

# THE 6-METHOXYMETHYL DERIVATIVE OF PYRROLO-dC FOR SELECTIVE FLUOROMETRIC DETECTION OF GUANOSINE-CONTAINING SEQUENCES

**Robert H. E. Hudson and Arash Ghorbani Choghamarani** Department of Chemistry, The University of Western Ontario, London, Ontario, Canada

□ The β-cyanoethyl phosphosphoramidite derivatives of 6-methyl- and 6-methylyd-3-(2-deoxy-β-D-ribofuranosyl)-3H-pyrrolo[2,3-d]pyrimidin-2-one have been synthesized. These monomers have been employed for oligodeoxynucleotide synthesis to evaluate their effect on duplex stability and ability to fluorometrically report on hybridization. The structurally conservative 6methoxymethyl-substitution results in a pyrrolocytidine that is stabilizing toward hybrid formation ( $\Delta Tm = +1.3$ °C) whereas the known 6-methylpyrrolocytidine is destabilizing ( $\Delta Tm = -4.7$ °C), in the sequence examined. The 6-methoxymethylpyrrolocytidine retains excellent mismatch discrimination and its fluorescence is selectively quenched when hybridized to a match oligodeoxynucleotide sequence. The quenching of fluorescence for an internal position is approximately three-fold, whereas a terminal position (5'-end or 3'-end) experienced approximately two-fold decrease in the fluorescence intensity.

Keywords DNA; fluorescence; pyrrolocytidine; base discriminating fluorophores

### INTRODUCTION

The use of fluorescently labeled nucleosides incorporated into oligonucleotides currently is finding applications in the development of single nucleotide polymorphism detection systems.<sup>[1,2]</sup> Another important application, for environmentally sensitive intrinsically fluorescent nucleobase analogs is the study of nucleic acid-protein interactions and nucleic acid dynamics.<sup>[3]</sup> Recently, 2'-deoxy-6-methylpyrrolocytidine (<sup>Me</sup>p-dC) has been exploited in the aforementioned contexts.<sup>[4–10]</sup> Although, <sup>Me</sup>p-dC has been adopted as a base-pairing competent, intrinsically fluorescent nucleobase for recognition of guanosine, its fluorescence properties and hybridization behavior in a well-defined chemical system have not been adequately

The authors wish to thank the Natural Sciences and Engineering Research Council of Canada (R.H.E.H.) and the Ministry of Higher Education and the Embassy of Iran in Canada for partial support of a visiting scientist (A.G.C.).

Address correspondence to Robert H. E. Hudson, Department of Chemistry, The University of Western Ontario, London, Ontario, N6A 5B7, Canada. E-mail: rhhudson@uwo.ca

described. Additionally, it has not been proven that <sup>Me</sup>p-dC is an optimal structure by evaluation and comparison to structural congeners.

Some of our earlier work on the direct synthesis of pyrrolocytosine (pC) derivatives from cytosine indicated that their fluorescence properties were sensitive to substitution on the pyrrole ring.<sup>[11,12]</sup> Pyrrolocytosine derivatives with an aromatic substituent at position six exhibited greater fluorescence intensity than pCs with an alkyl or other saturated group.<sup>[12]</sup> We also noted that the hydrophilicity of the pCs was affected by the nature of the substituent, for example, the methoxymethyl-substituent increased partitioning in to an aqueous phase as compared to the substituted phenyl groups.<sup>[12]</sup> Given that relatively minor structural alteration can give rise to such changes in the properties of pCs, and that little has been reported on structure-property relationships for pCs, we have started to investigate the effect of various substitutions on duplex stability and ability to fluorometrically report on hybridization. For this study, we compare the performance of 6-methoxymethyl- and 6-methyl-substitued 2'-deoxypyrrolocytosines.

## **RESULTS AND DISCUSSION**

Recently, 6-methyl-3-(2-deoxy- $\beta$ -D-ribofuranosyl)-3*H*-pyrrolo[2,3-*d*]-pyrimidin-2-one (<sup>Me</sup>p-dC), became commercially available and has been used as an base-stacking sensitive fluorophore.<sup>[14,15]</sup> We have used both purchased and synthesized <sup>Me</sup>p-dC in this investigation and have found that they perform identically. The novel 6-methoxymethyl-3-(2-deoxy- $\beta$ -D-ribofuranosyl)-3*H*-pyrrolo[2,3-*d*]pyrimidin-2-one (<sup>MMe</sup>p-dC) was synthesized as outlined in Scheme 1.

The synthesis follows that reported by Berry and coworkers,<sup>[16]</sup> and starts with the dimethoxytritylation of 5-iodo-2'-dexoyuridine followed by crosscoupling and annulation. The Sonogashira coupling of methylpropargyl ether with 5-iodo-2'-deoxyuridine gave lower yields because of the aqueous solubility of the product; the reaction was best approached by using the tritylated nucleoside. Subsequent steps, the O-to-N atom exchange by ammonia treatment,<sup>[17]</sup> and phosphitylation went smoothly to give the



SCHEME 1

	DNA sequence	Tm(°C) <sup>a</sup> Target Strand: 5'-GCG-TTA-X-ATT-GCG-3'			
		X=G	X=A	X=T	X=C
1	5'-CGC-AAT-C-TAA-CGC-3'	52.7	36.1	38.6	38.0
2	5'-CGC-AAT- <sup>Me</sup> pC-TAA-CGC-3'	$48.0^{b}$	37.0	$39.0^{c}$	37.5
3	5′-CGC-AAT- <sup>MMe</sup> pC-TAA-CGC-3′	54.0	41.6	41.0	34.0
4	5'-CGC-AAT-CTA-ACG- <sup>MMe</sup> pC -3'	53.0	_	_	
5	5'- <sup>MMe</sup> pC-GCA-ATC- TAA-CGC-3'	53.0	—	—	_

TABLE 1 Thermal denaturation of singly-modified DNA sequence\*

\*Oligomers were purified by RP-HPLC (DMT-on, DMT-off), desalted by size exclusion chromatography and identified by ESI-TOF MS.

<sup>a</sup>Ionic conditions: 100 mM NaCl, 10 mM PO<sub>4</sub><sup>3-</sup>, 0.1 mM EDTA, pH 7.0.

<sup>b</sup>Biphasic transition.

<sup>c</sup>Poorly defined transition.

phosphoramidite derivative in 42% overall chemical yield from the nucleoside. The same synthetic route was used for the synthesis of the <sup>Me</sup>p-dC phosphoramidite reagent.

The modified nucleosides were incorporated into oligodeoxynucleotides with coupling times prophylactically increased to 5 minutes. The coupling efficiency was observed to be same or better than the commercially supplied unmodified nucleoside phosphoramidites. Using a sequence recently reported by Sekine and coworkers for evaluation of a different bicyclic fluorescent cytidine analog,<sup>[2]</sup> we compared the hybridization and fluorescence properties of <sup>MMe</sup>p-dC and <sup>Me</sup>p-dC. The results for hybridization with match and mismatched DNA are presented in Table 1.

As shown, the <sup>MMe</sup>p-dC modification lead to a slight duplex stabilization when incorporated in a central and had a neutral effect when at terminal position, relative to cytosine, while <sup>Me</sup>p-dC was destabilizing ( $\Delta$ Tm = 4.7°C) when in a central position and also gave less well-behaved thermal denaturation curves in some instances. The <sup>MMe</sup>p-dC maintained excellent mismatch discrimination, equal to or better than <sup>Me</sup>p-dC, in the sequence examined. It is interesting to note, that a single central insert in the sequence used by Berry (5'-GCC-TAA-CTT-CXG-GAG-ATG-T-3', X = C, <sup>Me</sup>p-dC or <sup>MMe</sup>pdC), showed no difference in Tm between the two pyrrolocytosines, which may reflect the differences in the local (neighboring) sequence of the two oligomers (13-mer versus 19-mer).

Pyrrolocytosine has found use in fluorometrically reporting hybridization because the pC is quenched in the duplex as compared to the singlestranded species. As expected, <sup>MMe</sup>p-dC responds to hybridization as shown in comparison to <sup>Me</sup>p-dC for the 13-mer sequence (Table 1), with the fluorescence of the single strands compared to the matched duplex at 2  $\mu$ M, 20 °C, and excited at 350 nm, (Figure 1). Each of the pyrrolocytosines exhibited a 3-fold decrease in the fluorescence upon hybridization. Even though



**FIGURE 1** Comparison of the fluoresence response of single-stranded pC-containing DNA (broken line) versus double-stranded DNA (solid line). Left Panel: Oligomer **3**; Right Panel: Oligomer **2**. <sup>*a*</sup>Oligonucleotide concentration 2.0  $\mu$ M in 100 mM NaCl, 10 mM PO<sub>4</sub><sup>3–</sup>, 0.1 mM EDTA, pH 7.0. Excitation wavelength 350 nm, 4 nm bandwidth.

both oligomers are compared at equal concentration and under identical buffer and instrumental conditions, the <sup>MMe</sup>p-dC fluorophore gave a more intense signal. The difference in signal strength is surprising given the close structural similarity of the fluorophores and merits further investigation.

The <sup>MMe</sup>p-dC also responds to duplex formation when incorporated at terminal positions. Sequences **4** and **5** both exhibit a 2-fold decrease in fluorescence upon hybridization.

### CONCLUSIONS

We have described a modest structural change to the currently employed <sup>Me</sup>p-dC that resulted in a substitution (<sup>MMe</sup>p-dC) that gave more consistent hybridization behavior and excellent fluorescence response. Since pyrrolocytosine-containing oligomers are finding use as short probes, molecular beacons and in longer oligonucleotides, derivatives with improved properties could be of value in these studies. In addition, substitutions which improve aqueous solubility while preserving the fluorescence properties could find use in oligonucleotide analogs such as PNA where some conjugated fluorophores unfavorably influence solubility.

#### REFERENCES

- For a recent example see: Saito, Y.; Miyauchi, Y.; Okamoto, A.; Saito, I. Synthesis and properties of novel base-discriminating fluorescent (BDF) nucleosides: a highly polarity-sensitive fluorophore for SNP typing. *Tetrahedron Lett.* 2004, 45, 7827–7831.
- Miyata, K.; Tamamushi, R.; Ohkubo, A.; Taguchi, H.; Seio, K.; Santa, T.; Sekine, M. Synthesis and properties of a new fluorescent bicyclic 4-N-carbamoyldeoxycytidine Derivative. *Org. Lett.* 2006, 8, 1545–1548.

- Rist, M. J.; Marino, J. P. Fluorescent nucleotide analogs as probes of nucleic acid structure, dynamics and interactions. *Curr. Org. Chem.* 2002, 6, 775–793.
- 4. Although the use of pyrrolcytosine is a relatively recent phenomenon it was first described some time ago. The first synthesis of pyrrolcytidine from cytidine nucleoside was described by: Inoue, H.; Imura, A.; Ohtsuka, E. Synthesis of dodecadeoxyribonucleotides containing a pyrrolo[2,3-d]pyrimidine nucleoside and their base-pairing ability *Nippon Kagaku Kaishi* 1987, 7, 1214–1220.
- Martin, C.T.; Liu, C. Fluorescence characterization of the transcription bubble in elongation complexes of T7 RNA polymerase. *J. Mol. Biol.* 2001, 308, 465–475.
- Martin, C.T.; Liu, C. Promoter clearance by T7 RNA polymerase: initial bubble collapse and transcript dissociation monitored by base analog fluorescence. J. Biol. Chem. 2002, 277, 2725–2731.
- Chen, P.; He, C. A general strategy to convert the MerR family proteins into highly sensitive and selective fluorescent biosensors for metal ions. J. Am. Chem. Soc. 2004, 126, 728–729.
- Dash, C.; Raush, J. W.; Le Grice, S. F. J. Using pyrrolodeoxycytosine to probe RNA/DNA hybrids containing the human immunodeficiency virus type-1 3' polypurine tract. *Nucleic Acids Res.* 2004, 32, 1539–1547.
- Johnson, N. P.; Baase, W. A.; von Hippel, P. H. Investigating local conformations of double-stranded DNA by low-energy circular dichroism of pyrrolocytosine. *Proc. Nat. Acad. Sci. USA* 2005, 102, 7169– 7173.
- Marti, A. A.; Jockusch, S.; Li, Z.; Ju, J.; Turro, N. J. Molecular beacons with intrinsically fluorescent nucleotides. *Nucleic Acids Res.* 2006, 34, e50/1–e50/7.
- 11. Hudson, R. H. E.; Dambenieks, A. K.; Viirre, R. D. Fluorescent 7-deazapurine derivatives from 5iodocytosine via a tandem cross-coupling/annulation reaction. *Synlett.* **2004**, 13, 2400–2402.
- Hudson, R. H. E.; Dambenieks, A. K.; Moszynski, J. M. New fluorescent nucleobases derived from 5- alkynylpyrimidines. Proceedings of SPIE: Photonic Applications in Biosensing and Imaging, Toronto, 2005, 5969, 59690J1–J10.
- Hudson, R. H. E.; Dambenieks, A. K. Synthesis of N1-unsubstituted 5-alkynylcytosine and derivatives thereof. *Heterocycles* 2006, 68, 1325–1328.
- Thompson, K. C.; Norimune, M. Properties of a new fluorescent cytosine analogue, pyrrolocytosine. J. Phys. Chem. B 2005, 109, 6012–6019.
- 15. Mep-dC phosphoramidite reagent is available from Glen Research, Sterling, Virginia, USA 20164.
- Berry, D. A.; Jung, K.-Y.; Wise, D. S.; Sercel, A. D.; Pearson, W. H.; Mackie, H.; Randolph, J. B.; Somers, R. L. Pyrrolo-dC and pyrrolo-C: Fluorescent analogs of cytidine and 2'-deoxycytidine for the study of oligonucleotides. *Tetrahedron Lett.* 2004, 45, 2457–2461.
- A previous different synthesis has been reported: Woo, J.; Meyer, R. B.; Gamper, H. B. Jr. G/C-modified oligodeoxynucleotides with selective complementarity: synthesis and hybridization properties. *Nucleic Acids Res.* 1996, 24, 2470–2475.

Copyright of Nucleosides, Nucleotides & Nucleic Acids is the property of Taylor & Francis Ltd and its content may not be copied or emailed to multiple sites or posted to a listserv without the copyright holder's express written permission. However, users may print, download, or email articles for individual use.