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SYNTHESIS OF 6-AMINO-2'-O-METHYLCYTIDINE, A PROTONATED CYTIDINE ANALOG FOR TRIPLE HELIX BINDING STUDIES

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Abstract: The synthesis of 6-amino-2'-O-methylcytidine and its incorporation into an oligonucleotide is reported. Incorporation of this nucleoside required modified formamidine protection of the exocyclic amino groups. Binding to double-stranded DNA via triple helix formation was assayed by DNase I footprint analysis and revealed that this protonated C analog inhibited binding relative to the control 2'-O-methylcytidine.

The potential for the modulation of gene expression by targeting double stranded DNA (dsDNA) through triple helix formation has attracted significant attention.[1] Triple-stranded nucleic acids were first described in 1957[2] but only recently has this unique structure been pursued as a means of sequence specific DNA recognition with potential therapeutic applications. Previous reports have shown that a pyrimidine oligonucleotide (ON) can target a polypurine tract of duplex DNA in a sequence-specific manner.[3-5] In this binding motif, the incoming pyrimidine ON binds to the purine tract in a parallel orientation with thymidine (T) recognizing the adenine of an adenosine:thymidine (A:T) base pair and N^3-protonated cytidine (C^+) recognizing the guanine of a guanosine:cytidine (G:C) base pair. The pH dependence of this process,[6-8] coupled with ^1H NMR studies,[9,10] demonstrates that the C^+GC triplet requires the N^3-protonation of C.

In an effort to overcome the difficulty of protonating C at neutral pH, several groups, including ourselves, have designed neutral protonated C mimetics[11-13] which remove the pH dependence of triple helix formation at near physiological pH (*i.e.* pH=6-8). Modification of 2'-deoxy-5-methylcytidine (C^{me}) to raise the pKa of the critical N^3-position is another viable approach. Froehler *et. al.*[14] have shown that the carbocyclic ribose variant of C^{me} has a ca. 0.5 higher pKa than that of the parent deoxynucleoside and results in an enhanced triple helix formation at neutral pH. Similarly, substitution of the cytosine heterocycle can dramatically raise the pKa at the N^3-position. 6-Aminocytidine (6-amino-C) has been synthesized and the **pKa measured as 6.8 or a Δ pKa of 2.3 relative to 2'-deoxycytidine.**[15] This enhanced basicity could result in an enhanced triple helix formation at physiological pH.[16] We wish to report the synthesis of an oligonucleotide bearing this basic cytidine analog and its binding properties.

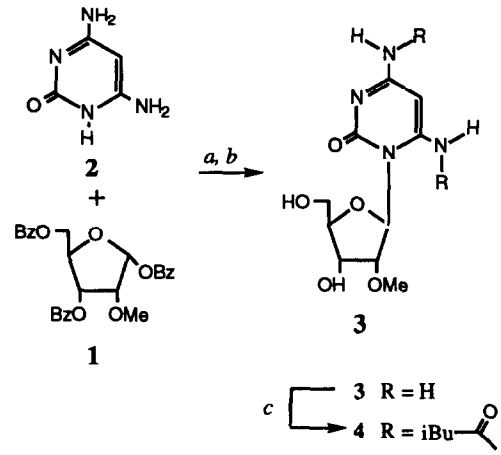
The synthesis of 6-amino-C deoxynucleoside has been reported,[15] but attempts to repeat the experimental detail proved fruitless in our hands. Therefore, an alternative route was pursued involving the glycosylation of 6-amino-C with 5',3'-O-dibenzoyl -1'-O-acetyl-2'-deoxyribose under standard Vorbruggen conditions (Scheme 1, reagents and conditions *a*) resulted in no detectable nucleoside formation. Ribonucleosides are known to possess more acid stable glycosidic bonds, when compared to the corresponding 2'-deoxy nucleosides.[17] Therefore, it was envisioned that the synthesis of the ribonucleoside could circumvent this problem.

The N4,N6-diisobutyryl protected 6-aminocytidine **4** was prepared as shown in Scheme 1. In order to ascertain the relative stability of this protected nucleoside to ON synthesis conditions, compound **4** was dissolved in 2.5% DCA in CH_2Cl_2. Unfortunately, the compound underwent complete deglycosylation to yield the heterocycle and sugar ($t_{1/2}$ was approximately 2 minutes). N-Substituted formamidines have been used for the protection of heterocyclic amino groups and are useful in stabilizing deoxyadenosine derivatives towards depurination.[18] N,N-Dibutylformamidine was identified as a first choice based upon previously reported studies examining the stability of N,N-disubstituted formamidines to ammonia deprotection.[18] This protecting group strategy was explored on the 2'-O-methyl derivative as shown in Scheme 2.

The 2'-O-Me sugar **1** was synthesized *via* a published procedure.[19] Commercially available 6-aminocytosine (**2**) was silylated and then allowed to react with sugar **1** under Vorbruggen coupling conditions to furnish compound **5** along with the α anomer (β:α ratio 9:1). Conveniently, the triflic acid salt of the β-nucleoside **5** precipitated from the MeOH-quenched reaction mixture, exclusively. Compound **5** was then treated with MeONa/MeOH followed by protection of the amine groups with N,N-dibutylformamide dimethylacetal in DMF to furnish the bis-formamidine protected compound **6a**. This compound is stable to standard detritylation condtions with no decomposition being observed in 2.5% DCA in CH_2Cl_2 over 24 hrs. Protection of the 5'-hydroxyl as a DMT ether followed by phosphonylation furnished compound **7a**. Attempts to incorporate this monomer into an ON using a H-phosphonate protocol resulted in no detectable ON being isolated. Strong bases such as TEA or DMAP are known to inhibit H-phosphonate couplings .[20] Assuming that the coupling failed due to the strong basicity of the bis-formamidine protected 6-amino-C heterocycle, a formamidine group of lower basicity was pursued. The N,N-di-(2-methoxy)ethylformamide dimethyl acetal was synthesized and allowed to react with debenzoylated compound **5** in DMF to yield compound **6b** (no decomposition observed in 2.5% DCA in CH_2Cl_2 over 24 hrs.) and was treated as before to yield compound **7b**. Incorporation of this monomer into the mixed nucleotide sequence 5' TC*TC*TC*TC*TC*TTTTT (where T=thymidine and C*=6-amino-2'-O-methylcytidine) was then accomplished by standard H-phosphonate methods to furnish ON **8**. Mixed oligonucleotide **8** was analyzed by exhaustive nuclease and phosphatase digestion followed by HPLC analysis of the resulting monomers and afforded the expected ratio of T : 6-amino-2'-O-methylcytidine.[21]

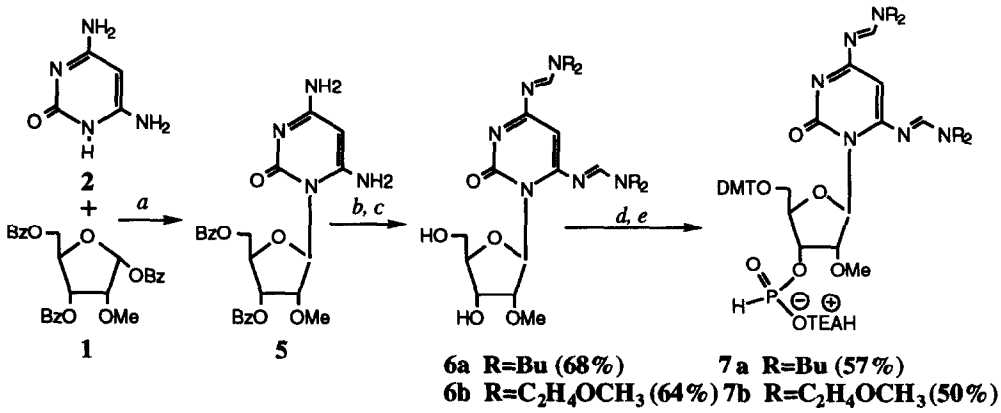
With the test ON (**8**) in hand, two additional control ONs were synthesized: 5' TC^TC^TC^TC^TC^TTTTT (**9**) and 5' $TC^{me}TC^{me}TC^{me}TC^{me}TC^{me}TTTTT$ (**10**), (where T=thymidine, C^=2'-O-methylcytidine, and C^{me}= 2'-deoxy-5-methylcytidine). Binding to dsDNA was determined by DNase I footprint analysis (as previously described)[22] with conditions selected to approximate the intracellular environment.[23] The control 2'-O-methylcytidine ON (**9**) afforded partial protection of the target duplex at 10 μM, while the 6-amino-2'-O-methylcytidine ON (**8**) did not show any detectable protection at that concentration. The 5-methyl-C containing ON (**10**) did bind to the target as has been previously observed[22] and afforded complete protection at a concentration of 0.1 μM, verifying the integrity of the target. The complete protection of the target by ON **10** and only partial protection by ON **9** is consistent with the enhanced affinity of C^{me} for dsDNA when compared to cytidine.[24,25] However, the substitution of an amino group at the 6-position of 2'-O-methylcytosine inhibits triple helix formation. The 6-amino modification could be perturbing the glycosyl

Scheme 1



a: TMSOTF, BSTFA, MeCN, 70°C; b: MeONa, MeOH, 20°C (68%, two steps);
c: 1. TMSCl, pyr, 20°C 2. isobutyryl chloride, pyr, 20°C 3. NH_4OH, 20°C (50%, three steps, one pot)

Scheme 2



6a R=Bu (68%) **7a R=Bu (57%)**
6b R=$C_2H_4OCH_3$ (64%) **7b R=$C_2H_4OCH_3$ (50%)**

a: TMSOTF, BSTFA, MeCN, 70°C (67%); *b*: MeONa, MeOH, 70°C (86%); *c*: $(R)_2NCH(OMe)_2$, DMF 20°C; *d*: DMTCl, pyr, 20°C; *e*: 2-chloro-4H-1,2,3-benzodioxaphosphorin-4-one, CH_2Cl_2, pyr, 0°C

conformation to disfavor binding to the target duplex. Triple helix structures require the protonated cytosine to adopt the anti conformation and the presence of a 6-substituent on 6-amino-2'-O-MeC within an ON could be preventing this. Previous ^{1}H NMR studies that were conducted on 6-amino-pyrimidine nucleosides (which bear a 5'-hydroxyl group) indicated that the glycosyl bond was adopting an anti conformer,[16] but perhaps the neighboring phosphate within the ON interacts with the 6-amino group of 6-amino-2'-O-Me-C resulting in a perturbation of conformation sufficient to preclude binding. Further NMR studies are required to elucidate this question.

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25. An additional variable between ON **9** and **10** is the presence of 2'-O-Me ribose sugars in ON **9**. 2'-O-Me sugars would be expected to enhance triple helix formation relative to the 2'-deoxyribose derivatives.[26,27] This is not observed, apparently the 5-methylcytosine stabilizing effect dominates in these sequences.
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