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Doubly thiazole orange-labeled cytidine for functional expansion of a hybridization-sensitive probe

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ABSTRACT

A 2′-deoxycytidine derivative modified by two thiazole orange dyes has been designed for functional expansion of hybridization-sensitive fluorescence probes. This nucleotide was synthesized by a procedure that included protection of the cytosine amino group by di(*n*-butyl)formamidine formation, followed by incorporation into DNA. The fluorescence of the synthesized DNA was controlled by excitonic interaction, showing strong fluorescence upon hybridization with the target nucleic acid and effective quenching in a single-stranded state of the probe.

A number of fluorescent DNA probes have been developed for observation of the functions of target nucleic acids.[1] In particular, much effort has been invested in the rational design of hybridization-sensitive fluorescent DNA probes to effectively detect the target nucleic acid, using on–off switching of fluorescence, depending on hybridization.[2] The functional fluorescent label of the probe is attached to either the strand ends or the internal nucleotide. Creation of the end label is synthetically easy, but it often exhibits relatively ineffective fluorescence switching due to low spatial restriction around the fluorescent dyes. The internal label exhibits effective fluorescence switching but sequence recognition is limited according to the type of modified nucleotide. The thymidine and uridine derivatives are the most common ones that have been developed as labeled nucleotides because protection of the base units during their synthesis is not required.

We have designed a new type of hybridization-sensitive probe.[3] This is an internal labeled probe that includes a 2′-deoxyuridine derivative modified by two thiazole orange dyes (X in Fig. 1) to achieve high fluorescence intensity for a hybrid with the target nucleic acid, such as single-stranded DNA, mRNA, and microRNA (miRNA), and effective quenching for a single-stranded state of the probe. The fluorescence is controlled by interdye excitonic interaction in the probe. However, the choices of the sequence, probe length and X-incorporation site in the probe design are limited when there are only a few adenine bases in the target sequence. Therefore, the synthesis of an alternative to X based on another nucleobase, for example, cytosine, is required. The development of a new fluorescent nucleotide will extend the choices of the design of hybridization-sensitive probes.

In this Letter we report on the synthesis and fluorescence of a doubly dye-labeled 2′-deoxycytidine. The efficient synthesis, with the choice of an appropriate protective group for an exocyclic amino group, afforded a fluorescence-labeled cytosine. Fluorescence from this cytosine derivative controlled by interdye excitonic interaction made possible the highly sensitive detection of nucleic acids (Schemes 1 and 2).

We designed a doubly dye-labeled 2′-deoxycytidine (Z) as an alternative to the uridine derivative X (Fig. 1). The feature of the synthesis of Z that differs from the synthesis of X is the existence of the exocyclic amino group of cytosine. For the successful synthesis of Z-containing DNA, protection of the cytosine amino group is required, and the protective group must be removed under only weak basic conditions after DNA synthesis. Initially, methyl acrylate was incorporated into 5-iodo-2′-deoxycytidine **1** in a Heck reaction (53%), and subsequently the ethyl ester was hydrolyzed to a carboxylic acid **2** (91%). We next protected the exocyclic amino group of **2**. Acetyl, benzoyl, and dimethylformamidine groups are often used as protective groups of a cytosine amino group. In our case, we chose not to use acetyl and benzoyl groups for the protection of the amino group because temporary protection with silyl groups is essential for the free hydroxyl groups at the 3′ and 5′ positions of the nucleoside. We opted to use the dimethylformamidine group to protect the cytosine amino group. The dimethylformamidine group is effective for

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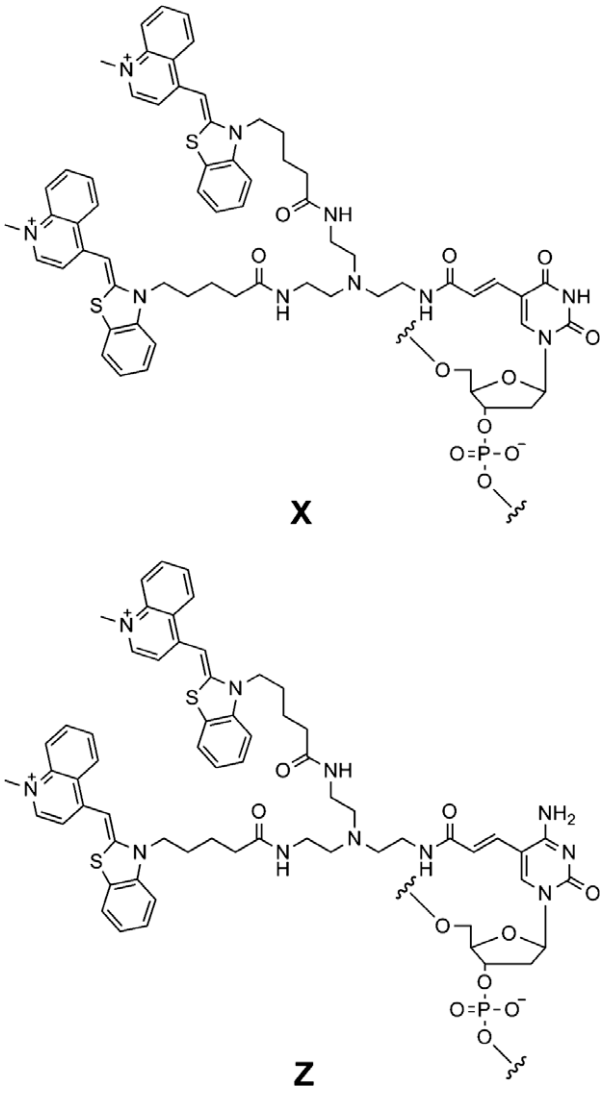


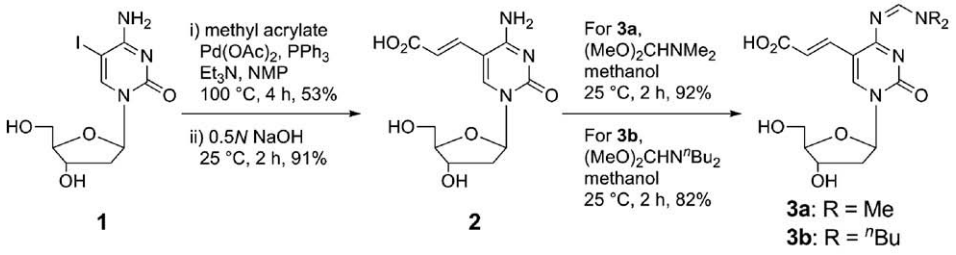
Figure 1. 2′-Deoxyuridine derivative (X) and a newly designed 2′-deoxycytidine derivative (Z), the fluorescence emission of which is controlled by interdye excitonic interaction.

the protection of the amino group without prior protection of hydroxyl groups, and it can be converted back to a free amino group under weak alkaline conditions. *N*,*N*-Dimethylformamide dimethyl acetal was added to a solution of **2** in methanol to afford **3a** after reaction at 25 °C for 2 h (92%). However, the subsequent coupling reaction of a carboxylic acid of **3a** and tri(2-aminoethyl)amine was inefficient, and the cytosine amino group was deprotected during the reaction.

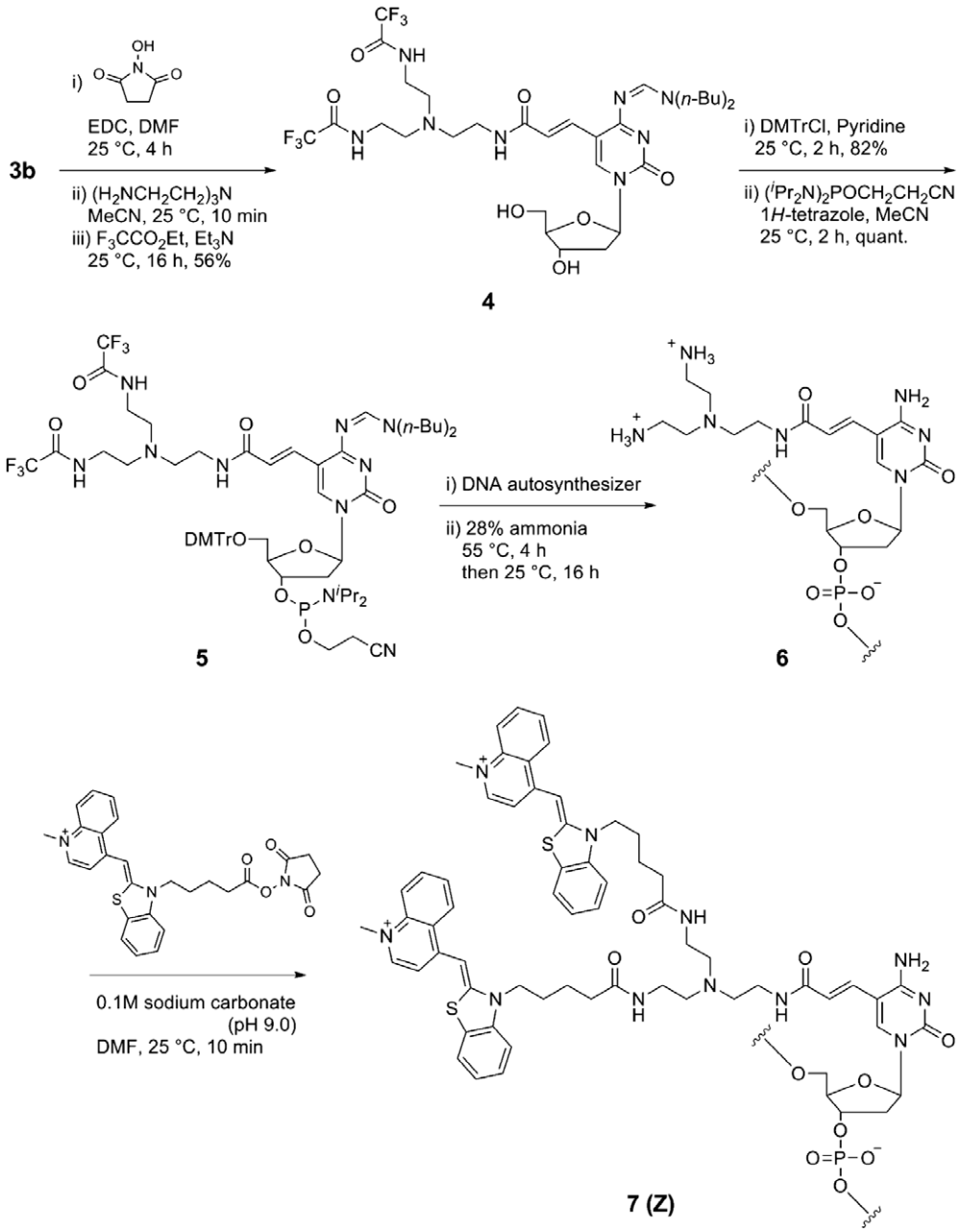
We next considered the formation of the more bulky amidines, dibenzylformamidine and di(*n*-butyl)formamidine, as candidate protective groups in order to avoid deblocking during the coupling reaction. The dibenzylformamidine group has been used for the protection of the primary amino group of guanine,[4] but in our case the yield of the incorporation of dibenzylformamidine in the preparation of Z was very low (trace). On the other hand, although there are only a few reports of the use of the di(*n*-butyl)formamidine group,[5] in our synthesis the di(*n*-butyl)formamidine group was successfully incorporated into the amino group of **2** and we obtained compound **3b** (82%). Compound **3b** was coupled with tri(2-aminoethyl)amine after succinimidylation, followed by the protection of two newly generated amino ends with a trifluoroacetyl group to afford compound **4** (56%). During these reactions, removal of di(*n*-butyl)formamidine attached to the cytosine amino group was not observed.

Compound **4** was protected with a 4,4′-dimethoxytrityl group (82%), and then converted quantitatively into phosphoramidite **5**. This compound was incorporated into DNA using a conventional phosphoramidite solid phase synthesis method (>90%). The synthesized DNA was subsequently deprotected in aqueous ammonia at 55 °C for 4 h and then at 25 °C for 16 h. During this reaction the di(*n*-butyl)formamidine of the Z precursor in the DNA was also converted into a free amino group. The fluorescent DNA probes containing a doubly fluorescence-labeled 2′-deoxycytidine were obtained by the covalent bonding of two molecules of thiazole orange derivatives[3a] with the two alkyl amino groups of the modified cytidine (98%). The synthesized DNA was purified by reverse-phase HPLC, and then used in the following experiments.

The fluorescence intensities of the Z-containing fluorescent probes were very different after hybridization with the complementary RNA strands.[6] For example, CGCAATZTAACGC displayed negligible fluorescence in the single-strand state in a phosphate buffer (pH 7.0). On the other hand, after mixing with 1 equiv of the complementary RNA, GCGUUAGAUUGCG, strong fluorescence of CGCAATZTAACGC appeared at 530 nm (Fig. 2). The reason for the hybridization-dependent change in the fluorescence intensity can be explained by the shift in the absorption maximum. The absorption spectrum before hybridization showed a stronger band at 478 nm and a weaker band at 508 nm ($A_{478}/A_{508} = 1.54$), whereas the band at 508 nm was predominant after hybridization ($A_{478}/A_{508} = 0.57$). The shift in the absorption maximum suggests that switching of the excitonic interaction between tethered thiazole orange dyes worked effectively, depending on hybridization with the RNA strand.[3a] The absorption at shorter wavelength before hybridization is caused by splitting of the excited state into two energy levels due to H-aggregation of the dyes[7] and excitation to the higher excitonic state. The excitation rapidly deactivates to the lower excitonic state, from which emission is forbidden. Hybridization with the complementary RNA induces dissociation of the dye aggregate and release from interdye excitonic interaction, resulting in a large enhancement of fluorescence. Other Z-containing fluorescent probes, TTTTTTZTTTTTT and TACCAGZCACCAT, also exhibited an absorption band shift and the switching of fluorescence emission, based on interdye excitonic interaction (Table S1).



Scheme 1. Protection of cytosine's exocyclic amino group.



Scheme 2. Synthesis of a DNA-containing doubly thiazole orange-labeled 2′-deoxycytidine.

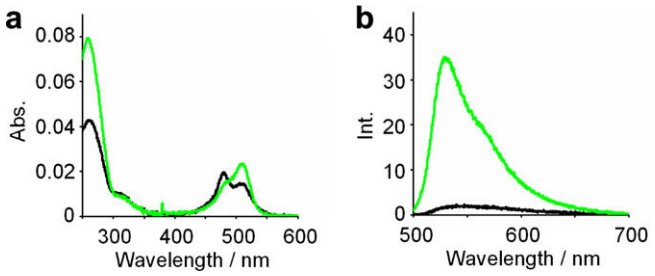


Figure 2. Absorption and emission spectra of CGCAATZTAACGC. (a) Absorption spectra. (b) Fluorescence emission spectra. Spectra of the probe (0.4 μM) were measured in 50 mM sodium phosphate (pH 7.0) containing 100 mM sodium chloride at 25 °C. λ_{ex} = 488 nm. Black, spectra before hybridization; green, spectra after hybridization with RNA, GCGUUAGAUUGCG.

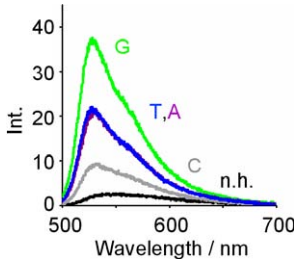


Figure 3. Emission spectra of CGCAATZTAACGC hybridized with DNA GCGTTA-NATTGCG (N = G (green), T (blue), A (purple), or C (gray)). Spectrum of nonhybridized state (n.h.) shows a black line. Spectra of hybrids (0.4 μM) were measured in 50 mM sodium phosphate (pH 7.0) containing 100 mM sodium chloride at 25 °C. λ_{ex} = 488 nm.

A significant change in fluorescence intensity was also observed for the hybrids with the target single-stranded DNA sequence. The hybrid of CGCAATZTAACGC with the complementary DNA showed strong fluorescence in contrast to the very weak fluorescence of the single-stranded state (Fig. 3). The hybrids with the DNA strand containing one base alternation at the site opposite to Z also emitted fluorescence. However, the fluorescence intensity was lower compared with that of the hybridization with fully matched DNA, and the quantum yields of mismatched hybrids decreased to 33–68% of that of the full-matched hybrid.

These fluorescence functions of Z-containing DNA probes are applicable to the efficient detection of short RNA. Use of the Z probe might be one of the most appropriate methods to detect short RNA strands, among the many RNA detection probes, such as molecular beacon,[8] MS2-GFP fusion proteins,[9] GFP reconstitution,[10] and quenched autoligation probes.[11] One type of short RNA, miRNA, is a 21- to 23-nucleotide noncoding RNA processed from hairpin precursors that have been identified in the genomes of a wide range of multicellular life forms, including plants and animals.[12] miRNA can regulate gene expression at the translational level through interactions with their target mRNAs. miR-375 is the highest expressed miRNA in pancreatic islets of humans.[13] Overexpression of miR-375 suppresses glucose-induced insulin secretion and, conversely, inhibition of the endogenous miR-375 function enhances insulin secretion. This miRNA, UUUGUUCGUUCGGCUCGCGUGA, has only one adenine base at the 3′ end. Therefore, probe design for miR-375 detection is very limited when the uridine derivative X is used for a hybridization-sensitive probe. Development of Z solved this problem, and extended the choice of probe design. In fact, the X-containing probe XCACGCGAGCCGAACGAACAA showed a low ratio of fluorescence intensities after and before hybridization (I_{ds}/I_{ss} = 2.5) (Fig. 4). The low I_{ds}/I_{ss} value is due to labeling at the strand end; inefficient binding of dyes to a hybrid structure results in weak fluorescence of the hybrid. Because the sequence of miR-375 includes only one adenine base, it is difficult to design another probe using X. On the other hand, when Z is available, we can select the best probe among many Z probe candidates. The I_{ds}/I_{ss} of a Z-containing probe, TCACGCGAGCCGAAZGAACAAA, was improved for miR-375 detection (I_{ds}/I_{ss} = 4.0). In addition, Z can be incorporated into several sites of the probe sequence. A probe in which two Z nucleotides were incorporated, TCACGZGAGCCGAAZGAACAAA, showed high fluorescence intensity of the probe in the presence of miR-375 and high quenching efficiency of the probe in the absence of miR-375 (I_{ds}/I_{ss} = 10). This high I_{ds}/I_{ss} value facilitated the observation of the clear yellow-green fluorescence of the Z-containing probe in the presence of miR-375 under white light illumination (Fig. 4c).

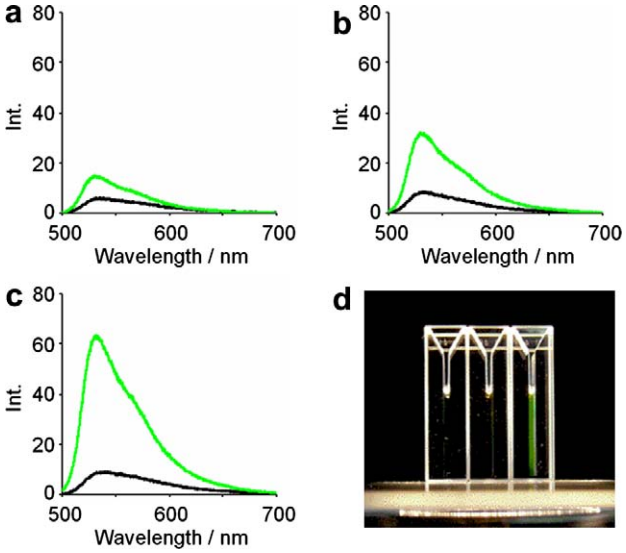


Figure 4. Detection of miR-375 by the fluorescence of hybridization-sensitive fluorescence probes. Emission spectra of probes (0.4 μM) were measured in 50 mM sodium phosphate (pH 7.0) containing 100 mM sodium chloride at 25 °C. (a) Emission spectra of an X-containing probe XCACGCGAGCCGAACGAACAA. Black, spectra before hybridization; green, spectra after hybridization with miR-375. λ_{ex} = 488 nm. (b) Emission spectra of a Z-containing probe TCACGCGAGCCGAAZGAACAAA. (c) Emission spectra of a Z-containing probe TCACGZGAGCCGAAZGAACAAA. (d) Fluorescence emission in cuvettes. A solution containing 2 μM probe TCACGZGAGCCGAAZGAACAAA in the absence (center) or presence (right) of 1 equiv of miR-375 containing 100 mM sodium chloride was irradiated with a 150 W halogen lamp. The left cuvette included neither probe nor miRNA.

In conclusion, we designed a new on–off fluorescent DNA probe based on 2′-deoxycytidine. The efficient synthesis, with the choice of an appropriate protective group for an exocyclic amino group, afforded a fluorescence-labeled cytosine. Fluorescence from this cytosine derivative is controlled by interdye excitonic interaction, which made highly sensitive nucleic acid fluorescent detection possible. Any cytosines in a sequence can be replaced by a newly labeled nucleotide, which extends the choices of probe design, as seen in the experiment using miR-375. The exciton-controlling probes are not only unique, but also applicable to RNA imaging for direct observation of gene expression.

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Supplementary data

Supplementary data (detailed synthetic procedures, HPLC profile, NMR data, T_m values, quantum yields, absorption, excitation, and emission spectra) associated with this paper can be found, in the online version, at doi:10.1016/j.tetlet.2009.10.037.

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