Synthesis of fluorescent cyclic cytosine nucleosides and their fluorescent properties upon incorporation into oligonucleotides

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ABSTRACT

We report here the synthesis and spectroscopic characterization of a new fluorescent pyrimidopyrimidoindole nucleoside derivative (dCPPP) modified at the cytosine base. The photophysical properties of dCPPP were examined by fluorescent spectroscopy and quantum chemical calculations. It was found that dCPPP-labeled oligonucleotides gave almost the same thermal stability as that of the corresponding unmodified sequences. In addition, the quantum yield of dCPPP in the double-stranded state was significantly higher than that in the single-stranded state.

INTRODUCTION

Fluorescence imaging is the most powerful technique currently available for continuous observation of the dynamic intracellular processes of living cells. The properties that are desirable in various applications are emission intensity, ease of preparation and conjugation, water solubility, varied excitation and emission wavelengths and large Stokes' shifts. As commercial available fluorophores, fluorescein and rhodamine derivatives are presumably the most widely used in biological research. Unfortunately, the synthesis of even simple derivatives involves often tedious multi-step procedures accompanied by low overall yields and is a serious limitation for tuning of their photophysical properties. Therefore, strategies for tuning the colours without reducing the fluorescence intensity are very important.

To image the behavior of DNA with a fluorescent molecule, a number of fluorescent nucleosides in place of the natural DNA bases have already been reported. These probes proved to be useful for understanding biological processes such as replication, transcription, recombination, and gene repair, because they minimally perturb the DNA structure. Among them, 2′-deoxy-2-aminopurine (2-AP) is the most popular. 2-AP has a high quantum yield (0.68) at physiological pH and a low excitation energy compared to the natural nucleobases and can therefore be selectively excited. Other commercially available fluorescent base analogs are pteridines 3-methylisoxanthopterin (3MI) and 6-methylisoxanthopterin (6MI). Saito et al reported the synthesis and fluorescent properties of benzopyridopyrimidine nucleoside (BPP). Recently, our group also reported a fluorescent pyrimidine nucleoside (a bicyclic 4-N-carbamoyldeoxycytidine derivative (dCPPP) and pyrolyrpidopyrimidine nucleoside (dCPPP)) having rather strong fluorescence (quantum yield: 0.10 in an aqueous buffer) and being quenched by the presence of guanine. Despite the report of such core skeletons of fluorescent nucleobases, the studies to modify the properties of these fluorescent nucleobases by introduction of substituents are scarce. Therefore, we decided to investigate the possibility to tune the photophysical properties of dCPPP by modifying the structure.

In this study, we report the synthesis and experimental studies on the photophysical properties of a pyrimidopyrimidoindole nucleoside derivative (dCPPP) as a ring expanded analog of dCPPP and dCPPP derivatives having various substituents at the indole ring. We tried to establish the fluorescent base analogue dCPPP as a fluorescent label of DNA probes with minimal disturbance of their overall structure. We also report the influence on the DNA stability when a normal cytosine base is replaced by dCPPP. The fluorescent measurement demonstrated that the relative fluorescent intensities of dCPPP derivatives in DNA duplexes increased markedly in comparison with those in the single strand state (Figure 1).

![Light emission](https://academic.oup.com/nass/article-abstract/50/1/19/1340657/bqfig1)

**Figure 1.** Light emitting DNA probe containing dCPPP upon hybridization with the complementary DNA strand.

Furthermore, *Tm* experiments were carried out to determine the stability of double-stranded DNAs containing a dCPPP derivative. From the data thus obtained, it was confirmed that the duplex containing a dCPPP is as thermally stable as the natural duplex.

RESULTS AND DISCUSSION
In the first step, 10-(2-deoxy-β-D-ribofuranosyl)-pyrimido[4',5':4,5]pyrimido[1,6-α]indole-6,9(7H)-dione (dC^{PP}) and its derivatives, dC^{R,PP} where R represents various substituents at the 3-position, were synthesized via a Suzuki-Miyaura coupling reaction of 5-iododeoxyctydine with 5-substituted N-Boc-indole-2-borates followed by spontaneous cyclization (Scheme 1). The dC^{PP} derivatives were obtained in satisfactory yields by use of Pd(OAc)$_2$ as a catalyst and TPPTS [tris(3-sulfonatophenyl)phosphine] as a ligand.

![Scheme 1. Synthesis of dC^{R,PP} derivatives.](image)

In the next step, the absorption and emission properties of dC^{PP} derivatives in 50 mM phosphate buffer, pH 7.0 were determined and compared to those of dC^{PP}. The absorption, excitation and emission spectra of dC^{PP} at room temperature are shown in Figure 2. The dC^{PP} exhibits a broad absorption spectrum, with a maximum absorption wavelength at 374 nm. The fluorescence spectrum consists of a broad band with maximum emission wavelength at 513 nm, which is more red-shifted than dC^{PP}. It was also found that dC^{PP} exhibited a much larger Stork's shift of 139 nm than that (121 nm) of dC^{PP}. By changing the substituents at the 3-position, it turned out that the electron-withdrawing groups increased the fluorescent intensity and the electron-donating groups having lone pairs of electrons reduced the intensity. Moreover, the Lippert-Mataga analysis of the Stokes’ shifts was also carried out to obtain estimated values of the dipole moments in the excited states of the derivatives.

The phosphoramidites were successfully prepared and incorporated into oligonucleotides having d(CGCAATNTACGC) sequences (N = dC^{PP} or its derivatives). To characterize the dC^{PP}-containing oligonucleotides, the thermodynamic stability of their duplexes was compared with those of the corresponding unlabeled duplexes by monitoring the absorbance changes at 260 nm. The $T_m$ analysis revealed the thermal stabilities of the dC^{PP}-containing duplexes were almost the same as that of the unlabeled duplex. These results suggested that dC^{PP} forms hydrogen bonds with guanine in the complementary strand without distorting the DNA conformation. To further assess the potential use of dC^{PP} as a fluorescent base, we characterized spectroscopic properties of the oligonucleotides incorporating dC^{PP} derivatives in their single-stranded or duplex states. From the fluorescent studies of a dC^{R,PP} (R = OMe)-containing single strand, it turned out that the quantum yield in the duplex state was almost four times larger than that in the single strand state.

![Figure 2. Absorption and emission spectra of dC^{PP} in 50mM phosphate buffer (pH 7.0). The fluorescence emission spectrum was obtained at $\lambda_{ex} = 366$ nm.](image)

**CONCLUSION**

We have synthesized a new fluorescent cytosine analog dC^{PP} and have showed the control of photophysical properties by changing the substituent at the 3-position. Some of the dC^{PP} derivatives showed interesting solvent dependent fluorescence enhancement and could be useful as the fluorescent structural probes for nucleic acids.

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**REFERENCES**