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Syntheses of 5-Formyl- and 5-Carboxyl-dC Containing DNA Oligos as Potential Oxidation Products of 5-Hydroxymethylcytosine in DNA

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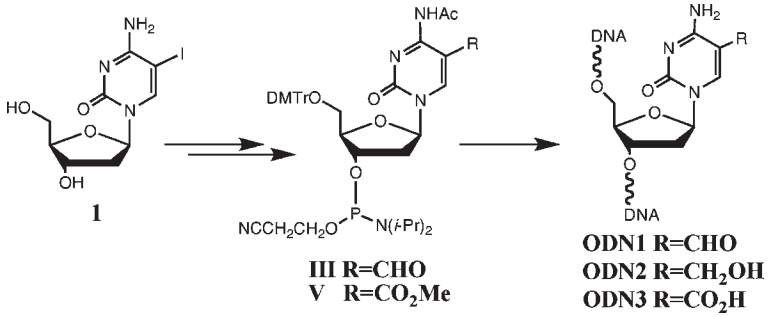
ABSTRACT



To investigate the potential oxidation products of 5-hydroxymethylcytosine (5-hmC)-containing DNA, we present here efficient syntheses of 5-formyl- and 5-methoxycarbonyl-2′-deoxycytidine phosphoramidites. The 5-formyl group in III was easy to introduce and was compatible with phosphoramidite and DNA syntheses. An additional treatment of ODN1 with $NaBH_4$ produced the corresponding ODN2 quantitatively. Phosphoramidite V was also incorporated into DNA, and the methyl ester could be hydrolyzed under mild basic conditions to afford ODN3.

As one of the best characterized epigenetic modifications, 5-methylcytosine (5-mC) plays an important role in many biological processes that include regulation of gene expression, genomic imprinting, X chromosome inactivation, and suppression of transposable element propagation.[1] Important progress in recent studies of DNA modification is the discovery of 5-hmC (the "sixth" base) in the mammalian genome, which is produced through the oxidation of 5-mC by a group of TET (ten eleven translocation) dioxygenases.[2] Recent studies have shown that 5-hmC is a widespread DNA modification in various tissues and cells.[3,4] We have developed a chemical-labeling method to enrich and sequence the distribution of 5-hmC in the mammalian genome.[4] Despite such exciting progress, little is known about how 5-hmC is metabolized in vivo. It has been hypothesized that the TET protein-catalyzed conversion of 5-mC DNA to 5-hmC may represent the first step of multiple-step reactions for DNA demethylation (Figure 1).[5] Further oxidation of 5-hmC in DNA may result in the formation of 5-formylcytosine (5-fC) DNA. It is possible that 5-fC is a transient intermediate, which can be quickly converted to 5-carboxylcytosine (5-caC) in vivo and cytosine by further decarboxylation (Figure 1).[6] Thus, it is important to

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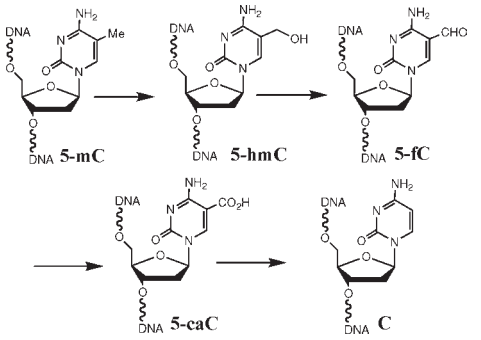


Figure 1. Possible demethylation pathway of 5-mC through 5-hmC, 5-fC, and 5-caC.

explore efficient and convenient syntheses of 5-fC and 5-caC-containing DNA as models for biological studies. These modified nucleosides can also serve as in vitro authentic standards for searching for 5-fC and 5-caC modifications in genomic DNA. Herein, we report the syntheses of two phosphoramidite building blocks **III** and **V** and their incorporation into DNA.

A literature search revealed that the synthesis of 5-fC-containing DNA had been accomplished by incorporating phosphoramidite **I**[7] into DNA (Figure 2). The structure of **I** does not contain 5-formyl functionality, but a postsynthetic NH_4OH treatment removes the acetyl protecting groups to yield a diol analogue, which is subsequently cleaved by treatment with $NaIO_4$ to generate the 5-formyl functionality. Although this approach can be used to prepare 5-fC in DNA, the synthesis of **I** is inconvenient. A ribo version phosphoramidite of 5-fC bearing an unprotected 5-formyl functionality (Figure 2, II) has recently been synthesized and incorporated into RNA,[8] suggesting that an unprotected 5-formyl group may be compatible with oligo synthesis. Therefore, we sought to synthesize phosphoramidite **III** (Figure 2) as the building block to prepare 5-fC in DNA.

5-Formyl-2′-deoxycytidine has been prepared by direct oxidation of 5-methyl-2′-deoxycytidine with $K_2S_2O_8$, but the yield is rather low.[9] Catalanotti et al. reported that 3′,5′-silyl-protected thymidine analogue can be oxidized to the corresponding 5-formyl-2′-deoxyuridine in a reasonable yield,[10] but its conversion to the corresponding 5-formyl-2′-deoxycytidine analogue requires multiple steps including possible protection of the 5-formyl functionality. We chose to prepare 5-formyl-2′-

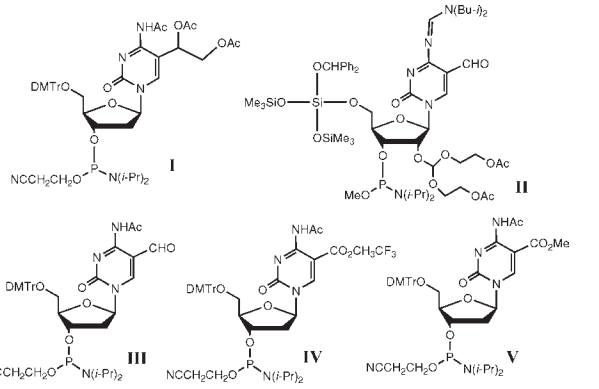
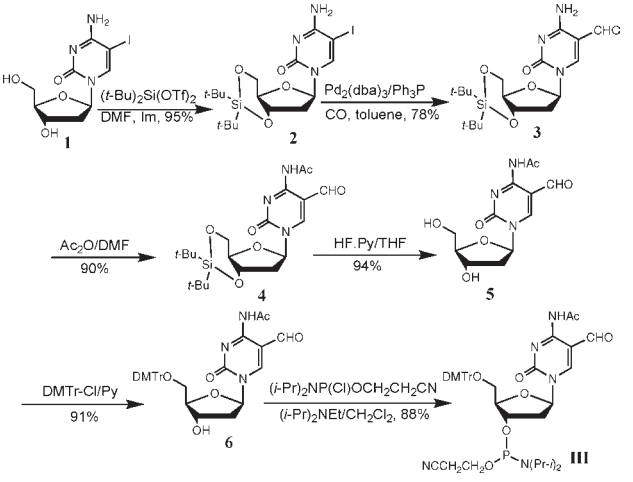


Figure 2. Structures of phosphoramidites for synthesis of DNA or RNA containing 5-fC or 5-caC modifications.

deoxycytidine using the Stille reaction to introduce the 5-formyl group.[11] Thus, the 3′- and 5′-hydroxyls of the commercial 5-iodo-2′-deoxycytidine (**1**) were first protected with di-*tert*-butylsilyl group to give **2** in 95% yield (Scheme 1).[12] Pd-catalyzed Stille reaction of **2** afforded 5-formyl-dC analogue **3** in 78% yield. Protection of the 4-amino group by treating **3** with acetic anhydride in DMF furnished **4** in 90% yield. Removal of the 3′,5′-silyl protecting group by treating **4** with hydrogen fluoride–pyridine gave **5** in 94% yield. To test the compatibility of the 5-formyl group during DNA synthesis and the subsequent deprotection conditions, we treated **5** with the deblocking, coupling, capping, and the oxidation reagents that are used for DNA synthesis, respectively. TLC showed that **5** stayed intact in all cases, confirming that the 5-formyl group could survive DNA synthesis. When we treated **5** under mild deprotection conditions (0.1 M K_2CO_3 in MeOH for 2 h at rt), quantitative conversion of **5** to 5-formyl-2′-deoxycytidine free nucleoside was observed, confirming that **5** is

Scheme 1. Synthesis of Phosphoramidite **III**



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also compatible with the mild deprotection conditions. Protection of 5′-OH of **5** with DMTr (91%) followed by 3′-phosphitylation afforded the phosphoramidite **III** (88%). The synthesis entails six steps in 50% overall yield starting from **1**.

Phosphoramidite **III** was then incorporated into a model sequence 5′-TCXGA (X = 5-fC) using ultramild reagents with the modified phosphoramidite **III** using double coupling to give resin-attached **ODN1**. We divided the resin into two parts and deprotected them with the following two procedures, respectively. The first part was treated with 0.1 M K_2CO_3 in MeOH/H_2O (1:1 v/v) at rt for 2 h followed by addition of AcOH to neutralize the final solution to neutral pH; the second part was treated with concentrated NH_4OH at rt for 2 h. The two samples were then subjected to reverses-phase HPLC analysis. In both cases, the desired 5mer was produced as the only major product (peak a, Figure 3A,B). The small peak b in Figure 3B is probably the imine intermediate; this peak disappeared completely after overnight standing, suggesting that the imine intermediate was cleanly hydrolyzed into aldehyde (Figure 3C).

Recently, we reported the synthesis of 5-hmC phosphoramidite with TBDMS as a 5-CH_2OH protecting group.[13] Although the synthesis is highly efficient and entailed eight steps in 32% overall yield, the generation of 5-CH_2OH was accomplished through the reduction of the corresponding 5-CHO analogue, and the resulting 5-CH_2OH was subsequently protected as a TBDMS ether. Since 5-fC phosphoramidite **III** could be synthesized more efficiently in six steps in 50% overall yield and it is completely compatible with DNA synthesis and the subsequent deprotection, we reasoned that 5-fC-containing DNA might be converted to 5-hmC-containing DNA by postsynthetic reduction so that two steps (introduction and removal of TBDMS) could be spared.

To facilitate isolation of the DNA, we chose to reduce the 5-fC-containing DNA when it was still attached with resin. Thus, resin-attached **ODN1** was treated with 0.1 M $CeCl_3 \cdot 7H_2O$ in MeOH (1 mL) followed by addition of 1 mg of $NaBH_4$. After 15 min, the resin was treated with the same two mild conditions as those used for deprotection of **ODN1** to give **ODN2**. The HPLC analysis showed that in both cases the peak of 5-fC-containing oligo **ODN1** was shifted to peak c (Figure 3D,E), which is the corresponding 5-hmC-containing oligo **ODN2**. This new method provides a more efficient and convenient approach for synthesis of 5-hmC-containing DNA.

To prepare 5-caC in DNA, we searched for the oxidation conditions that can postsynthetically oxidize the 5-formyl group of 5-fC in DNA to the corresponding 5-carboxyl functionality. Although we have screened various oxidizing reagents, we have yet to identify the proper oxidizing conditions. As an alternative approach, we sought to synthesize the phosphoramidite that contains a 5-ester group that can be hydrolyzed to 5-carboxylic acid in postsynthetic deprotection. Nomura et al. reported the synthesis of the phosphoramidite **IV** (Figure 2) with a 5-trifluoroethyl ester functionality.[14] After incorporation into DNA, it was used to react with amines to generate amide analogues with trifluoroethoxy as the leaving group. We prepared phosphoramidite **IV** by following the literature procedure and found that, for unknown reasons, the palladium-catalyzed carbonylation reaction always led to formation of a significant amount of reduced byproduct, N^4-Ac-2′-deoxycytidine. In addition, we found that the trifluoroethyl ester could not be hydrolyzed efficiently under mild basic conditions. After phosphoramidite **IV** was incorporated into a short oligo, 5-TCXGA (**ODN3**), and deprotected with 0.1 M K_2CO_3 in 1:1 MeOH/H_2O overnight at rt, HPLC analysis showed that only 65% of the fully deprotected DNA (peak d, Figure 3F) was obtained in addition to another 35% oligo (peak e, Figure 3F) still containing trifluoroethyl ester.

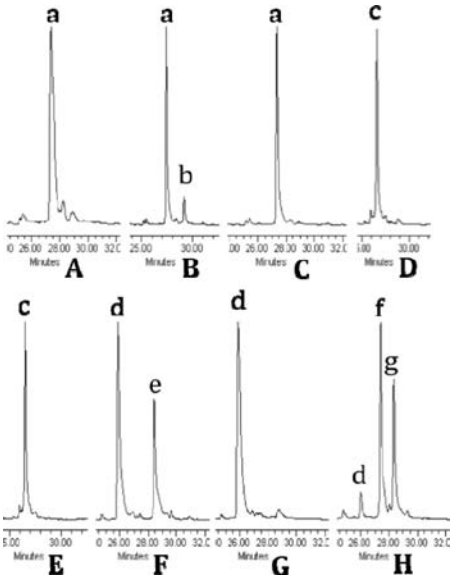

Figure 3. (A) **ODN1** was deprotected by treatment of 0.1 M K_2CO_3 in MeOH/H_2O (1:1 v/v) at rt for 2 h. Peak a is the fully deprotected 5mer, $[MH]^+ = 1491$. (B) **ODN1** was deprotected by treatment of NH_4OH at rt for 2 h; peak b is the proposed imine intermediate. (C) **ODN1** obtained in the same way as (B) was dissoved in H_2O and allowed to stand overnight at rt, after which peak b disappeared. (D) **ODN2** was deprotected by treatment of 0.1 M K_2CO_3 in MeOH/H_2O (1:1 v/v) at rt for 2 h; peak c is the fully deprotected 5mer, $[MH]^+ = 1493$. (E) **ODN2** was deprotected by NH_4OH at rt for 2 h. (F) **ODN3** made from phoshoramidite **IV** was deprotected by treatment of 0.1 M K_2CO_3 in MeOH/H_2O (1:1 v/v) at rt overnight. Peak d is the fully deprotected 5mer, $[MH]^+ = 1507$; peak e is the 5mer with 5-trifluoroethoxycarbonyl-dC modification, $[MH]^+ = 1589$. (G) **ODN3** made from phoshoramidite **V** was deprotected by treatment of 0.1 M K_2CO_3 in MeOH/H_2O (1:1 v/v) at 40 °C overnight. (H) **ODN3** made from phoshoramidite **V** was treated with NH_4OH at rt for 2 h. Peak f is the 5mer with a 5-formamide modification, $[MH]^+ = 1506$; peak g is the 5mer with a 5-methoxycarbonyl-dC modification, $[MH]^+ = 1521$.

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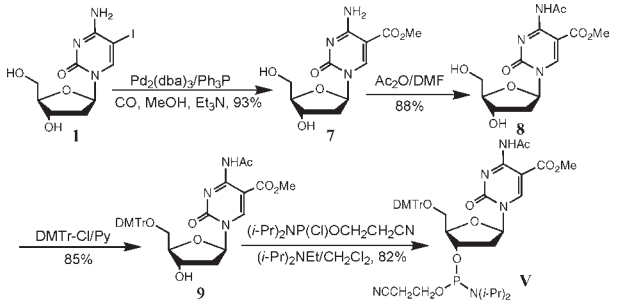
Our past experience that methyl ester could be readily hydrolyzed under mild basic conditions prompted us to synthesize the corresponding phosphoramidite **V** (Figure 2). Our initial attempt to introduce the 5-methoxycarbonyl group into dC by treating 5-iodo-N^4-2′-deoxycytidine with CO in the presence of Et_3N, $(PhCN)_2PdCl_2$, and MeOH in DMF at 50 °C, similar to the procedure used previously for preparation of the corresponding CF_3CH_2 ester,[14] gave only a very low yield of the desired methyl ester. The byproduct, N^4-acetyl-2′-deoxyctidine, was always isolated in high yields. However, we found that when 5-iodo-dC (**1**) was used as substrate and $Pd_2(dba)_3$ as catalyst, **1** was quantitatively converted into the corresponding methyl ester **7** and no reduction byproduct was detected as indicated by TLC (93% isolated yield, Scheme 2). Protection of the 4-amino group with Ac gave **8** in 88% yield. Protection of 5′-hydroxyl of **8** with DMTr (85%) followed by 3′-phosphitylation produced the phosphoramidite **V** (82%). The synthesis entails four steps from **1** in 57% overall yield.

Scheme 2. Synthesis of Phosphoramidite **V**



Phosphoramidite **V** was then incorporated into the sequence 5′-TCXGA (X = 5-methoxycarbonyl-dC) to give the polymer-attached **ODN3**. When the resin was treated under mild basic conditions (0.1 M K_2CO_3 in $MeOH/H_2O$ overnight at 40 °C), HPLC showed that the methyl ester was cleanly hydrolyzed to afford 5-caC-containing DNA as the major product (peak d, Figure 3G). In contrast, when polymer-attached **ODN3** was treated under the other mild conditions (concentrated NH_4OH at rt for 2 h), HPLC showed that the major product is an amide (peak f, Figure 3H) with only a small amount of desired 5-caC in DNA formed (peak d, Figure 3H), suggesting that NH_4OH treatment is not suitable in this case.

In order to further confirm the application of phosphoramidites **III** and **V**, we incorporated them into 20mer oligos (5′-TTTCAGCTCXGGTCACGCTC). Using the same deprotection conditions, we purified the desired modified DNA oligos by HPLC and characterized them by MALDI-Tof MS (X = 5-fC, $[MK]^+$ = 6080; X = 5-caC, $[MK]^+$ = 6096).

In summary, we have achieved an efficient synthesis of the phosphoramidite of 5-formyl-2′-deoxycytidine **III** without protection of the 5-formyl group. The synthesis entails six steps in 50% overall yield. The 5-formyl group can survive all transformations during the phosphoramidite and subsequent DNA syntheses and two different mild deprotection conditions to give 5-fC-containing DNA **ODN1**. We also demonstrate that an additional treatment of the resin-attached 5-fC-containing DNA with $NaBH_4$ could postsynthetically convert the 5-fC-containing DNA into the corresponding 5hmC-containing DNA quantitatively, which provides a more convenient alternative synthesis of 5-hmC-containing DNA. We have also developed an efficient synthesis of the 5-caC phosphoramidite **V** with methyl ester as the protecting group in four steps in 57% overall yield and demonstrated that **V** is compatible with subsequent DNA synthesis. We believe these synthetic procedures will provide sufficient materials for future investigation of 5-hmC-containing DNA and 5-fC- and 5-caC-containg DNA oligos in biological investigations.

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Supporting Information Available. Analytical data for compounds **2**–**9**, **III**, and **V** including their ^1H and ^{13}C NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.