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Synthesis and properties of microenvironment-sensitive oligonucleotides containing a small fluorophore, 3-aminobenzonitrile or 3-aminobenzoic acid

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ABSTRACT

Two C-nucleosides bearing small fluorescent groups as a base were synthesized by Heck-type coupling reaction and incorporated into DNA. They exhibited environment-sensitive fluorescence and opposite solvatochromic properties. The modified DNAs containing 3-aminobenzonitrile or 3-aminobenzoic acid retained the duplexes and their fluorescence reflected the microenvironment.

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Fluorescent nucleic acids are attractive molecules for reading genetic records and an important analytical tool for studying structures of nucleic acids and intermolecular interactions with other molecules. Many fluorescent nucleic acid probes have been developed so far. Especially, fluorescent nucleoside analogs have been used for the labeling at arbitrary positions in nucleic acids. These fluorophores were attached at various positions to the nucleoside, such as C5-position of pyrimidine nucleoside,[1] C2 of purine nucleoside,[2] and 2′ of deoxyribose.[3] They were used for the incorporation of fluorophores into DNAs or RNAs. Moreover, nucleoside analogs, which were replaced by nucleic acid bases in fluorophores, have been reported.[4] Okamoto et al. developed a microenvironment-sensitive fluorescent probe using the Neil Red nucleoside.[4a] Kool et al. reported nucleosides bearing polycyclic aromatics as bases, such as naphthalene, phenanthrene, and pyrene.[5,6] A small fluorescent group is preferred as the modified group in DNA because it is not expected to disrupt the DNA duplexes significantly.

3-Aminobenzonitrile (*m*-cyanoaniline) was reported as a fluorescent molecule that is sensitive to several parameters of the surrounding environment, such as polarity and water content.[7] 3-Aminobenzoic acid is also a fluorescent molecule, in which the fluorescence of the molecule depends on the polarity of the solvent and the pH of its aqueous solutions.[8] The molecular size, like that of a fluorescent molecule, is relatively small. The modified amino acid was derived using 3-amonobenzonitrile derivatives to produce a novel fluorescent probe for studying living systems.[9] We also reported a fluorescent C-nucleoside bearing 3-aminobenzonitrile as the nucleobase.[10] The fluorescence intensity and emission wavelength of this nucleoside analog were found to be strongly dependent on the polarity of the solvents. These nucleoside analogs are expected to not form a Watson–Crick base pair with specific natural bases, but the oligodeoxyribonucleotide bearing these nucleoside analogs can be expected to form duplexes since the bases are small. Such bases will be equally affected by four natural bases. When the bases have an environmental sensitive fluorescent property, they will be responsive to perturbation raised from the surrounding environment. Such a fluorescent base is useful for the analysis of any single-base mutations.[11]

Here, we report the synthesis of fluorescent C-nucleoside analogs containing a relatively small and microenvironment-sensitive fluorophore, their incorporation into DNA, and their fluorescent properties. We describe in detail the synthesis and fluorescence properties of two of these nucleoside analogs, which have 3-aminobenzonitrile or 3-aminobenzoic acid as a nucleobase, as

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shown in Fig. 1. These nucleosides are incorporated into DNA, and their properties, such as the stability and fluorescence in the duplexes, are studied.

1. Results and discussion

Fluorescent aromatic groups were attached to glycal by a Heck-type coupling reaction to produce C-nucleosides. For the coupling reaction, iodophenyl derivatives were prepared by iodination of the corresponding aniline derivatives. The 3-aminobenzonitrile derivative, which is the nucleobase moiety of compound **1**, and the 3-aminobenzoic acid derivative, which is the nucleobase moiety of compound **2**, were prepared as shown in Scheme 1. The 3-aminobenzonitrile derivative was synthesized by diazotization and the Sandmeyer reaction of 2-amino-5-nitrobenzonitrile to obtain 2-iodo-5-nitrobenzonitrile (**3**), and then reduced to give 5-amino-2-iodobenzonitrile (**4**). The 3-aminobenzoic acid derivative was synthesized by diazotization and the Sandmeyer reaction of 2-amino-5-nitrobenzoic acid to obtain 2-iodo-5-nitrobenzoic acid sodium salt (**5**), and then subjected to esterification and reduction to give methyl 3-amino-6-iodobenzoate (**7**) in good yield. The amino group of **7** was protected by a phenoxyacetyl (Pac) group before coupling with glycal. The coupling reaction of a halogenated aromatic compound with glycal has been reported in the literature.[12] Both iodo-derivated 3-aminobenzonitorile (**4**) and protected 3-aminobenzoic acid (**8**) were coupled by a Heck-type reaction with protected glycal, as shown in Scheme 2. The reaction proceeded by palladium-catalyzed couplings followed by deprotection of silyl groups and the reduction in moderate yields totally. A part of compound **10** was deprotected in the amino group to obtain compound **2** for the measurement of its fluorescent spectrum. The anomeric configurations of compound **1** and compound **2** were determined by coupling constants of H1′ and H2′ protons. Ren et al. reported that H1′− H2′coupling constants were 6–8 Hz for α-C-nucleosides, and 5 and 10 Hz for β-C-nucleosides.[13] Experimental H1′− H2′coupling constants of compound **1** and compound **2** were J = 5.4 and 10.5 Hz, and J = 5.9 Hz and 10.0 Hz, respectively. Therefore, both compounds were assigned to be β-anomers. Compound **1** and compound **10** were derived to protected nucleosides to be incorporated into DNA. The amino group of compound **1** was protected by the Pac group. Initially, the amino group was protected by the acetyl group, but the protection group was not efficiently removed after the oligonucleotide assembling. Therefore, the Pac group was used as an amine-protecting group since it could be easily removed by mild conditioning. To assemble oligonucleotides containing fluorescent C-nucleoside analogs, protected C-nucleoside 3′-phosphoramidites were synthesized, as shown in Scheme 3. The 5′-hydroxy groups of compounds **9** and **10** were protected with a dimethoxytrityl (DMTr) group (**11** and **12**), and then, the 3′-hydroxy group was derived to cyanoethyldiisopropylphosphoramidite (**13** and **14**). The compounds were characterized by their melting temperatures, ^{1}H NMR, ^{13}C NMR, and ESI mass spectrometry. Also, compound **1** and compound **2** were characterized by their specific rotations. In addition, 5′-DMTr-nucleoside 3′-phosphoramidite analogs, **13** and **14**, were identified by ^{31}P NMR and high-resolution mass spectroscopy.

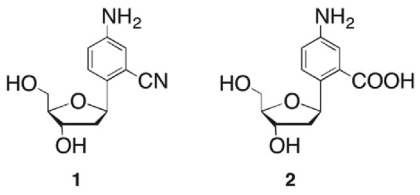
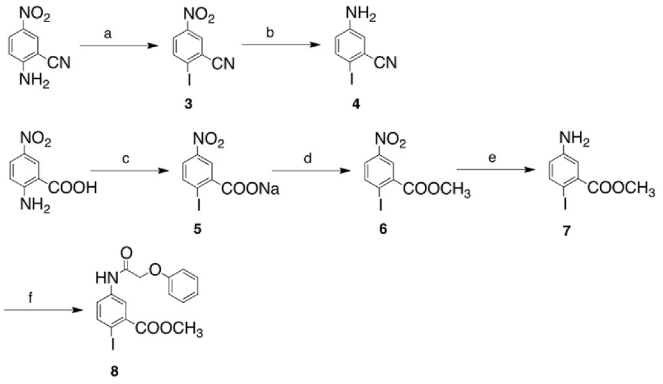
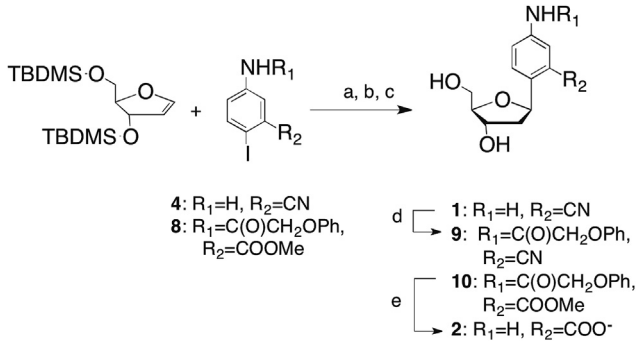


Fig. 1. Structures of fluorescence C-nucleosides in this study.



Scheme 1. Reagents and conditions: (a) (i) $NaNO_2$, conc. HCl, (ii) KI; (b) $SnCl_2$, EtOH; (c) (i) $NaNO_2$, conc. HCl, (ii) KI; (d) (i) $SOCl_2$, (ii) MeOH; (e) $SnCl_2$, EtOH; (f) $(Pac)_2O$, DMF.



Scheme 2. Reagents and conditions: (a) $Pd(OAc)_2$, $As(Ph)_3$, n-Bu_3N, DMF, 90 °C; (b) AcOH, n-TBAF; (c) $NaBH(OAc)_3$, CH_3CN, AcOH; (d) $(Pac)_2O$, DMF; (e) 1 M NaOH aq.

These modified oligodeoxyribonucleotides (ODNs) and other unmodified ODNs were synthesized by the solid-phase phosphoramidite method using a DNA synthesizer. The modified C-nucleoside phosphoramidites were coupled with prolonged coupling time (600 s). These synthesized ODNs were deprotected and purified in an ordinary manner; their sequences and abbreviations are listed in Table 1 **mDNA-1** and **mDNA-2** contain nucleoside **1** and nucleoside **2**, respectively. Both nDNA and cDNA are natural oligodeoxyribonucleotides. nDNA has the same sequence as that of mDNA, but contains thymidine instead of a modified nucleoside. cDNA is a complementary ODN against mODN and nODN, and A, G, C, or T is contained as a complementary base to the modified nucleoside.

It was reported that 3-aminobenzonitrile exhibited a shorter fluorescence wavelength in a hydrophobic than that in a hydrophilic solvent and fluorescence quenching in hydrogen-bond forming solvents, especially water.[7] In addition, the fluorescence of 3-aminobenzoic acid depended on the solvent and solution pH.[8] Fluorescence spectra of their attached C-nucleosides were measured for different solvents and pH values (Fig. 2). Nucleoside **1** showed strong emission in hydrophobic solvents and poor emission in water, similarly to 3-aminobenzonitrile. The pH of the solutions did not affect the fluorescence intensity at pH 4–9. On the other hand, nucleoside **2** showed strong emission in hydrophilic solvents and poor emission in hydrophobic solvents. Also, as the pH increased from 4 to 9, the fluorescence intensity increased, markedly changing between pH 4 and 5, corresponding the pKa of the 3-aminobenzoic acid moiety. This suggested that fluorescence properties depended on the dissociation of the carboxy group.

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