RNA containing pyrrolocytidine base analogs: good binding affinity and fluorescence that responds to hybridization

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ABSTRACT

6-Phenylpyrrolocytidine and 6-methoxymethylene-pyrrolocytidine are base-modified nucleosides with remarkable fluorescence properties. When incorporated into RNA, these analogs enhance binding affinity towards RNA and DNA targets with a concomitant change in their fluorescence upon duplex formation. The fluorescence response depends on the nature of the 6-substituent and the sequence position of the modified nucleoside. The fluorescence response of these structurally conservative, well-tolerated fluorescent nucleosides may be exploited as probes in the study of nucleic acid processing enzymes.

INTRODUCTION

Fluorescent reporter groups incorporated into oligonucleotides are proving to be very useful as tools to study nucleic acids. In light of this utility, elegant base modifications which impart fluorescence yet do not cause significant structural perturbation of the nucleic acids and preserve the base-pairing properties have gained increasing interest as opposed to traditional bulky fluorophores.\(^1\) The pyrroloctosine base analog possesses these desirable properties.\(^2,4\) It does not disrupt normal base pairing and has a fluorescent excitation/emission that does not overlap with those of proteins.\(^5,6\) Until recently its major drawback compared to traditional fluorophores, such as fluorescein, was their lower degree of fluorescence emission. Increased fluorescence was made possible by substituting the 6-position of the base of 2'-deoxypyrroloctidine with aromatic residues without compromising other desirable properties.\(^9,10\) We now report the incorporation of 6-phenylpyrroloctidine (\(^{\text{Ph}}\)pC) 1 and 6-methoxymethylene-pyrroloctidine (\(^{\text{MeOMe}}\)pC) 2 into RNA and their fluorescence and hybridization properties, Figure 1.

RESULTS AND DISCUSSION

The strategy for the synthesis of 6-phenylpyrroloctidine (\(^{\text{Ph}}\)pC) and 6-methoxymethylene-pyrroloctidine (\(^{\text{MeOMe}}\)pC) phosphoramidites was based on previously described methods for the synthesis of PhdpC\(^{11}\) and pC.\(^5\) 5-Iodouridine was first dimethoxytritylated which was followed by Sonogashira coupling with the appropriate alkyne. The 5-alkynyluracil moiety of the resulting nucleoside was subsequently subjected to Ag\(^{+}\)-mediated cyclization to form a furanouracil nucleoside derivative. This was then converted to the corresponding pyrroloctidine by treatment with aqueous ammonia. Standard conditions were used to install the 2'-O-tertbutyldimethylsilyl group\(^11,12\) which was separated from the 3'-O-regioisomer by silica gel column chromatography. Finally, the 5'-O-DMT-3'-O-phosphoramidite reagents were prepared according to literature methods.\(^11\)

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'→3')</th>
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<tbody>
<tr>
<td>ORN-1</td>
<td>GAUCUGAGCCUCUGGAGCU</td>
</tr>
<tr>
<td>ORN-2</td>
<td>GAUCUGAGCCUGGAG1U</td>
</tr>
<tr>
<td>ORN-3</td>
<td>GAUCUGAGCCUGGAGACU</td>
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<td>ORN-4</td>
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</tr>
<tr>
<td>ORN-5</td>
<td>GAUCUGAGCG2UGGAGCU</td>
</tr>
</tbody>
</table>

Table 1. Sequences of oligonucleotides in this study.

Oligoribonucleotides (ORN) containing a single insert of either \(^{\text{Ph}}\)pC or \(^{\text{MeOMe}}\)pC in an 18-mer were prepared, Table 1. ORNs were synthesized on an ABI 3400 (Applied Biosystems) instrument using standard solid-phase synthesis protocols and commercially available reagents.\(^11\) Couplings for the modified phosphoramidites were manually carried out in anhydrous DCM in a concentration of 0.15 mM with coupling times extended to 1 h. Deprotection from the solid support was performed in a 3:1 mixture of conc. aqueous ammonia and ethanol for 48 h at room temperature followed by desilylation by treatment with distilled Et\(_3\)N:HF for 48 h at room temperature. The crude ORNs were analyzed by 20% denaturing PAGE to reveal that coupling of the \(^{\text{Ph}}\)pC was similar in yield to those of commercial 2'-O-silyl amides, while \(^{\text{MeOMe}}\)pC couplings fell to about 30%. All oligonucleotides were

![Figure 1. 6-Phenylpyrroloctidine 1 (\(^{\text{Ph}}\)pC) and 6-methoxymethylene-pyrroloctidine 2 (\(^{\text{MeOMe}}\)pC).](https://example.com/figure1.png)
purified by 24% denaturing PAGE, desalted on NAP-25 Sephadex columns, and characterized by ESI-TOF.

Thermal denaturation curves were obtained using both UV and fluorescence (1 μM in oligonucleotide, 10 mM sodium phosphate pH 7.0, 50 mM NaCl and 0.1 mM EDTA). UV measurements were performed on a Cary-3 Spectrophotometer (Varian Inc.) and fluorescence experiments were performed on a Cary Eclipse Fluorescence Spectrophotometer (Varian Inc.).

The T_m data for the sequences examined show that \textsuperscript{3p}pC and \textsuperscript{9p}pC modifications do not impair binding to DNA and RNA targets compared to the RNA control (ORN-1) (data not shown). Modifications placed in the middle of the oligonucleotide improved binding more than ones close to the 3'-end.

The \textsuperscript{3p}pC modified RNA showed fluorescence excitation maxima at 380 nm and emission maxima at 463 nm, whereas \textsuperscript{9p}pC RNA showed excitation and emission maxima at 360 and 455 nm, respectively. The relative fluorescence of \textsuperscript{3p}pC is much stronger than that of \textsuperscript{9p}pC, the fluorescence of the former being readily visible to the naked eye. Upon addition of a complementary oligonucleotide (RNA or DNA), the fluorescence is dramatically decreased due the quenching that occurs in the duplex. Interestingly, this quenching effect is more pronounced with the oligonucleotides containing fluorescence modifications closer to the 3’-terminus (ORN-2 and ORN-4), while ORN-3 showed hardly any quenching upon hybridization. ORN-2 displayed the best quenching at ca. 80 % (Figure 2). At this point it is not clear if the differences in the degree of quenching of \textsuperscript{3p}pC and \textsuperscript{9p}pC is a consequence of their location in an oligonucleotide duplex or the nature of their neighbouring nucleotides. Further studies are aimed at elucidating potential sequence effects.

Thermal denaturation profiles were also monitored using the fluorescent quenching from hybridization, with oligonucleotide ORN-2 displaying the greatest increase in fluorescence upon denaturation when bound to complementary DNA (Figure 3) and RNA. In all cases, well-behaved monophasic transitions were observed and the T_m values obtained by fluorescence corresponded well with those obtained by UV.

**CONCLUSION**

We have demonstrated the RNA containing \textsuperscript{3p}pC or \textsuperscript{9p}pC shows good duplex stability and bestows useful fluorescent signals to an oligonucleotide. More importantly, their fluorescence responds very strongly to changes in hybridization state, especially when they are placed at the 3’-terminus of a duplex. We are now exploiting the fluorescence response of these structurally conservative, well-tolerated labels in the study of NA-processing enzymes.

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**REFERENCES**


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