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Pyrrolo-dC and pyrrolo-C: fluorescent analogs of cytidine and 2′-deoxycytidine for the study of oligonucleotides ☆

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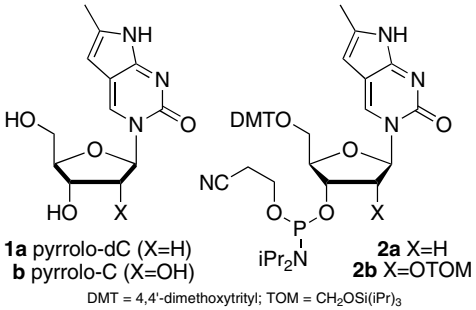
Abstract—Pyrrolo-dC (**1a**, 6-methyl-3-(2-deoxy-β-D-ribofuranosyl)-3*H*-pyrrolo[2,3-*d*]pyrimidin-2-one) and its cyanoethyl phosphoramidite **2a** were synthesized. The latter was incorporated into oligodeoxyribonucleotides by standard automated synthesis techniques, where pyrrolo-dC was found to serve as a fluorescent analog of deoxycytidine. The cyanoethylphosphoramidite (**2b**) of pyrrolo-C (**2a**, 6-methyl-3-(β-D-ribofuranosyl)-3*H*-pyrrolo[2,3-*d*]pyrimidin-2-one) was also synthesized and may find use for the site-specific incorporation of a fluorescent cytidine analog into oligoribonucleotides.

Fluorescence spectroscopy is useful for the detection and quantification of nucleic acids as well as the study of their structure, dynamics, hybridization, and interaction with other molecules, for example, proteins.[1] Applications of this sensitive technique include DNA sequencing, fluorescence in situ hybridization (FISH), fluorescence resonance energy transfer (FRET), and molecular beacons. Typically, a fluorophore is attached to the nucleic acid via a linker.[1] However, the freedom of movement of linker-based fluorophores precludes gathering a full complement of data. The measurement of fluorescence due to sites within an unmodified nucleic acid (e.g., due to the nucleobases) would be a desirable complement to the linked fluorophore approach.[2,3] For example, fluorescent bases may serve as sensitive real-time probes of base stacking and base pairing in their vicinity and may prove useful as probes for diagnostic, sequencing, and molecular recognition applications. The fluorescence of natural nucleotide bases is weak, fluorescent decay times are short, and the signals are averaged over all of the bases in the oligonucleotide, resulting in little information of use. The lack of significant natural fluorescent reporters may also be seen as an advantage, since fluorescent nonnatural nucleotide base analogs may be observed without interference.

Herein we report the synthesis of the cyanoethyl phosphoramidites **2a** and **2b** of pyrrolo-dC (**1a**) and pyrrolo-C (**1b**), respectively, for site-specific incorporation of these fluorescent deoxycytidine and cytidine analogs into oligonucleotides, as well as a synthesis of pyrrolo-dC (**1a**) itself. The 6-methyl-3*H*-pyrrolo[2,3-*d*]pyrimidin-2-one heterocycle embodies a very conservative structural change to the cytosine ring, and is stable to automated oligonucleotide synthesis. It is highly fluorescent, exhibiting an emission maximum at 473 nm in a 19-mer oligodeoxyribonucleotide, well away from emission by proteins, and its fluorescence is sensitive to the state of hybridization of the oligonucleotide, making it ideally suited for probing DNA structure and dynamics. Importantly, it base pairs normally as a cytosine, does not disrupt the DNA helix, and is tolerated by DNA and RNA polymerases.



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The search for nonnatural nucleotide base analogs with good fluorescence properties has resulted in several useful probes for site-specific incorporation into oligonucleotides by automated synthesis[3] or polymerases.[2] These are typically planar polycyclic aromatic heterocycles that are designed to retain the base stacking and hydrogen bonding interactions of the natural base.[4] Key examples of fluorescent analogs that are known in a form suitable for solid-phase oligonucleotide synthesis (e.g., as their 3′-phosphoramidites) are shown in Figure 1. 2-Aminopurine (2-AP)[5] is a popular analog of the purine bases adenine and guanine. It is available as a deoxyriboside (**3a**) or riboside (**3b**) in phosphoramidite[6] and triphosphate[2] form, and when incorporated into oligonucleotides, forms base pairs with T, U, and C.[2,3] Based on Leonard's work on etheno analogs of natural nucleobases,[7] etheno-dA (**4a**), etheno-A (**4b**), etheno-dC (**11a**), and etheno-C (**11b**) have been incorporated into DNA and RNA oligonucleotides via standard cyanoethyl phosphoramidite chemistry.[8] Note that while these structures are similar to dA/A and dC/C, the etheno bridge precludes proper hydrogen bonding for hybridization to complementary bases. Pfleiderer's fluorescent pteridine analogs of dA, 6-MAP (**5a**), and DMAP (**6a**), have been incorporated into DNA oligonucleotides via phosphoramidite chemistry,[9,10] as have the analogs of dG, 3-MI (**7a**) and 6-MI (**8a**).[10–12] These substitutions were found to be minimally disruptive of DNA structure and useful for real time measurement of changes in base stacking or pairing in their vicinity.[11,13] Benzo[*g*]quinazoline-2,4-(1*H*, 3*H*)-dione 2′-deoxyriboside (**9a**)[14] and 5-methyl-2-pyrimidinone 2′-deoxyriboside (**10a**)[15,16] are fluorescent thymidine analogs that have been incorporated into DNA oligonucleotides by phosphoramidite and phosphite triester chemistry, respectively. The latter does not hybridize successfully with adenosine. The 2′-*O*-methylriboside of 4-amino-1*H*-benzo[*g*]quinazoline-2-one (**12c**) has been incorporated into oligonucleotides via its amidite and serves as a fluorescent cytosine analog.[17] Using the H-phosphonate method, oligonucleotides containing the tricyclic cytidine analogs **13a** and **14a** were made.[18,19] These bases were found to hybridize successfully with guanosine, but apparently their fluorescent nature was not studied. In later studies, Wilhelmsson incorporated these nucleosides into peptide nucleic acids (PNAs), where they were found to hybridize with guanosine residues and exhibit useful fluorescence properties.[20] The riboside of 2-pyrimidinone (**15b**) is fluorescent and has been incorporated into oligonucleotides using cyanoethyl phosphoramidite chemistry, where it was used to study the importance of the exocyclic amino group in cytidine during catalytic cleavage of a ribozyme.[21] Finally, Inoue et al. building on work done in Bergstrom's labs, have incorporated the fluorescent pyridopyrimidine dF (**16a**) into a dodecamer via the phosphodiester method on solid support and found this fluorescent nucleotide to pair well with dG.[22] It can also pair to a dA via an alternate tautomer. Oligodeoxyribonucleotides containing **16a** have now been synthesized using the cyanoethyl phosphoramidite method and used to study DNA triple helices.[23,24] Considering the cytosine-containing analogs **11**–**16**, we felt that the 3*H*-pyrrolo[2,3-*d*]pyrimidin-2-one ring present in **1** and **2** (see above) would retain the full base-pairing and hydrogen bonding capabilities of dC and C with minimal structural change, providing a potentially more useful fluorescent analog.

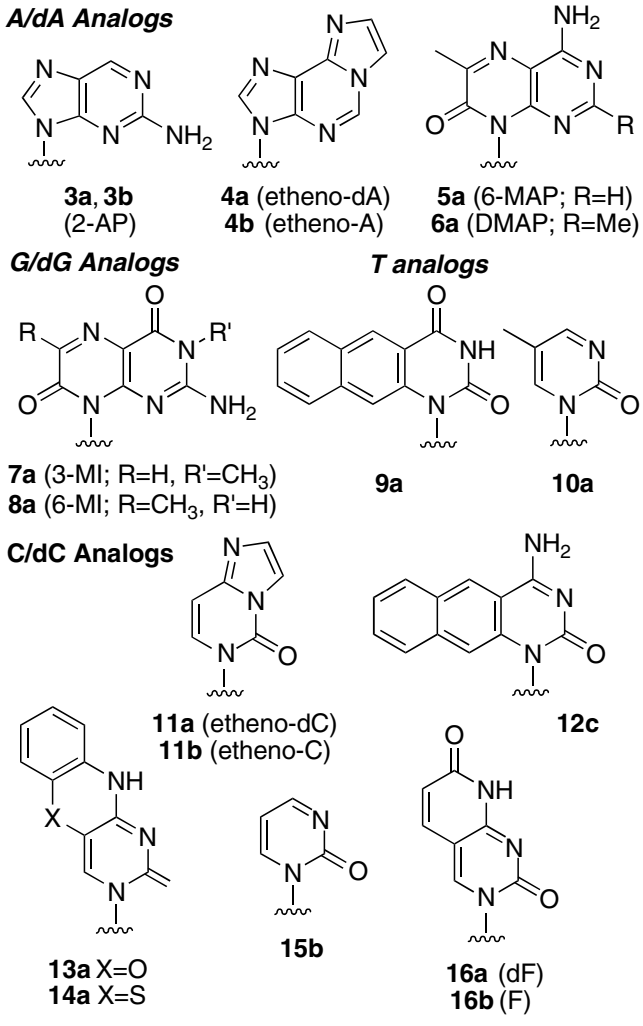


Figure 1. Representative fluorescent nucleosides that have been incorporated into oligonucleotides (**a**: 2′-deoxyribosyl; **b**: ribosyl; **c**: 2′-*O*-methylribosyl).

Of particular relevance to the current work on pyrrolo-dC (**1a**) and pyrrolo-C (**1b**) is a 1987 report by Inoue and co-workers,[25] who were following up on their earlier work on dF (**16a**).[22] The fluorescent 6-unsubstituted 3*H*-pyrrolo[2,3-*d*]pyrimidin-2-one derivative **17** (dF*, Fig. 2) was used to prepare dodecadeoxyribonucleotides using the phosphotriester solid-phase approach. Duplexes containing dF* indicated that an F* • G base pair had stability similar to that of a C • G base pair.[25] In 1996, Gamper and co-workers prepared the cyanoethyl phosphoramidite **18** and attempted to incorporate it into an oligonucleotide using automated solid-phase synthesis, but the phosphite oxidation step caused degradation of the 2*H*-pyrrolo[2,3-*d*]pyrimidin-2-one ring, presumably by oxidation at the 6-position by iodine.[26] Alternatively, they were able to incorporate furo[2,3-*d*]-pyrimidin-2(3*H*)-one into a 28-mer using the amidite **19**

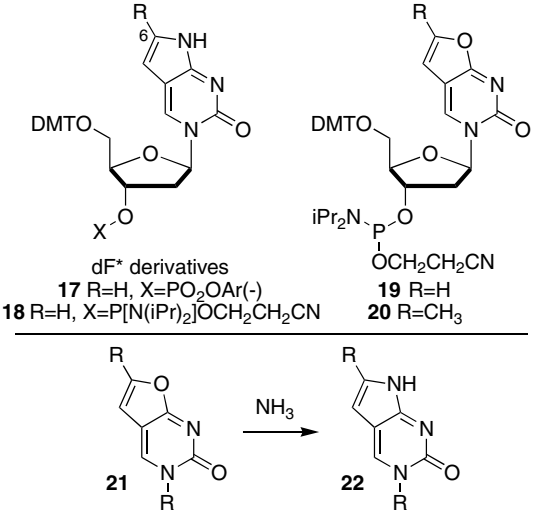
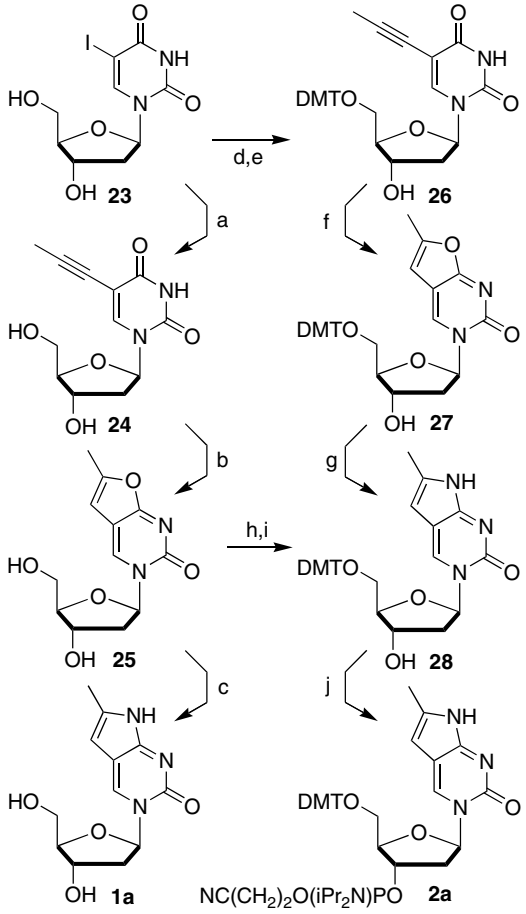


Figure 2. Prior work on 3*H*-pyrrolo[2,3-*d*]pyrimidin-2-one nucleosides.

and found that the ammonia treatment encountered in the final step of DNA synthesis led to the formation of the 2*H*-pyrrolo[2,3-*d*]pyrimidin-2-one (dF*).[26] Thus, the transformation **21** to **22** had occurred on the newly synthesized oligonucleotide. Gamper also reported the conversion of the simple nucleoside **21** (R = 2′-deoxyribosyl) to the pyrrolo version **22** with ammonia, illustrating a useful new route to 3*H*-pyrrolo[2,3-*d*]-pyrimidin-2-ones.[26,27] In related unpublished work from our labs,[28] we synthesized **20** and incorporated this fluorescent nucleoside into oligodeoxyribonucleotides. As found by Gamper, ammonia deblocking during solid-phase synthesis caused conversion to the furan ring to a pyrrole ring, resulting in pyrrolo-dC incorporation. Liu and Martin have also used **20** to incorporate pyrrolo-dC into an oligonucleotide via ammonia treatment at the final stage of DNA synthesis.[29,30]

We now report the synthesis of the cyanoethyl phosphoramidites of pyrrolo-dC (**2a**) and pyrrolo-C (**2b**) for site-specific incorporation of these fluorescent materials into oligodeoxyribo- and oligoribonucleotides using standard solid-phase amidite-based chemistry without the need to employ the furo-to-pyrrolo transformation. We chose to incorporate a C-6 alkyl group into the heterocycle in order to avoid the oxidative degradation observed above during solid-phase oligonucleotide synthesis.[26] Although 6-alkyl-3*H*-pyrrolo[2,3-*d*]pyrimidin-2-one nucleosides are known,[25,27] they are not found in amidite form and do not include what we considered to be the best choice, a C-6 methyl group, which would minimize steric disruption of base-pairing and stacking. Further, no ribonucleosides containing the 3*H*-pyrrolo[2,3-*d*]pyrimidin-2-one ring system are known, and we postulate that pyrrolo-C (**2b**) would be useful for the study of oligoribonucleotides.

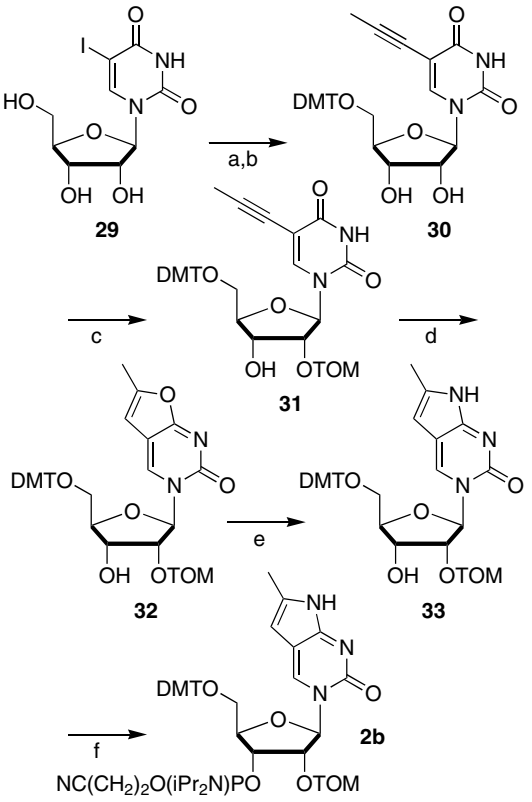
The synthesis of pyrrolo-dC **1a** and its cyanoethyl phosphoramidite **2a** is shown in Scheme 1. Using chemistry analogous to that reported by Robins and



Scheme 1. Synthesis of pyrrolo-dC **1a** and its cyanoethyl phosphoramidite **2a**. Reagents and conditions: (a) propyne (12 equiv), NEt_3 (2 equiv), CuI (0.07 equiv), $Pd(PPh_3)_4$ (0.04 equiv), DMF (65%); (b) NEt_3, CuI (0.12 equiv), MeOH, reflux, 4 h (95%); (c) satd NH_3 in MeOH, pressure bottle, 50 °C, 20 h (95%); (d) 4,4′-dimethoxytrityl chloride (DMT-Cl, 1.2 equiv), NEt_3 (5 equiv), THF; (e) propyne (14 equiv), NEt_3 (22 equiv), CuI (0.09 equiv), $Pd(PPh_3)_4$ (0.04 equiv), acetone (75%, two steps); (f) NEt_3, CuI (0.1 equiv), MeOH; (g) satd NH_3 in MeOH, THF, pressure bottle, 50 °C, 22 h (73%, two steps); (h) DMT-Cl, pyr, CH_2Cl_2 (87%); (i) satd NH_3 in MeOH, pressure bottle, 50 °C (49%); (j) $(i\text{-}Pr_2N)_2PO(CH_2)_2CN$, 1*H*-tetrazole, CH_2Cl_2, rt, 4.5 h (53%).

others,[31] Sonogashira coupling[32] of propyne with 5-iodo-2′-deoxyuridine (**23**) or its 5′-DMT-protected version gave the alkynes **24** and **26**, respectively, which were cyclized to the furo[2,3-*d*]pyrimidin-2(3*H*)-ones **25** and **27** by the method of Robins and Barr.[33–35] The ammonia exchange method of Woo et al.[26] (e.g., **21** to **22**, Fig. 2) was employed to give pyrrolo-dC (**1a**) and **28**. Alternatively, tritylation of **25** followed by treatment with ammonia also afforded **28**. Phosphitidylation of **28** gave the phosphoramidite **2a**.

For automated solid-phase synthesis of oligoribonucleotides (RNA), we chose TOM chemistry[36,37] for protection of the 2′-hydroxyl group of the requisite phosphoramidite, that is **2b** (Scheme 2). Thus, 5-iodouridine was tritylated and propynylated to give **30**,



Scheme 2. Synthesis of pyrrolo-C cyanoethyl phosphoramidite **2b**. Reagents and conditions: (a) 4,4′-dimethoxytrityl chloride (DMT-Cl, 1.2 equiv), pyridine, rt, 19 h (92%); (b) propyne (17 equiv), NEt_3 (2 equiv), CuI (0.07 equiv), $Pd(PPh_3)_4$ (0.04 equiv), acetone, rt, 40 h (73%); (c) Bu_2SnCl_2 (1.0 equiv), *i*-Pr_2NEt (3.5 equiv), $Cl(CH_2)_2Cl$, rt, 1.5 h, add *i*-Pr_3SiOCH_2Cl (TOM-Cl, 1.28 equiv), reflux, 20 min, separate (30% 2′-OTOM; 17% 3′-OTOM); (d) NEt_3, CuI, MeOH, reflux, 1 h (85%); (e) satd NH_3 in MeOH, pressure bottle, 42 °C, 22 h (69%); (f) Cl(*i*-Pr_2N)$PO(CH_2)_2CN$ (2 equiv), NEt_3 (10 equiv), CH_2Cl_2, rt, 2 h (35%).

which was converted into its 2′-*O*-[(triisopropylsilyl)oxy]methyl (2′-*O*-TOM) derivative **31**.[36,37] Cyclization, ammonia exchange, and phosphitidylation as in Scheme 1 gave **2b** via the intermediates **32** and **33**.

We have used pyrrolo-dC phosphoramidite **2a** to make oligodeoxyribonucleotides using standard automated synthesis techniques, where the only modification necessary is to use a more dilute iodine oxidizer (0.02 M). While a full report on the structure and function of these oligomers is forthcoming, a brief overview of our preliminary results follows. Oligodeoxyribonucleotides bearing pyrrolo-dC were synthesized and annealed with complementary strands containing each of the four natural deoxyribonucleotides. Pyrrolo-dC was found to hybridize selectively with dG (dG ≫ dT > dA > dC). Melting temperatures of fully complementary duplex oligodeoxyribonucleotides bearing one to four pyrrolo-dC residues were found to be identical to dC-bearing controls, indicating that a pyrrolo-dC • dG base pair is equivalent to a dC • dG base pair. Oligonucleotides containing pyrrolo-dC act as efficient primers and the PCR products appear to be identical for primers with 1 – 5 pyrrolo-dC residues replacing dC. Preliminary data indicate that pyrrolo-dC codes as dC in PCR experiments and that pyrrolo-dC triphosphate is incorporated efficiently by Taq polymerase specifically opposite dG. Regarding fluorescence, the 19-mer 5′-GCC TAA CTT CXG GAG ATG T-3′ (X = pyrrolo-dC) showed excitation maxima at 272 and 345 nm and emission at 473 nm, far from nucleic acids and proteins. The quantum yield of fluorescence for pyrrolo-dC is quite sensitive to its hybridization state, making it ideally suited for probing the dynamic structure of DNA. In work by Liu and Martin,[29] the transcription bubble in elongation complexes of T7 RNA polymerase was characterized by observing a 2-fold increase in fluorescence as the polymerase induces melting. By starving the polymerase of specific nucleoside triphosphates, the enzyme could be stalled at specific sites, producing fluorescence snapshots of the complex. Further work in the same lab[30] has shown that when pyrrolo-dC is mismatched with a dA in an otherwise complementary duplex, fluorescence is higher than in single-stranded DNA, which is in turn higher than in a fully complementary DNA duplex. Fluorescence is quenched in DNA/RNA heteroduplexes. This unusual behavior allows differentiation in situ between a DNA–DNA duplex and a DNA–RNA heteroduplex. We expect that pyrrolo-C cyanoethyl phosphoramidite **2b** will show similar utility for RNA structural analysis.

Supplementary data: Selected characterization data for compounds **1a**, **2a**,**b**, **28**, **31**, and **33**. The supplementary data is available online with the paper in ScienceDirect.

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36. Pitsch, S.; Weiss, P. A.; Jenny, L.; Stutz, A.; Wu, X. *Helv. Chim. Acta* **2001**, *84*, 3773–3795.
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