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4-Cyanoindole-2′-deoxyribonucleoside (4CIN): A Universal Fluorescent Nucleoside Analogue

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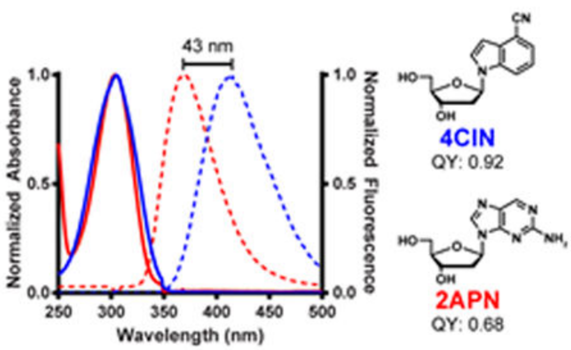
Abstract

The synthesis and characterization of a universal and fluorescent nucleoside, 4-cyanoindole-2′-deoxyribonucleoside (**4CIN**), and its incorporation into DNA is described. **4CIN** is a highly efficient fluorophore with quantum yields >0.90 in water. When incorporated into duplex DNA, **4CIN** pairs equivalently with native nucleobases and has uniquely high quantum yields ranging from 0.15–0.31 depending on sequence and hybridization contexts, and surpassing that of 2-aminopurine, the prototypical nucleoside fluorophore. **4CIN** constitutes a new isomorphic nucleoside for diverse applications.

Graphical abstract



Fluorescent nucleosides are powerful tools for a variety of applications in chemical biology.[1,2] The isomorphic and fluorescent nucleoside analogue, 2-aminopurine (**2APN**, Figure 1), is arguably the most widely utilized of such compounds. **2APN**'s environment sensitive fluorescence has been used to study polymerases,[3–5] base-flipping enzymes,[6] DNA dynamics,[7] DNA mismatches,[8] riboswitches,[9,10] and electron transfer in DNA,[11] and its continual use has sustained experimental and computational studies of the molecule itself.[12–19] Limitations of **2APN** include a 5-step chemical synthesis from guanosine (one step

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.Supporting figures and table, experimental procedures, and spectral characterization data (PDF) synthesis from thioguanosine),[20,21] a modest Stokes shift, and strong fluorescence quenching in DNA.[11,18,22] Consequently, highly fluorescent isomorphic nucleosides with improved fluorescence properties in DNA are desired, with notable recent advances.[1,2,23–25]

Indole is a privileged sc affold for native nucleobases (*e.g.,* adenine and guanine),[26,27] and amino acids (*e.g.*, tryptophan). Recently, cyano-functionalized indoles have proven interesting as fluorescent tryptophan mimics.[28–31] 4-cyanoindole (**4CI**, Figure 1) has demonstrated the most impressive properties among the cyanoindoles in terms of fluorescence intensity, red-shifted excitation and emission wavelengths, and Stokes shift.[28] Herein, we report the application of the **4CI** fluorophore to the development of a fluorescent, isomorphic nucleoside analogue, 4-cyanoindole-2′-deoxyribonucleoside (**4CIN**, Figure 1), with significantly enhanced fluorescence properties compared to **2APN**.

4CIN is synthesized in two steps starting from **4CI** and commercially available 3,5-di-O-toluoyl-α−1-chloro-2-deoxy-D-ribofuranose (**1**) (Scheme 1).[33] Elaboration to the phosphoramidite for oligonucleotide synthesis follows a two-step sequence of 4,4′-dimethoxytrityl protection of the 5′-alcohol to yield **3**, followed by formation of phosphoramidite **4** using 2-cyanoethyl-*N,N′*-diisopropylchlorophosphoramidite. The **4CIN** phosphoramidite can be synthesized in four steps at 35% overall yield.

We first studied **4CIN** and benchmarked it to **2APN** by evaluating absorption and emission spectra (Figure 2, maxima in Table 1). Nucleobases **4CI** (Figure 2A) and 2-aminopurine base (Figure S1A) share similar maximum absorbance wavelengths (304 nm and 305 nm, respectively), but **4CI** emits at 393 nm in comparison to 2-aminopurine base at 373 nm. In a nucleoside context, **2APN** (Figure 2C) has no change in its absorbance or emission properties relative to the nucleobase, whereas **4CIN** has a large emission shift with a maximum at 412 nm.[18,34]

4CIN was then evaluated to determine molar extinction coefficients (ε), fluorescence lifetimes (τ), and relative quantum yields (QYs) in multiple solvents. The extinction coefficient at 305 nm for **4CI** was previously reported as 8056 $M^{-1}cm^{-1}$.[31] We calculated the ε_{305} for **4CIN** (H_2O) to be 7790 ± 320 $M^{-1}cm^{-1}$ (Table 1). We also calculated ε_{305} for **4CIN** in different solvents (*e.g.,* PBS and THF), finding similar absorbance properties. Fluorescent lifetimes were measured on a time-resolved fluorometer[35] by exciting at 355 nm, and relative to **4CI**[31] we found **4CIN** observed only a small lengthening of τ to approximately 10 ns in aqueous solutions, although in THF, the lifetime drops to 5.6 ns. Relative QYs were collected for **4CI**, **4CIN**, and **2APN** utilizing previously described protocols and L-tryptophan (**Trp**, Figure S1B) and quinine sulfate (**QS**, Figure S1C) as standards.[36] Our measurements for **4CI** (0.86 at 270 nm; 0.73 at 305 nm; 0.76 at 325 nm) were consistent with a previous report (0.85 at 270 nm; 0.78 at 325 nm).[31] Commercial **2APN** was also subjected to this procedure yielding a relative QY of 0.61 at 305 nm, which agrees well with the accepted value of 0.68.[18] **4CIN** yielded remarkably improved QY properties over **4CI**, with marked increases in QY at both 305 nm and 325 nm to 0.92, while at 270 nm no change was observed (0.85 at 270 nm). In PBS-buffered conditions, we found minimal changes relative to deionized water and again in THF we observed fluorescence quenching (Table 1).

Phorphoramidite **4** was next utilized to synthesize **4CIN**-functionalized oligonucleotides **ODN1** and **ODN2** (Figure 3A), where sequences were chosen to complement our previous studies of other nucleotides.[37] First, the relative thermal stabilities of model DNA sequences containing X = **4CIN**, **2APN**, and 5-NO_2-indole and Y = A, C, G, or T were determined using UV thermal melting experiments (data shown in Table S1).[37] While no **ODN1** DNA duplex was more stable than those containing either a central G-C or A-T pair (T_ms = 57.3 °C and 52.6 °C, respectively), modified **ODN1** duplexes showed similar thermal stabilities to that of G- and A- mismatches which range from 42.5 – 47.4 °C. **4CIN** modified **ODN1** duplexes showed thermal stability in the narrow range of 41.4 – 43.5 °C, while **2APN**-modified **ODN1** duplexes yielded a similar melting temperature range of 43.4 – 44.5 °C, with the exception of **2APN**'s preferred pair with Y = T (T_m = 49.8 °C).[38,39] These data suggests that **4CIN** exhibits similar, non-discriminatory hybridization properties as 5-NO_2-indole, a universal hydrophobic base,[40] which hybridizes with all base pairs in a similar thermal range (T_m range: 44.7 – 49.8 °C). Given the similarities between **4CI** and 5-NO_2-indole nucleobases (*e.g.* hydrophobic and lack of H-bond donors and acceptors), we hypothesize that **4CI** adopts similar duplex DNA conformations as 5-NO_2-indole,[26] but with the nitrile positioned towards the DNA major groove. **ODN2** was evaluated similarly (Figure 3A, Table S1), but modifications were made only on the 5′-terminus and paired only against T. All modified **ODN2** duplexes were similarly stable to that containing a natural A-T pair (T_m range: 52.0 – 54.9 °C)

The fluorescent properties of nucleosides can change in a DNA context, and thus we analyzed modified duplex **ODN1** and **ODN2** for changes in emissive properties. **2APN** is known to have shifted emission properties when incorporated into oligonucleotides,[18] and we observed similar results with **4CIN**. While both single- and double-stranded **ODN2** modified with **4CIN** saw little change in fluorescence maxima, **4CIN**-modified **ODN1** observed a lowered fluorescence maximum to 403 nm in single-stranded DNA and between 380–390 nm in duplex DNA depending on the base pair (Figure S2). The fluorescent DNAs were then evaluated to ascertain relative QYs at 305 nm in PBS buffer, both as a single-strand and in a double-stranded helix (Figure 3B). **2APN**, like most fluorescent nucleoside analogues, observes significant quenching in DNA,[18] lowering its nucleoside QY of 0.68 to 0.029 and 0.098 depending on whether it is placed centrally or terminally in our model single-stranded DNAs. In duplex DNA, a similar trend follows, where quenching is enhanced, resulting in lowered QYs for both **2APN** modified duplexes. **4CIN** modified single-stranded DNAs follow a similar trend, with QYs lowered to 0.072 and 0.168 in **ODN1** and **ODN2**, respectively, although these values are a two-fold enhancement relative to those modified with **2APN** (Figure 3B). In a DNA duplex, however, **4CIN** follows a remarkably different trend. While both modified **ODN2** duplexes observe an expected QY reduction, **4CIN**-modified **ODN1** (duplex) observes a QY increase to 0.30. Investigation of the sequence dependence of this trend, pairing **ODN1** (X = **4CIN**) against Y = A, C, G, and T (Figure 3B, **right**), revealed the QY remains constant, with the exception of when **4CIN** is paired against G, which is consistent with other reports that G can quench fluorescence.[43,44] Against a 2′-deoxyribose spacer meant to mimic an abasic site (Y = Ab, see Supporting Information; T_m = 39.9 °C for **ODN1** X = **4CIN**, Y = Ab), **4CIN** remains highly fluorescent. Given the expected trend of enhanced quenching in duplex DNA, these properties of **4CIN** are unique. Further evaluations of sequence effects on **4CIN**'s fluorescence properties are needed in future studies since the 3′ and 5′ nucleotides to a fluorophore in an oligonucleotide can have various effects on the fluorescent reporter.[22,45–48]

To rule out gross changes in duplex DNA structure as a result of **4CIN** incorporation, we performed circular dichroism (CD) measurements of modified **ODN1** (Figure 3C). CD analysis complements the thermal stability data and again shows these DNAs are quite stable and their overall helicity is not changed. The CD spectra of duplex **ODN1** (X = A, Y = T) resembles that of a typical poly[A]poly[T] sequence (deep 245 nm band and weaker 260–280 nm bands),[49] and with only minor perturbations observed between **ODN1** modified with either X = **2APN** or **4CIN** paired against Y = T. We conclude that no gross changes in DNA structure occurred with these modifications. However, more quantitative studies of **4CIN** akin to those reported with **2APN**[50] would be useful to characterize those perturbations to duplex DNA structure resulting from **4CIN** incorporation that are not evident in CD and thermal melting experiments. Nonetheless, our investigation has demonstrated that **4CIN** modified oligonucleotides may be able to discriminate between single- and double-stranded DNA, as **4CIN** fluorescence increases when hybridized (and not positioned at a terminus). Additional studies are needed to further interrogate this promising application of **4CIN** since our conclusions are based on data from two **4CIN-**modified DNA sequences.

In summary, we have shown that **4CIN** is a readily synthesized, highly fluorescent nucleoside analogue with a red-shifted maximum emission (412 nm) and high quantum yield (>0.90 at 305 nm). In addition, **4CIN** has unique fluorescence characteristics when incorporated (non-terminally) into oligonucleotides, including enhanced QYs (range: 0.15 – 0.31) when incorporated into our model double-stranded sequences in comparison to single-stranded sequences. Taken together, **4CIN**'s impressive fluorescence properties give it the potential to serve as a useful probe for various chemical and biological studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

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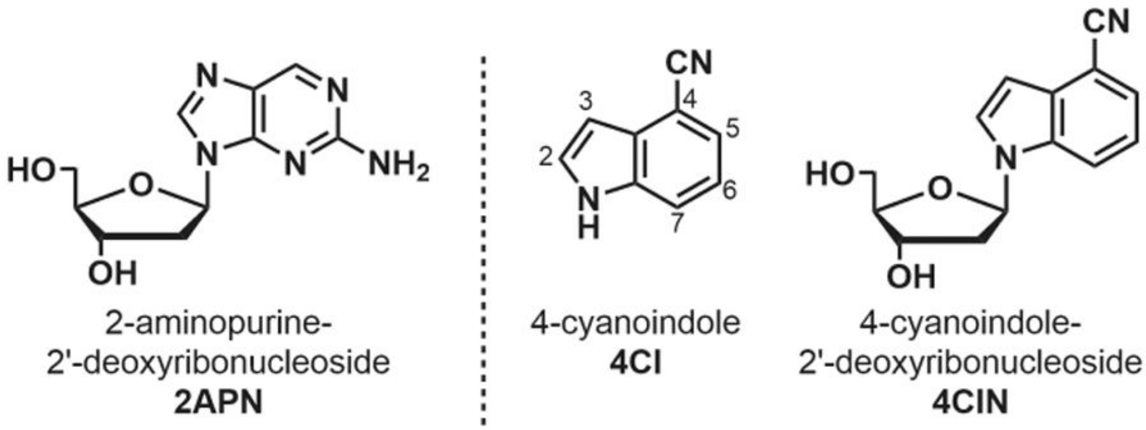


Figure 1.
Structures of **2APN**, **4CI** (with indole numbering) and **4CIN**

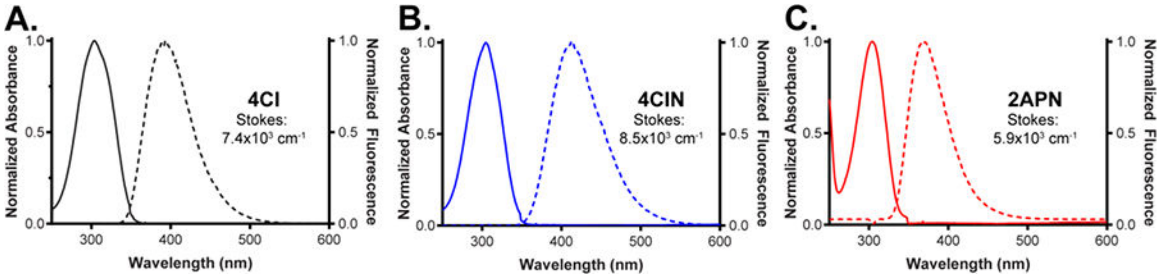


Figure 2.

Normalized absorbance (left axis, solid curve) and fluorescent emission (right axis, dotted curve) spectra of compounds and Stokes shifts.[32] Fluorescence spectra were collected by exciting at the absorption maximum. All compounds were dissolved in distilled and deionized H_2O.

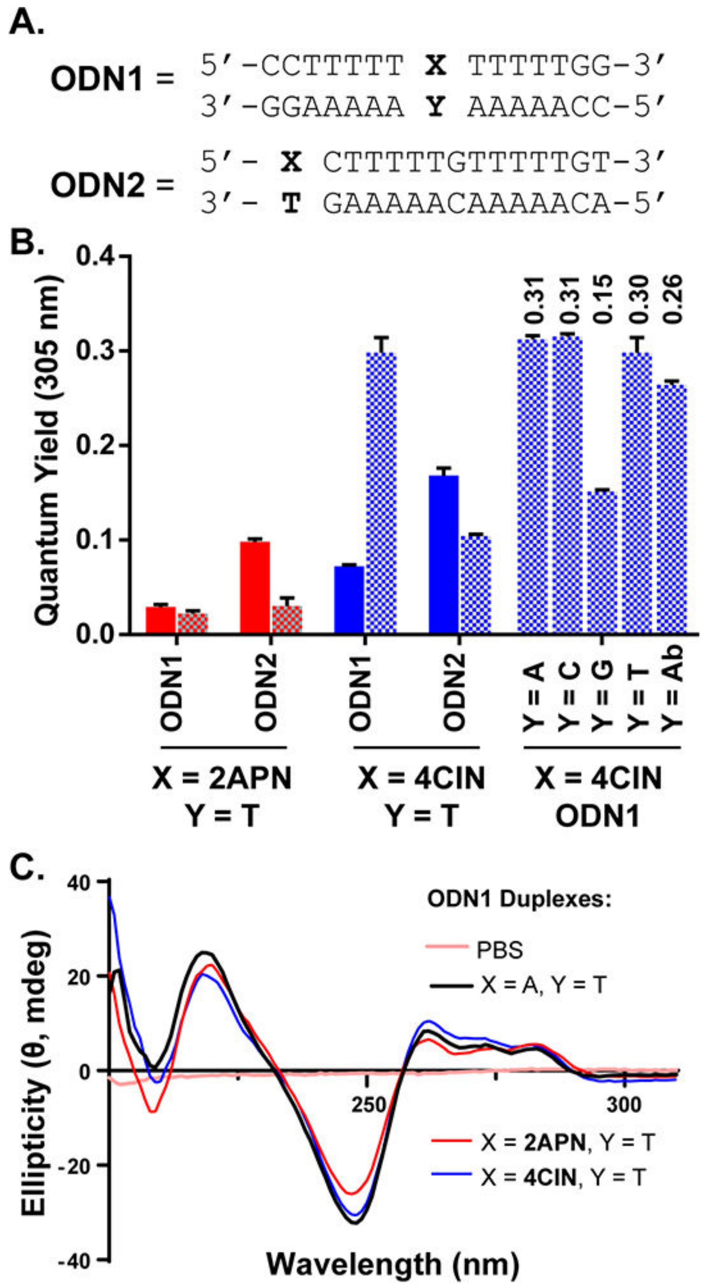
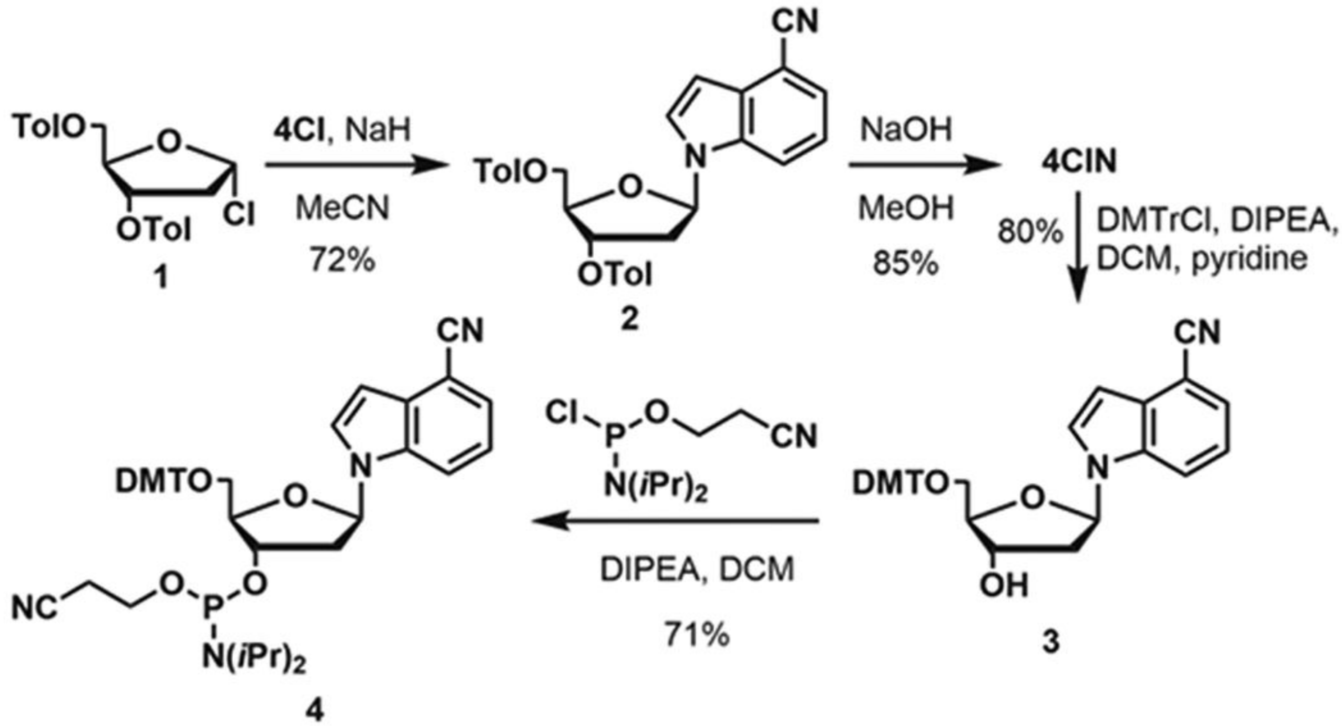


Figure 3.

A. DNA utilized as either single- or double-stranded. Double-stranded DNA was annealed in PBS (pH = 7). **B.** Quantum yields (305 nm) of **ODN1** and **ODN2** single-strands (top strand only) and duplexes. QYs measured in PBS (pH = 7) at 25 °C relative to **QS** in aqueous 0.105 M $HClO_4$ (pH = 1). Mean ± SD (n = 3) is shown. Single-stranded DNA in solid bars. Double-stranded DNA in patterned bars. **C.** Circular dichroism spectra (200 nm – 310 nm) of **ODN1** duplexes (15 nmol) in PBS (pH = 7).



Scheme 1.
Synthesis of 4CIN and phosphoramidite 4.

Table 1.

Spectral and fluorescent properties of 4CI, 4CIN, and 2APN.

sample	solvent	$\lambda_{ex, max}$ [a]	$\lambda_{em, max}$	$\varepsilon_{305\ nm}$ ($M^{-1}cm^{-1}$)	τ (ns)	QY (270 nm) [b]	QY (305 nm) [c]	QY (325 nm) [c]	Φe [d]
4CI	H_2O	304	393	8056 [e]	9.0 [f]	0.86 ± 0.03	0.73 ± 0.01	0.76 ± 0.04	5880
4CIN	H_2O	305	412	7790 ± 320	10.1	0.85 ± 0.01	0.92 ± 0.02	0.92 ± 0.01	7160
4CIN	PBS	305	412	7880 ± 120	10.0	ND	0.90 ± 0.01	ND	7100
4CIN	THF	305	381	8030 ± 180	5.6	ND	0.64 ± 0.02	ND	5140
2APN	PBS	303	369	5000–6000 [g]	11 [h]	ND	0.61 ± 0.01 [i]	ND	ND

[a.] Only local maxima greater than 250 nm are listed.

[b.] Calculated relative to **Trp** in water.

[c.] Calculated relative to **QS** in aqueous 0.105 M $HClO_4$ (pH =1).

[d.] Brightness is calculated by multiplying ε_{305} and QY_{305}.

[e.] Literature value for **4CI** in water.[31]

[f.] Our measurement is listed. Literature value for **4CI** in water is 9.1 ns.[31]

[g.] Literature values for the nucleobase and nucleoside vary[19,41,42]

[h.] Literature value for **2APN** in water.[13]

[i.] Our measurement is listed. Literature value in buffered solution is 0.68.[18]

ND = not determined. PBS used at pH = 7. See Supporting Information for experimental details.