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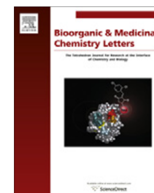

Improved synthesis of 5-hydroxymethyl-2′-deoxycytidine phosphoramidite using a 2′-deoxyuridine to 2′-deoxycytidine conversion without temporary protecting groups

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ABSTRACT

5-Hydroxymethylcytosine has recently been characterized as the ‘sixth base’ in human DNA. To enable research on this DNA modification, we report an improved method for the synthesis of 5-hydroxymethyl-2′-deoxycytidine ($^{5\text{-HOMe}}$dC) phosphoramidite for site-specific incorporation into oligonucleotides. To minimize manipulations we employed a temporary protecting group-free 2′-deoxyuridine to 2′-deoxycytidine conversion procedure that utilizes phase transfer catalysis. The desired $^{5\text{-HOMe}}$dC phosphoramidite is obtained in six steps and 24% overall yield from 2′-deoxyuridine.

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The canonical ‘ATCG’ bases of DNA can undergo covalent modifications on their purine and pyrimidine rings (Fig. 1). Such modifications[1,2] can arise from random base damage, or from enzyme-catalyzed modification, as in the case of 5-hydroxymethyl-2′-deoxyuridine[3] ($^{5\text{-HOMe}}$dU) and its hypermodified glycosylated derivative, base J[4–6] (dJ). In human cells, *S*-adenosylmethionine-dependent DNA methyl transferases[7] catalyze the C-5 methylation of cytosine bases to generate 5-methylcytosine ($^{5\text{-Me}}$dC). The presence of $^{5\text{-Me}}$dC in CpG islands of promoter regions of genes can result in inhibition of transcription through epigenetic modulation of the binding of chromatin-associated proteins.[8] Aberrant DNA methylation patterns are linked to diseases including cancer.[9–11]

Recently, Kriaucionis and Heintz reported the presence of a novel human DNA modification, 5-hydroxymethyl-2′-deoxycytidine ($^{5\text{-HOMe}}$dC) in Purkinje neurons. In some regions, $^{5\text{-HOMe}}$dC comprises up to ~20% of the total $^{5\text{-Me}}$dC content.[12] $^{5\text{-HOMe}}$dC is also enriched in areas of the human brain associated with higher cognitive function.[13] Tahiliani et al. found that the 2-oxoglutarate and Fe(II)-dependent hydroxylase[14] TET1 catalyzes the conversion of $^{5\text{-Me}}$dC to $^{5\text{-HOMe}}$dC in human cell lines.[15] These findings were recently extended to the catalytic domains of the homologous oxygenases TET2 and TET3.[16] TET2 is proposed to be a tumor

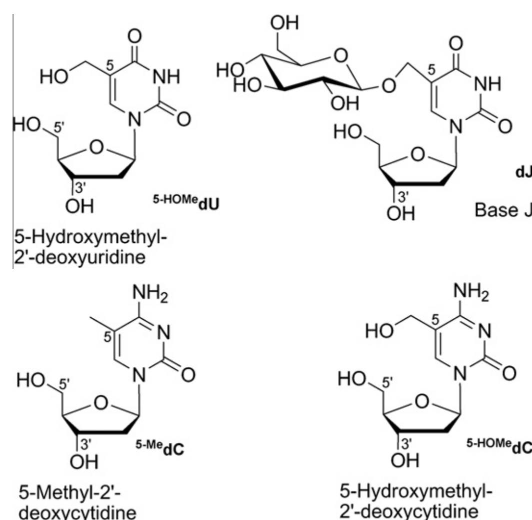

Figure 1. Selected known modifications at the 5-position of pyrimidine bases in DNA.

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suppressor gene and a candidate prognostic marker in myeloid malignancies.[17,18] To enable studies on the effect of 5-hydroxymethylcytosine on the biochemical and biophysical properties of DNA, an efficient route to $^{5\text{-HOMe}}$dC phosphoramidite **9** is required. We describe an improved synthesis of **9** that is suitable for the preparation of gram-scale quantities for use in automated DNA synthesis.

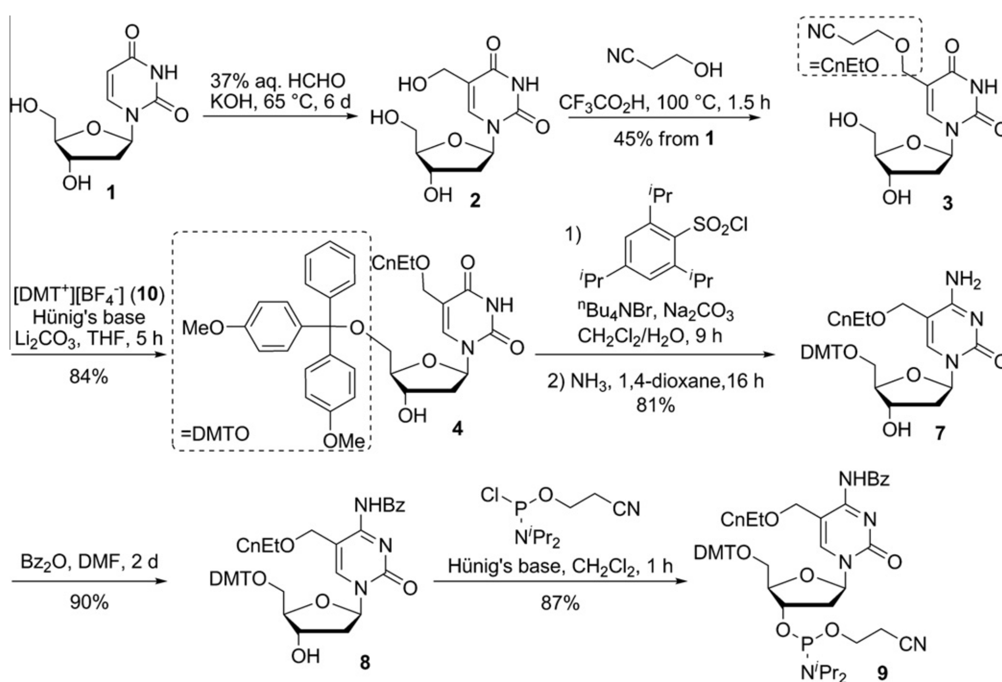
Reported approaches to the preparation of $^{5\text{-HOMe}}$dC phosphoramidite from 2′-deoxyuridine[19] or 2′-deoxythymidine[20] involve the introduction and selective protection of the pyrimidine 5-hydroxymethyl group, 2′-deoxyuridine to 2′-deoxycytidine interconversion requiring protection/deprotection of the 3′- and 5′-hydroxyl groups, and installation of dimethoxytrityl (DMT) and benzoyl groups on the 5′-hydroxyl and exocyclic N^4-amino groups, respectively, followed by phosphoramidite formation. de Kort et al.[20] have reported a synthesis of 5-acetyl-protected $^{5\text{-HOMe}}$dC via radical bromination of 3′,5′-di-TBDMS-protected 2′-deoxythymidine, followed by a nucleophilic substitution with cesium acetate to yield a 5-acetyl-protected phosphoramidite. In our hands, the bromination step in this sequence gave only moderate yields. Another recent synthesis relies on an elegant concomitant protection of the 5-hydroxymethyl and exocyclic N^4-amino groups resulting in a cyclic structure, yet requires expensive starting materials and multiple protecting group manipulations.[21]

In an attempt to devise an efficient and economical synthetic route in which the use of temporary protecting groups is avoided, we then investigated the direct hydroxymethylation[22] of 2′-deoxyuridine **1** (Scheme 1) with a view to subsequent chemoselective protection[19] of the 5-hydroxymethyl group with 2-cyanoethanol under S_N1 conditions. Protection with the 2-cyanoethyl group also avoids the problem of rearrangement during cleavage of oligonucleotides, as observed with the acetyl group.[20]

Acid- or base-catalyzed hydroxymethylations[23,22] are often low-yielding, due to limited substrate conversion[23] and competing Cannizarro reactions. We found that isolation of 2-cyanoethyl-protected **3** was difficult when using crude hydroxymethylation product mixtures, due to similar retention times of product **3** and starting material **1** on silica gel. However, reaction of sufficiently purified (>90%) $^{5\text{-HOMe}}$dU **2** with 2-cyanoethanol and catalytic CF_3CO_2H gave **3** in 45% yield from dU **1**. The use of catalytic amounts of HCl or *p*-toluenesulfonic acid rather than CF_3CO_2H in the protection step lead to partial degradation. In a reported preparation[19] of phosphoramidite **9**, Reese's reagent[24] ($POCl_3$/1,2,4-triazole) was used to convert cyanoethyl-protected 2′-deoxyuridine to the corresponding cytidine in a three-stage reaction, comprising temporary protection of the 3′- and 5′-hydroxyl groups by acetylation, followed by triazolide formation and ammonolysis with concomitant acetyl deprotection.[19] In our hands, purification of the acetyl-protected 2′-deoxyuridine intermediate was required to achieve efficient conversion with Reese's reagent and selective removal of the acetyl protecting groups in the presence of the base-labile cyanoethyl group was problematic, resulting in low overall yields.

We then considered the early introduction of a 5′-DMT protecting group (Table 1), both to facilitate the purification of otherwise polar intermediates, and to avoid the need for temporary 5′-protection. Reported reagent combinations that are applicable to the DMT protection of 5-methyl-2′-deoxycytidine,[25,26] such as DMT-Cl/DMAP/pyridine or $AgNO_3$/DMT-Cl,[27] resulted in incomplete conversion of **3** to **4** and low yields (Table 1). The limited reactivity of the 5′-hydroxyl group may be due to steric hindrance by the 5-cyanoethoxymethyl group (compared to a 5-methyl substituent).

In an attempt to improve conversion of **3** to **4**, we investigated use of DMT tetrafluoroborate **10**, a reactive reagent that has been used in 'difficult' nucleoside protections.[28,29] Compound **10** was synthesized from DMT chloride in a one-pot procedure without chromatographic purification (Supplementary data). When a suspension of **3** in THF was treated with **10**, complete consumption of starting material was observed. However, the isolated yields of **4** were moderate (54%, Table 1, entry 3) due to competing formation of the 3′,5′-di-DMT-protected compound **5** (~30%). In part, formation of **5** may be attributed to the higher solubility of **4** in THF as compared to the starting material **3**. The yield of DMT-protected 2′-deoxyuridine **4** was substantially improved (84%, entry 4) by performing the reaction at higher dilution, employing incremental addition of DMT tetrafluoroborate **10** and switching from



Scheme 1. Improved procedure for the preparation of $^{5\text{-HOMe}}$dC phosphoramidite **9**.

Table 1
Optimization and substrate scope of 5′-DMT protection with DMT tetrafluoroborate **10**

	Conditions	Substrate	R^1	Product	Yield[a] (%)
(1)	DMT-Cl/DMAP[b]	**3**	CH_2OEtCN	**4**	33
(2)	DMT-Cl/$AgNO_3$[c]	**3**	CH_2OEtCN	**4**	29
(3)	**10** (1.8 equiv), Li_2CO_3, 2,6-lutidine	**3**	CH_2OEtCN	**4** + **5**	54[d]
(4)	**10** (1.7 equiv)[e]	**3**	CH_2OEtCN	**4**	84
(5)	**10** (2 equiv)[e]	**11**	H	**17**	89
(6)	**10** (1.5 equiv)[e]	**12**	CH_3	**18**	74
(7)	**10** (2 equiv)[e]	**13**	Br	**19**	88
(8)	**10** (1.5 equiv)[e]	**14**	I	**20**	84
(9)	**10** (1.8 equiv)[e]	**15**	CF_3	**21**	87
(10)	**10** (1.8 equiv)[e]	**16**	(2′-Deoxyinosine)	**22**	76

[a] Isolated yields.
[b] DMT-Cl (1.5 equiv), DMAP (0.05 equiv), pyridine, 7 d.
[c] DMT-Cl (1.15 equiv), $AgNO_3$ (1.15 equiv), pyridine, THF, 2 d.
[d] Byproduct 5′,3′-di-DMT-protected **5** also isolated (yield 30%).
[e] Incremental addition of **10**, Li_2CO_3 (5 equiv), Hünig's base (3 equiv).

2,6-lutidine to Hünig's base, which was more amenable to removal in the workup.

To test the substrate scope of our optimized conditions for DMT tetrafluoroborate-based protection, the reaction was performed on a set of 2′-deoxyuridine derivatives (**11**–**15**, Table 1) with diverse 5-substituents and 2′-deoxyinosine **16**. The desired products **17**–**22** were obtained in consistently high yields (74–89%). In particular, this method appears to be useful for selective 5′-DMT protection of less reactive 5-substituted 2′-deoxyuridine derivatives.

A bottleneck in the subsequent steps was the conversion of 2′-deoxyuridine **4** to 2′-deoxycytidine **7**. This is because the reaction conditions need to be compatible with both the acid-labile DMT group and the base-labile cyanoethyl moiety. Conversion of **4** to **7** was initially attempted using Reese's reagent[24,26,30,31] with transient TMS protection of the 3′-hydroxyl group (Table 2). However, these reagents gave incomplete conversion and low isolated yields (30–40%) along with recovered starting material (50–60%). Alternative reaction conditions employing TPSCl/DMAP/Et_3N in CH_3CN[30,32] required long reaction times and did not improve the outcome (Table 2). Use of excess reagents caused purification problems.

Phase transfer catalysis has been used for the efficient preparation of 3′,5′-diprotected 5-azidomethylcytidine[33] under mild conditions, including under circumstances where other 2′-deoxyuridine to 2′-deoxycytidine conversion methods were unsuccessful. Encouraged by previous studies on 3′,5′-disilylated O^4-arylsulfonylthymidine derivatives,[34] which are valuable intermediates for Pd-catalyzed cross-coupling reactions, we considered that the bulky 5′-DMT group of 2′-deoxyuridine **4** and the sterically demanding 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl) activating agent might enable selective activation of the pyrimidine carbonyl via intermediate **6** (indicated by mass spectrometric analysis of TLC spots), even in the presence of a free 3′-hydroxyl group. Indeed, reaction of **4** with TPSCl using TBAB as phase-transfer catalyst in a biphasic aqueous sodium carbonate–CH_2Cl_2, followed by ammonolysis gave 2′-deoxycytidine **7** in high yields (81% after chromatography).

The higher reactivity of the pyrimidine O^4-oxygen as compared to the unprotected 3′-hydroxyl group may reflect deprotonation of **4** at the acidic N^3-nitrogen in the aqueous phase, followed by TBAB-mediated phase transfer of the anion of **4** into the organic phase where reaction with TPSCl occurs. It is known that the use of TBAB as phase transfer catalyst can often require careful product purification to ensure its complete removal. Indeed, we were initially unable to fully remove TBAB from **7** by chromatography. We found that the addition of DMF (~5–10% v/v) in the extractive workup before the ammonolysis step considerably facilitated the removal of TBAB.

This procedure for 2′-deoxyuridine to 2′-deoxycytidine conversion of **4** to **7** is rapid, robust, and does not depend on toxic and moisture-sensitive reagents such as $POCl_3$. This is a rare example of an efficient chemoselective conversion of 2′-deoxyuridines to 2′-deoxycytidines in the absence of a 3′-protecting group. In

Table 2
Development of a protecting group free phase-transfer-catalyzed method for conversion of 2′-deoxyuridine **4** to 2′-deoxycytidine **7** using tetrabutylammonium bromide (TBAB)

Conditions			Time	Yield[a] (%)
Activating agent	Additives	Solvent		
(1) TMSCl protection, then $POCl_3$	1,2,4-Triazole, Et_3N	CH_3CN	2 h	40
(2) TMSCl protection, then TPSCl (10 equiv)	DMAP, Et_3N	CH_3CN	2.5 d	<60
(3) TPSCl (3 equiv)	TBAB, Na_2CO_3	CH_2Cl_2/H_2O	2.5 h	72
(4) TPSCl (1.3 equiv)	TBAB, Na_2CO_3	CH_2Cl_2/H_2O	8.5 h	81

[a] Isolated yields.

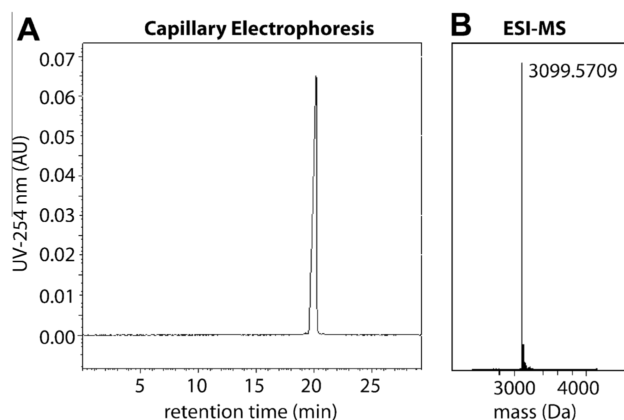


Figure 2. (A) Capillary electrophoresis and (B) electrospray mass spectrum (negative ion mode) of HPLC-purified oligonucleotide 5′-T**X**GT**X**GT**X**GT-3′ which contains three $^{5\text{-HOMe}}$dC (**X**) nucleotides. ESI-MS accurate mass required 3100, found 3099.57.

addition, the conditions are sufficiently mild for the reaction to be performed in the presence of the 5′-DMT group, which can directly be used in solid-phase supported DNA synthesis, as compared to silyl protecting groups.

Attempts to *N*-benzoylate **7** with benzoyl chloride following TMS protection at the 3′-hydroxyl group[35] afforded complex mixtures. However, use of benzoic anhydride in dry DMF[25,26] allowed for the chemoselective protection of the exocyclic amino group of **7** to give **8** (highly acid-sensitive) in 90% isolated yield, again without reaction at the 3′-hydroxyl group.

Compound **8** was then 3′-phosphorylated[19,20] to afford the $^{5\text{-HOMe}}$dC phosphoramidite **9** (87% isolated yield, >99% pure by ^{31}P NMR). The ^{1}H NMR of the product was identical to that of a commercial sample (Glen Research Corporation, Sterling, Virginia, USA). Phosphoramidite **9** was used in the solid-phase synthesis of a series of oligonucleotides containing up to three $^{5\text{-HOMe}}$dC nucleotides. All oligonucleotides were analyzed by capillary electrophoresis and characterized by mass spectrometry (Fig. 2 and Supplementary data).

In summary, we have developed a substantially improved synthetic route to a protected precursor of 5-hydroxymethylcytosine and have demonstrated that the product can readily be incorporated into oligonucleotides for biological applications.

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Supplementary data

Supplementary data (synthetic protocols and analytical data, and copies of ^{1}H and ^{13}C NMR spectra for all new compounds. Oligonucleotide synthesis protocol, analysis and characterization of oligonucleotides) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.12.098.

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