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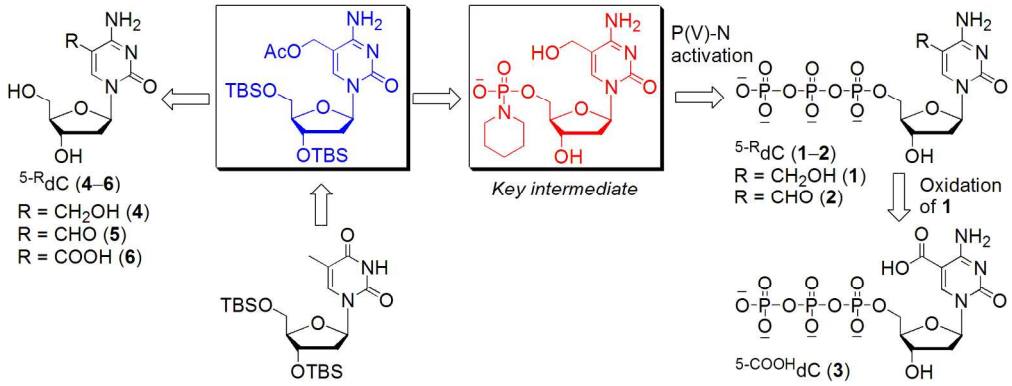

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Efficient synthesis of 5-hydroxymethyl-, 5-formyl-, and 5-carboxyl-2′-deoxycytidine and their triphosphates

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An efficient P(V)–N activation strategy for the preparation of high-quality 5-hydroxymethyl-, 5-formyl-, and 5-carboxyl-2′-deoxycytidine triphosphates has been developed. The method was also optimized for gram-scale synthesis of the corresponding parent nucleosides from 2′-deoxythymidine.

During the past few decades, it has been unravelled that DNA methyltransferases-mediated methylation of cytosine in eukaryotic genomes is one of the most important epigenetic marks for transcriptional gene silencing.[1] In mammalian DNA, cytosines are predominantly methylated within CpG sites, and the methylation patterns are heritable as stable epigenetic signals during cell divisions.[2] Meanwhile, DNA demethylation in specific contexts is required for the recovery of cytosine bases, enabling flexible and dynamic regulation of gene expression during cellular development.[3]

Recently, a series of 5-methylcytosine (5-mC) oxidation products, 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine (5-fC), and 5-carboxycytosine (5-caC) have been detected in mammalian DNA.[4] The ten eleven translocation proteins (TET1–3) were identified as the corresponding oxidases with molecular oxygen and 2-ketoglutarate as cofactors. The new research evidence reported by He et al.[6] and Zhang et al.[7] strongly supported that TET-mediated oxidation of 5-mC leads to active DNA demethylation in epigenetic programming of cells.[8] While thymine-DNA glycosylase (TDG) was determined as an enzyme for 5-caC excision repair,[6] other enzymes involved in either base excision repair (BER) or decarboxylation pathways remain to be elucidated.

In the past few years, several phosphoramidite-based solid phase approaches for the preparation of 5-hmC-, 5-fC-, and 5-caC-containing oligodeoxynucleotides (ODNs) have been developed.[9] To advance the investigation of the mechanisms and enzymes related to 5-mC oxidation, longer DNA fragments with 5-hmC-, 5-fC-, and 5-caC bases are highly desired but hard to be synthesized by the tedious solid phase methods. More recently, Carell and his co-workers reported an expeditious synthesis of long 5-hmC-, 5-fC-, and 5-caC-containing ODNs (150 bp) from 5-hydroxylmethyl-, 5-formyl-, and 5-carboxyl-2′-deoxycytidine triphosphates ($^{5\text{-HOMe}}$dCTP (**1**), $^{5\text{-CHO}}$dCTP (**2**), and $^{5\text{-COOH}}$dCTP (**3**)) by polymerase chain reaction (PCR).[10] However, the oxidative modifications of cytosine posed a huge challenge for the preparation of the corresponding triphosphates (Fig. 1). Though the reported yield for $^{5\text{-CHO}}$dCTP was 70%, the disproportional peaks on its ^{31}P NMR spectrum revealed that the sample was contaminated with a significant amount of polyphosphate impurities.[10] Similar issues were also found in the low-yielding synthesis of $^{5\text{-COOH}}$dCTP (7% yield) and the protected $^{5\text{-HOMe}}$dCTP (1% yield).[10] In this paper, we report an efficient preparation of the triphosphates of all three $^{5\text{-Me}}$dC oxidation products (**1**–**3**) on the basis of the P(V)–N activation strategy we established for nucleoside polyphosphate synthesis.[11] The optimized method for gram scale synthesis of the parent nucleosides, $^{5\text{-HOMe}}$dC (**4**), $^{5\text{-CHO}}$dC (**5**), and $^{5\text{-COOH}}$dC (**6**), from dT is also described.

Currently, there are two major synthetic routes for the preparation of $^{5\text{-Me}}$dC oxidation derivatives. The one utilizing 5-iodo-2′-deoxycytidine ($^{5\text{-I}}$dC) starting material directly installed the 5-formyl or 5-methoxycarbonyl group by the Pd-catalyzed Stille reaction.[9a–d] The reduction of $^{5\text{-CHO}}$dC (**5**) afforded $^{5\text{-HOMe}}$dC (**4**).[9a,10] However, the cost of $^{5\text{-I}}$dC and the use of pressurized reactor for gaseous CO limited its application. Therefore, we employed the other approach starting from dT.[4c]

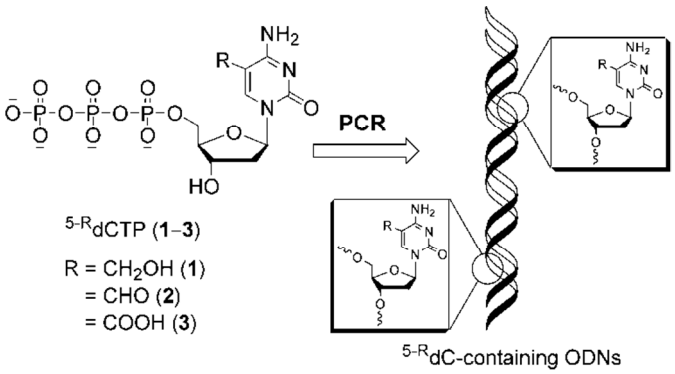
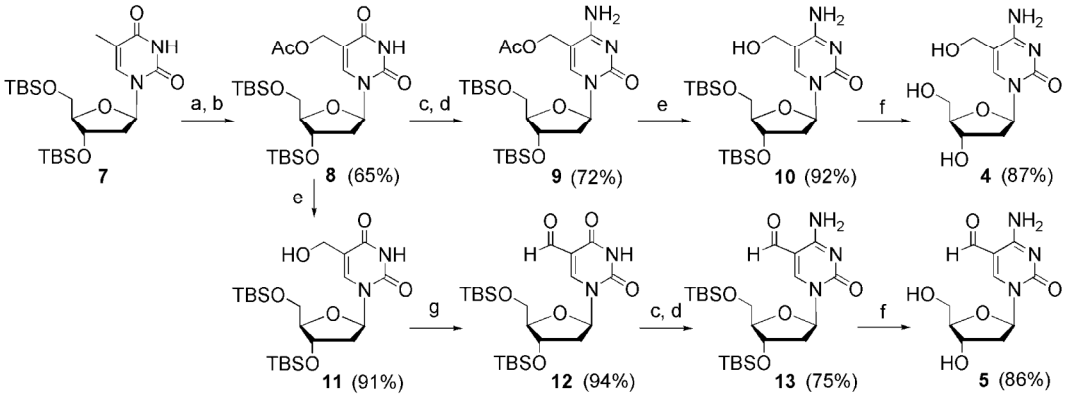
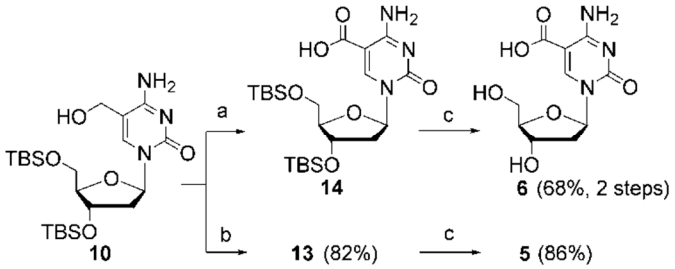


Fig. 1 The $^{5\text{-R}}$dCTP-based PCR technology for the expeditious preparation of long 5-hmC-, 5-fC-, and 5-caC-containing ODNs.[10]



Scheme 1 Synthesis of $^{5\text{-HOMe}}$dC (**4**) and $^{5\text{-CHO}}$dC (**5**). Reagents and conditions: a. NBS, AIBN, CCl_4, 80 °C, 1.5 h; b. CH_3COOK, 40 °C, 30 min; c. TsCl, *N*-methylpiperidine, Et_3N, 4 h; d. 28% NH_4OH, 30 min; e. K_2CO_3/MeOH/H_2O, 20 °C, 2 h; f. THF/H_2O/TFA (2:1:1), 20 °C, 1 h; g. activated MnO_2, CH_2Cl_2, 30 °C, 48 h.

As shown in Scheme 1, 3′,5′-diTBS-protected dT (**7**) which could be easily prepared from dT was brominated with NBS in CCl_4, and then treated with potassium acetate to afford the acetylated intermediate **8** in 65% yield.[12] After **8** was deacetylated with K_2CO_3, mild oxidation of the protected $^{5\text{-HOMe}}$dU (**11**) with activated MnO_2 in CH_2Cl_2 furnished clean conversion to the silylated $^{5\text{-CHO}}$dU (**12**) in 94% yield.[9e,13]



Scheme 2 Synthesis of $^{5\text{-COOH}}$dC (**6**) and $^{5\text{-CHO}}$dC (**5**). Reagents and conditions: a. TEMPO/BAIB (0.2/2.5 equiv), CH_2Cl_2/H_2O, 20 °C, 8 h; b. TEMPO/BAIB (0.2/1.1 equiv), CH_2Cl_2/H_2O, 20 °C, 2 h; c. THF/H_2O/TFA (2:1:1), 20 °C, 1 h.

As Carell mentioned, the major challenge for $^{5\text{-HOMe}}$dCTP (**1**) synthesis is associated with the 5-hydroxylmethyl group in $^{5\text{-HOMe}}$dC (**4**), which makes it difficult to selectively phosphorylate the OH group at C5′ position to access $^{5\text{-HOMe}}$dC 5′-monophosphate (**16**).[10] To address this issue, we attempted to conduct selective amination at C4 position of **8** to obtain the acetylated $^{5\text{-HOMe}}$dC intermediate **9,** which could be later utilized for the synthesis of **16**. However, the conventional $POCl_3$/1,2,4-triazole/ methanolic NH_3 method only gave **9** in low yield (12%) with significant amount of deacetylated product.[4c,14] Switching to 28% NH_4OH largely reduced the undesired deacetylation on the 5-hydroxylmethyl group, and afforded **9** in 60% yield.[12a,15] To further improve the synthetic efficacy and simplify the procedures, TsCl was used with 1-methylpiperidine as the activator to form a more reactive quaternary ammonium intermediate.[16] The in situ aminolysis selectively afforded $^{5\text{-HOMe}}$dC precursor **9** in 72% yield within 30 min. More interestingly, this one-pot reaction with TsCl/1-methylpiperidine/28% NH_4OH exhibited excellent compatibility with the 5-formyl group in **12**, and yielded the protected $^{5\text{-CHO}}$dC precursor **13** in high conversion rate (75%). Final deprotection (deacetylation and desilylation for **9**/desilylation for **13**) gave $^{5\text{-HOMe}}$dC (**4**) and $^{5\text{-CHO}}$dC (**5**) in high yields.

Due to the presence of the TBS groups and glycosidic bond in $^{5\text{-HOMe}}$dC precursor **10**, most conventional strong oxidizing methods are too harsh to transform the hydroxyl group to carboxylic acid with high chemoselectivity. To obtain $^{5\text{-COOH}}$dC (**6**), **10** was oxidized with TEMPO/BAIB (0.2/2.5 equiv) under mild conditions.[17] The subsequent removal of the TBS groups with TFA afforded **6** in nearly 70% yield over two steps (Scheme 2). It is noteworthy that the outcomes of TEMPO/BAIB-mediated oxidation are strongly correlated with the amount of BAIB.[18] When stoichimetric amount of BAIB was applied (TEMPO/BAIB (0.2/1.1 equiv)), **10** could be efficiently transformed into the corresponding aldehyde **13**, providing an alternative approach to $^{5\text{-CHO}}$dC (**5**).

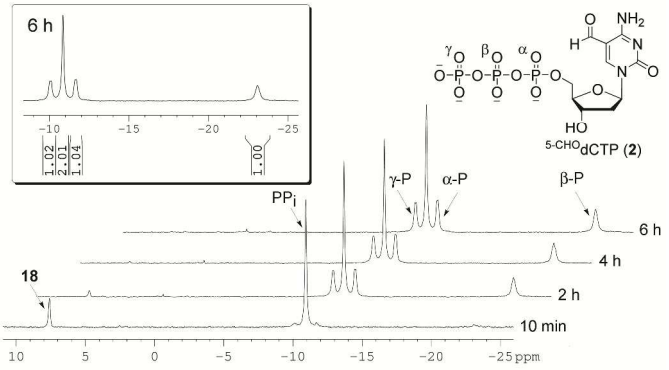
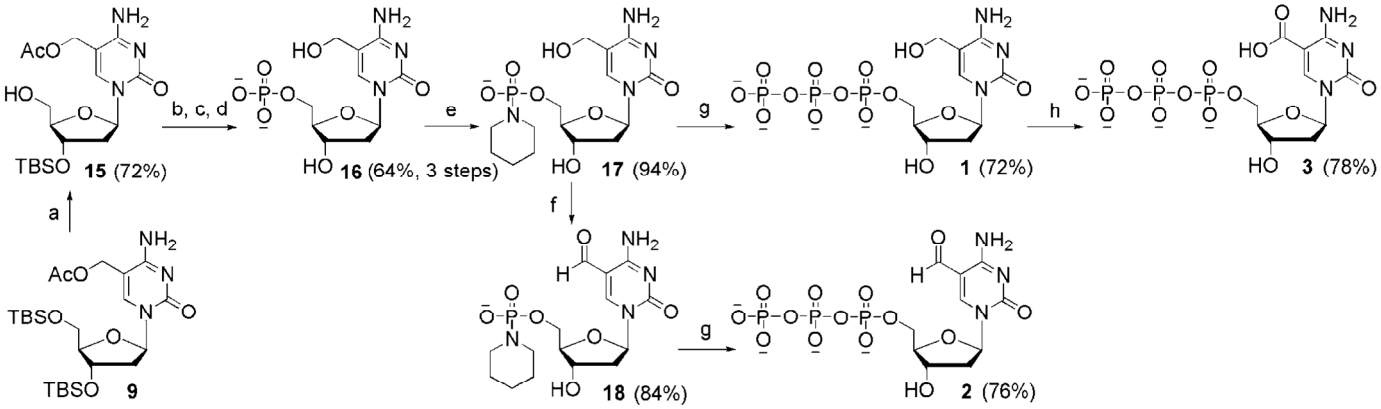


Fig. 2 The stacked ^{31}P NMR tracing spectra of $^{5\text{-CHO}}$dCTP (**2**) synthesis.

Our synthetic route to $^{5\text{-HOMe}}$dCTP (**1**) and $^{5\text{-CHO}}$dCTP (**2**) started from acetylated $^{5\text{-HOMe}}$dC intermediate **9** (Scheme 3). Efficient regioselective desilylation at 5′ position was achieved by lowering the concentration of TFA (THF/H_2O/TFA, 4:1:1) and reaction temperature (0 °C).[19] Treatment of **15** with $POCl_3$ in $PO(OMe)_3$ followed by sequential deacetylation and desilylation afforded the $^{5\text{-HOMe}}$dC 5′-monophosphate (**16**) in 64% yield. Our observation that no proton sponge was required for the phosphorylation of **15** was in agreement with previous reports on the synthesis of cytosine-containing nucleoside



Scheme 3 Synthesis of $^{5\text{-HOMe}}$dCTP (**1**), $^{5\text{-CHO}}$dCTP (**2**), and $^{5\text{-COOH}}$dCTP (**3**). Reagents and conditions: a. THF/H_2O/TFA (4:1:1), 0 °C, 2 h; b. $POCl_3$, $PO(OCH_3)_3$, 0 °C, 2 h; c. TFA/H_2O (1:1), 20 °C, 1 h; d. K_2CO_3/MeOH/H_2O, 20 °C, 1 h; e. 2,2′-dithiodianiline, PPh_3, piperidine, DMSO, 20 °C, 8 h; f. activated MnO_2, MeOH, 50 °C, 24 h; g. $(n\text{Bu}_4\text{N})_3\text{HP}_2\text{O}_7$, DCI, 20 °C, 6 h; h. TEMPO/BAIB (0.4/2.5 equiv), *t*BuOH/CH_2Cl_2/H_2O (4:4:1), 20 °C, 48 h.

monophosphates.[20] In the following step, **16** was converted to $^{5\text{-HOMe}}$dC 5′-phosphoropiperidate (**17**) by the redox condensation method in excellent yield (94%).[11c] Treatment of **17** with activated MnO_2 smoothly oxidized 5-hydroxylmethyl group to 5-formyl group to give $^{5\text{-CHO}}$dC 5′-phosphoropiperidate (**18**). Finally, **17** and **18** were subjected to the 4,5-dicyanoimidazole (DCI)-promoted P(V)–N activation strategy to synthesize $^{5\text{-HOMe}}$dCTP (**1**) and $^{5\text{-CHO}}$dCTP (**2**).[11a,c] ^{31}P NMR tracing experiments showed that both **1** and **2** were obtained with high conversion efficacy as exemplified by the reaction of **2** (Fig. 2), indicating that the P(V)–N activation method well tolerated the hydroxylmethyl and formyl modifications on cytosine. Ethanol precipitation followed by ion exchange chromatography afforded **1** and **2** in high isolated yields.

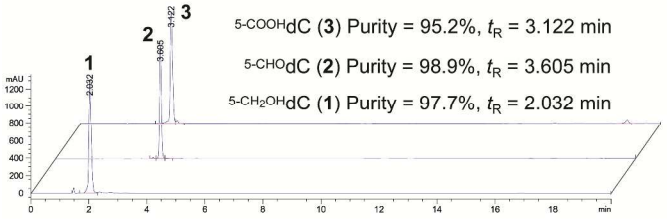


Fig. 3 The HPLC traces of $^{5\text{-HOMe}}$dCTP (**1**), $^{5\text{-CHO}}$dCTP (**2**), and $^{5\text{-COOH}}$dCTP (**3**).

The attempt to oxidize **17** with TEMPO/BAIB (0.2/2.5 equiv) system only afforded the desired $^{5\text{-COOH}}$dC 5′-phosphoropiperidate (**19**) in low yield (<20%) due to the labile nature of phosphoropiperidate under even weakly acidic conditions. Therefore, we directly oxidized $^{5\text{-HOMe}}$dCTP (**1**) with TEMPO/BAIB (0.4/2.5 equiv) to yield $^{5\text{-COOH}}$dCTP (**3**). ^{31}P NMR tracing results showed that the oxidation process in monophasic *t*BuOH/CH_2Cl_2/H_2O (4:4:1) solvent system[21] was smooth and clean. After 48 h, **3** was isolated in 78% yield. The quality of **3** was determined with analytic RP-HPLC along with **1** and **2**. The HPLC traces in Fig. 3 showed that triphosphates **1**–**3** prepared by our method were of high purity (>95%). But it is worth noting that the solvent system also played a key role in the TEMPO/BAIB oxidation. When monophasic CH_3CN/H_2O (1:1) or biphasic CH_2Cl_2/H_2O (1:1) was used, the oxidation of **1** was extremely slow. While the TEMPO/BAIB oxidation of **10** in biphasic CH_2Cl_2/H_2O (1:1) afforded **14** within 8 h, the oxidation in monophasic *t*BuOH/CH_2Cl_2/H_2O (4:4:1) required much longer time (48 h).

Conclusions

In summary, we have developed an efficient method for the preparation of high-quality 5-hydroxylmethyl-, 5-formyl-, and 5-carboxyl-2′-deoxycytidine triphosphates (**1**–**3**) on the basis of the P(V)–N activation strategy. The synthesis of the parent nucleosides (**4**–**6**) were also optimized to provide facile access to all three oxidation products of $^{5\text{-Me}}$dC. The P(V)–N activation method described in this paper along with the $^{5\text{-R}}$dCTP-based PCR technology may greatly facilitate the investigation of $^{5\text{-Me}}$dC-related epigenetic regulations and development of regenerative drugs.

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† Electronic Supplementary Information (ESI) available: Experimental procedures and NMR spectra of intermediates and products are included. See DOI: 10.1039/c000000x/

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