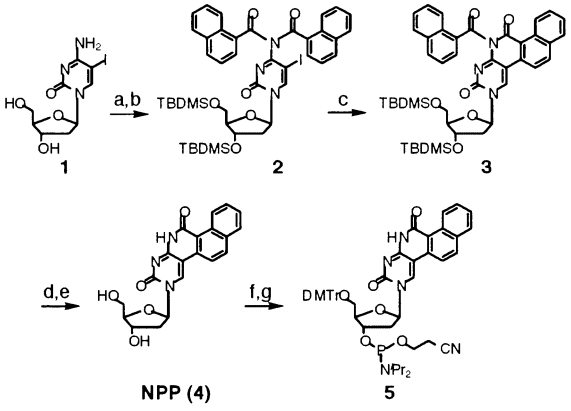
Abstract—A new base-discriminating fluorescent nucleoside, NPP, that can sharply distinguish between A and G bases opposite NPP is described. The hybridization of an ODN probe containing NPP with a target DNA facilitates the judgment of the type of purine base located at a specific site on the target DNA.

Synthetic fluorescent nucleosides for studying structures and dynamics of nucleic acids have attracted much attention. Nucleosides possessing various fluorophores have been explored, including fluorescent nucleoside analogs and fluorophore-linked base conjugates.[1–4] Such fluorescent nucleosides are valuable as sensitive reporter probes for detecting the change in the microenvironment of DNA, such as occurs in hybridization and conformational change. In our continuing efforts to develop base-discriminating fluorescent (BDF) nucleobases, we have recently demonstrated an effective method that provides a clear distinction of the type of base on the complementary strand by the fluorescence change caused by the DNA microenvironment.[5] An oligodeoxynucleotide (ODN) containing an artificial nucleoside, benzopyridopyrimidine (BPP), can be used as an effective BDF probe for A/G single nucleotide polymorphisms (SNPs) typing. However, the quantum yield of BPP is relatively small. In addition, the fluorescence of a single-stranded BPP BDF probe is stronger than that of the probe hybridized with the target DNA possessing an adenine opposite BPP. Thus, the fluorescence property of BPP has prompted us to explore a fluorescent nucleoside for use as a more effective BDF probe for SNP typing.

Here, we report a novel BDF nucleoside, naphthopyridopyrimidine (NPP) and its use for the distinction of purine bases on the complementary strand. NPP showed a strong fluorescence when the base opposite NPP was adenine. The quantum yield of NPP hybridized with an adenine base was greatly enhanced as compared with that of BPP hybridized with an adenine base.

The synthesis of NPP is outlined in Scheme 1.[6] The hydroxy groups of 5-iodo-2′-deoxycytidine **1** were protected by silyl groups, and then two naphthoyl groups were incorporated into the amino group to give **2**.



Scheme 1. *Reagents and conditions*: (a) TBDMSCl, imidazole, DMF, room temperature, 5 h; (b) naphthoyl chloride (3 equiv.), pyridine, room temperature, 14 h, 74% (two steps); (c) *hν* (high pressure Hg lamp), propylene oxide, benzene, 7 min, 18%; (d) *conc*. ammonia–chloroform–methanol (1:1:1), 50°C, 18 h, 86%; (e) TBAF, THF, room temperature, 2 h, 60%; (f) 4,4′-dimethoxytrityl chloride, pyridine, room temperature, 6 h, 91%; (g) $(^{i}Pr_2N)_2PO(CH_2)_2CN$, 1*H*-tetrazole, acetonitrile, room temperature, 2 h, *quant*.

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Table 1. The oligodeoxynucleotides (ODNs) used in this study

	Sequences
ODN(NPP)	5′-d(CGCAAT[**NPP**]TAACGC)-3′
ODN(BPP)	5′-d(CGCAAT[**BPP**]TAACGC)-3′
ODN(A)	5′-d(GCGTTA**A**ATTGCG)-3′
ODN(G)	5′-d(GCGTTA**G**ATTGCG)-3′
ODN(T)	5′-d(GCGTTA**T**ATTGCG)-3′
ODN(C)	5′-d(GCGTTA**C**ATTGCG)-3′

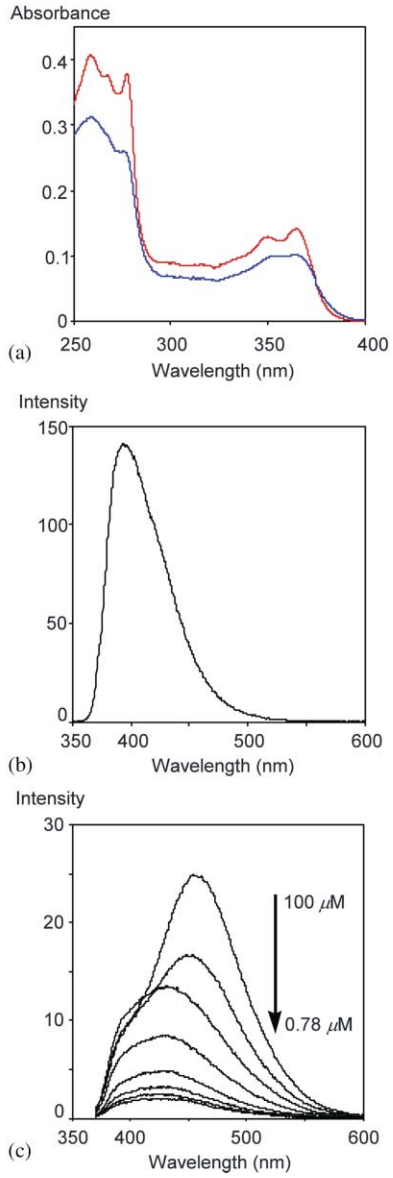

Figure 1. Absorption and fluorescence spectra of NPP. (a) Absorption spectra of 10 μM NPP in methanol (red) and 50 mM sodium phosphate, 0.1 M sodium chloride, pH 7.0 (blue). (b) Fluorescence spectra of 25 μM NPP in methanol. Excitation wavelength was 373 nm. (c) Fluorescence spectra of different concentrations of NPP in 50 mM sodium phosphate, 0.1 M sodium chloride, pH 7.0. Excitation wavelength was 362 nm.

Photoirradiation with a high pressure Hg lamp in the presence of propylene oxide produced the cyclized product **3**. The deprotection of **3** gave NPP (**4**), which was subsequently converted to the phosphoramidite **5**[7] for use in a DNA synthesizer. The ODNs synthesized are summarized in Table 1.[8]

We initially measured the absorption and fluorescence spectra of NPP in methanol and sodium phosphate buffer (pH 7.0). The absorption maxima for NPP were observed at 365 nm for methanol and at 364 nm for sodium phosphate buffer, where natural nucleosides have no absorption (Fig. 1a). However, the absorbances are quite different in different solvents ($\varepsilon_{365}=14{,}800$ for methanol, $\varepsilon_{364}=10{,}200$ for sodium phosphate buffer). With excitation of NPP at 373 nm in methanol, we observed strong fluorescence at 395 nm ($\Phi=0.26$) (Fig. 1b). In contrast, the fluorescence of NPP in phosphate buffer was very weak ($\Phi=0.013$ for 100 μM NPP and $\Phi=0.13$ for 0.78 μM NPP) and the maximum wavelength was dependent on the concentration of NPP (452 nm for 100 μM NPP and 432 nm for 0.78 μM NPP) (Fig. 1c). The interesting solvent- and concentration-dependent fluorescence changes suggest an aggregation of NPP possessing a hydrophobic aromatic system in aqueous media.

Next, the fluorescence spectra of NPP-containing ODN (**ODN(NPP)**) and the duplexes containing different bases opposite NPP were measured with excitation at 370 nm. When fluorescent nucleobase NPP was incorporated into ODN, a relatively weak fluorescence was observed at 421 nm ($\Phi=0.052$), as shown in Figure 2. The fluorescence of **ODN(NPP)** is probably quenched by the hydrophobic aggregation of nucleobases in aqueous media. On the other hand, the fluorescence property of **ODN(NPP)** forming duplexes was strongly dependent on the purine bases opposite NPP. The fluorescence spectra of **ODN(NPP)**/**ODN(A)** had a strong peak at 422 nm, whereas the fluorescence of the

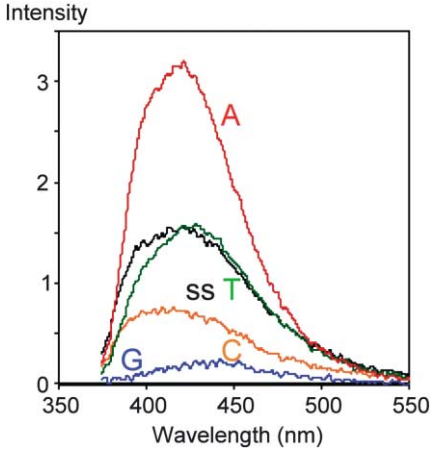

Figure 2. Fluorescence spectra of 2.5 μM **ODN(NPP)** hybridized with 2.5 μM **ODN(T)**, **ODN(C)**, **ODN(G)** or **ODN(A)** (50 mM sodium phosphate, 0.1 M sodium chloride, pH 7.0, room temperature). Excitation wavelength was 370 nm.

ODN(NPP)/ODN(G) duplex was almost negligible. The fluorescence quantum yield of **ODN(NPP)/ODN(G)** ($\Phi = 0.007$) was approximately 14 times less than that observed for **ODN(NPP)/ODN(A)** ($\Phi = 0.096$). For the other mismatched duplexes **ODN(NPP)/ODN(C)** and **ODN(NPP)/ODN(T)**, the fluorescence intensities were weak compared with that for **ODN(NPP)/ODN(A)** ($\Phi = 0.021$ and 0.051, respectively). The base-selectivity of the fluorescence emission of NPP was very similar to the selectivity observed for BPP, but the quantum yield of NPP hybridized with an adenine base was greatly enhanced as compared with that of BPP hybridized with an adenine base ($\Phi = 0.035$).[5] These fluorescence properties of NPP are favorable for the application as an SNP typing probe.

The fluorescent nucleobase NPP can form stable base pairs with both A and G. In melting temperature (T_m) measurements of the duplex,[9] high duplex stabilities were observed for both **ODN(NPP)/ODN(A)** and **ODN(NPP)/ODN(G)** duplexes ($T_m = 55.3$ and 56.1°C, respectively), which were similar to those of the natural base pairs T/A ($T_m = 52.5$°C) and C/G ($T_m = 56.0$°C). Thus, NPP is an effective degenerate base like BPP. As observed for BPP,[5] NPP probably forms a stable base pair in the Watson–Crick pairing mode for NPP/G and in the wobble mode for NPP/A. The T_m of **ODN(NPP)/ODN(T)** and **ODN(NPP)/ODN(C)** was 50.4 and 52.0°C, respectively, indicating that the base pair between NPP and pyrimidines is loose.[10]

This clear change in fluorescence that depends on the type of nucleobases opposite NPP, particularly purine bases, will be useful for the detection of the difference of single nucleotides, such as SNPs and point mutations of genes. We examined the usefulness of NPP BDF probes for A/G SNP typing, and compared it with that of BPP BDF probes. We added BDF probes, **ODN(NPP)** or **ODN(BPP)**, to solutions of the target sequence, **ODN(A)** or **ODN(G)**, and then incubated the mixture at room temperature for one minute. The sample solutions were illuminated at 365 nm, and the fluorescence images were taken through a 380 nm filter (Fig. 3). The fluorescence emission from **ODN(NPP)/ODN(A)** was very strong and clearly distinguishable from the poor fluorescence from **ODN(NPP)/ODN(G)**. In addition, the adenine-selective emission by an NPP BDF probe was much stronger than that of a BPP BDF probe. It is noteworthy that the fluorescence of single-stranded **ODN(NPP)** was strongly suppressed compared with the duplex with **ODN(A)**, whereas the fluorescence intensity of **ODN(BPP)/ODN(A)** was smaller than that of the single-stranded state. The fluorescence property of an NPP BDF probe is superior to that of a BPP BDF probe.[11]

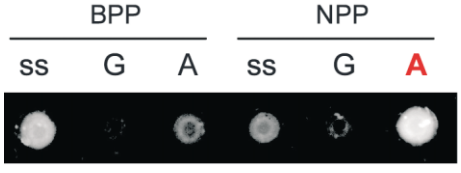

Figure 3. Fluorescence differences among the purine bases opposite BPP and NPP (2.5 μM strand concentration, 50 mM sodium phosphate, 0.1 M sodium chloride, pH 7.0, room temperature). The sample solutions were illuminated with a 365 nm transilluminator. The image was taken through a 380 nm long pass emission filter.

In summary, we have devised a new fluorescent nucleoside, NPP, that can sharply distinguish the nucleobases opposite NPP, particularly between A and G bases. The hybridization of an NPP BDF probe with a target DNA facilitates judgment of the type of purine base located at a specific site on the target DNA, and is very effective for A/G SNP typing.

Acknowledgements

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6. Spectroscopic data for selected compounds are provided. **2**: ^{1}H NMR ($CDCl_3$) δ 8.59 (s, 1H), 8.27 (d, 2H, $J = 8.4$ Hz), 8.00 (d, 2H, $J = 7.2$ Hz), 7.60 (d, 2H, $J = 7.6$ Hz), 7.54 (d, 2H, $J = 8.0$ Hz), 7.51–7.46 (m, 2H), 7.44–7.39 (m, 2H), 7.08 (t, 2H, $J = 7.2$ Hz), 6.16 (t, 1H, $J = 6.0$ Hz), 4.40 (dt, 1H, $J = 6.0$, 3.6 Hz), 4.07 (quartet, 1H, $J = 2.8$ Hz), 3.96 (dd, 1H, $J = 11.6$, 2.4 Hz), 3.77 (dd, 1H, $J = 11.6$, 2.4 Hz), 2.64 (ddd, 1H, $J = 13.2$, 6.0, 3.6 Hz), 2.11 (dt, 1H, $J = 13.2$, 6.4 Hz), 0.94 (s, 9H), 0.90 (s, 9H), 0.15 (d, 6H, $J = 4.4$ Hz), 0.09 (d, 6H, $J = 3.2$ Hz); ^{13}C NMR ($CDCl_3$) δ 171.1, 167.1, 153.8, 151.3, 133.2, 132.2, 132.1, 130.1, 128.0, 127.7, 127.2, 126.6, 125.1, 123.8, 89.0, 88.4, 71.7, 64.9, 62.5, 42.5, 26.2, 25.7, 18.5, 18.0, −4.6, −4.9, −5.1, −5.2; MS (FAB, NBA/CH_2Cl_2) m/z 890 [$(M+H)^+$]; HRMS (FAB) calcd for $C_{43}H_{53}IN_3O_6Si_2$ [$(M+H)^+$] 890.2518, found 890.2517. **3**: ^{1}H NMR ($CDCl_3$) δ 9.79 (d, 1H, $J = 8.4$ Hz), 9.36 (d, 1H, $J = 8.4$ Hz), 9.27 (s, 1H),

8.16 (d, 1H, $J=9.2$ Hz), 8.10 (d, 1H, $J=8.0$ Hz), 7.94–7.90 (m, 4H), 7.81–7.76 (m, 1H), 7.69–7.58 (m, 3H), 7.39 (t, 1H, $J=8.0$ Hz), 6.32 (t, 1H, $J=6.4$ Hz), 4.36 (dt, 1H, $J=9.6$, 2.4 Hz), 4.21 (quartet, 1H, $J=2.4$ Hz), 4.11 (dd, 1H, $J=11.2$, 2.4 Hz), 3.89 (dd, 1H, $J=11.6$, 3.2 Hz), 2.77 (ddd, 1H, $J=13.2$, 6.0, 2.4 Hz), 2.01 (dt, 1H, $J=14.4$, 7.2 Hz), 0.90 (s, 18H), 0.13 (d, 6H, $J=8.0$ Hz), 0.09 (d, 6H, $J=3.6$ Hz); ^{13}C NMR ($CDCl_3$) δ 169.9, 163.6, 157.5, 153.2, 141.2, 136.1, 135.8, 134.1, 133.1, 132.8, 132.7, 132.0, 131.9, 129.7, 129.4, 128.7, 128.3, 127.2, 127.1, 127.0, 126.2, 124.5, 117.5, 117.0, 100.6, 89.6, 89.1, 72.7, 63.4, 43.1, 26.0, 25.8, 18.5, 18.1, –4.5, –4.9, –5.2; MS (FAB, NBA/$CHCl_3$) m/z 762 [(M+H)$^+$]; HRMS (FAB) calcd for $C_{43}H_{52}N_3O_6Si_2$ [(M+H)$^+$] 762.3395, found 762.3392. **4**: ^{1}H NMR (DMSO-d_6) δ 10.14 (d, 1H, $J=8.8$ Hz), 9.39 (s, 1H), 8.11 (d, 1H, $J=8.8$ Hz), 8.04 (d, 1H, $J=9.2$ Hz), 7.89 (d, 1H, $J=7.6$ Hz), 7.57–7.55 (m, 1H), 7.50–7.47 (m, 1H), 6.28 (t, 1H, $J=6.0$ Hz), 4.41 (quartet, 1H, $J=4.8$ Hz), 3.88–3.87 (m, 1H), 3.85–3.77 (m, 2H), 2.31 (quintet, 1H, $J=6.4$ Hz), 2.19 (quintet, 1H, $J=6.4$ Hz); ^{13}C NMR (DMSO-d_6) δ 164.0, 157.6, 153.4, 142.7, 134.8, 134.2, 132.2, 131.2, 128.8, 126.35, 126.27, 119.2, 116.2, 100.2, 87.7, 87.1, 79.2, 68.3, 59.8, 41.3; MS (FAB, NBA/DMSO) m/z 380 [(M+H)$^+$]; HRMS (FAB) calcd for $C_{20}H_{18}N_3O_5$ [(M+H)$^+$] 380.1247, found 380.1247.

7. To a solution of **4** (12 mg, 0.032 mmol) in pyridine (1 mL) was added 4,4′-dimethoxytrityl chloride (14 mg, 0.041 mmol), and the mixture was stirred for 6 h at ambient temperature. The reaction mixture was concentrated and purified by column chromatography on silica gel, eluting with chloroform–methanol (20:1) to give dimethoxytrityl product (20 mg, 91%): ^{1}H NMR ($CDCl_3$) δ 9.82 (d, 1H, $J=8.8$ Hz), 9.14 (s, 1H), 7.68–7.11 (m, 13H), 6.72 (d, 4H, $J=8.8$ Hz), 6.46–6.42 (m, 1H), 4.56–4.50 (m, 1H), 4.30–4.26 (m, 1H), 3.63 (s, 6H), 3.59–3.56 (m, 1H), 3.32–3.28 (m, 1H), 3.00–2.95 (m, 1H), 2.42–2.38 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 163.3, 158.6, 158.1, 157.4, 154.2, 144.2, 140.6, 135.4, 135.3, 135.2, 132.9, 132.5, 131.6, 129.9, 129.2, 129.4, 128.4, 128.0, 127.9, 127.8, 127.1, 127.0, 126.9, 126.5, 117.6, 117.0, 113.3, 113.1, 101.3, 88.6, 87.0, 86.9, 71.5, 63.3, 55.2, 45.8, 42.4; MS (FAB, NBA/$CHCl_3$) m/z (%) 682 [(M+H)$^+$]; HRMS (FAB) calcd for $C_{41}H_{36}N_3O_7$ [(M+H)$^+$] 682.2553, found 682.2558. To a solution of dimethoxytrityl–NPP (31 mg, 0.046 mmol) in acetonitrile (2 mL) was added 2-cyanoethyl tetraisopropyldiphosphoramidite (18 μL, 0.055 mmol) and tetrazole (4 mg, 0.055 mmol), and the mixture was stirred for 2 h at ambient temperature. The reaction mixture was filtrated and used with no further purification to the ODN synthesis step.
8. **ODN(NPP)**, MALDI-TOF [(M−H)$^-$] calcd 4054.71, found 4055.33; **ODN(BPP)**, MALDI-TOF [(M−H)$^-$] calcd 4004.65, found 4005.03.
9. All T_m 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 2 to 80°C using a heating rate of 1°C/min.
10. T_ms of the natural base pairs A/T and G/C were 52.6 and 57.4°C, respectively.
11. The general utility of our method is limited by the flanking base pair of NPP. When the flanking base pair is a G/C base pair, the fluorescence of NPP is considerably suppressed. Thus, the SNP typing method would be inaccurate for the sequence containing a G/C base pair flanking the SNP site.