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A chemical photo-oxidation of 5-methyl cytidines

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Abstract. A new approach for the oxidation of 5-methyl cytidine modification in nucleic acids to 5-formyl cytidine is reported. This method displays excellent chemo-/bio-selectivity, from which the methyl group in cytidine nucleosides could be selectively oxidized to biologically important formyl functionality without interfering with other cellular biomolecules.

Keywords: 5-methylcytidine; 5-formylcytidine; photochemical oxidation; artificial enzymes; epigenetic modifications

Introduction

Oxidation phenomenon is involved in various biochemical pathways and plays a crucial role in numerous pathological events and clinical situations.[1] The precise oxidation states in biological macromolecules (nucleic acids, proteins, etc.) accurately controls cellular signaling pathways and has been recognized as a major target for potential pharmaceutical intervention.[2] Consequently, the precise chemical regulation of these redox change under the assistance of small organic molecules (SOM) have attracted increased attention in recent years. These chemical motifs or artificial enzymes[3] with the capacity of mimicking redox pathways could potentially be utilized as biological probes or pharmaceutical candidates that regulate cellular fate and differentiation by controlling the redox state within the cells.

5-Methylcytidine[4] and its oxidation intermediates (5-hydroxymethylcytidine,[5] 5-formylcytidine[6] and 5-carboxylcytidine[7]) are important epigenetic modifications in nucleic acids,[8] which plays pivotal roles in accurate and efficient transcription and translation of genetic information. In recent years, 5-formylcytidine has attracted significant attention as a distinctive oxidative derivative of 5-methylcytidine demethylation. In DNA, 5-formylcytidine affects the rate and specificity of RNA polymerase II (RNAPII) and causes increased RNAPII backtracking, increased pausing, and reduced fidelity in nucleotide incorporation.[9] 5-Formylcytidine in RNA also plays an essential role through its ability to pair with G and A in the third position of the codon, thus furnishing the decoding of non-universal AUA codon as Met.[10] Previous reports have suggested that the oxidative step from 5-methylcytidine to 5-formylcytidines is catalyzed by TET and ABH1 enzymes[11] (Scheme 1A). Owing to the inertness of the methyl substituent and fragility of glyosidic bond, the oxidation of the C-H bond in 5-methylcytidine is always difficult to achieve with external chemical reagents.[12] An early attempt was made by Itahara *et al.*, who treated 5-methylcytidine with peroxosulfate ions at high temperature (75 °C) to produce 5-formylcytidine in 23% yield.[13] Recently Cadet and Nishimoto *et al.* applied a photochemical reaction into the oxidation of 5-formylcytidine. 5-Formyl-2'-deoxycytidine was obtained under the irradiation of UV light (312 nm) with Menadione in a low yield (13%), which unfortunately limited the method only to the mechanistic study.[13] Recently we demonstrated that the demethylation process of N^6-methyladenosine (m^6A) in live cells could be achieved by a bioorthogonal photo-oxidation with Flavin mononucleotide.[14] This finding represents the first example of *in vitro* modulating RNA epigenetic modification with SOMs.[15] Herein, we present a facile opto-transformation under mild conditions to produce 5-formylcytidine and its analogous from 5-methylcytidine under the promotion of a small organic molecular photosensitizer without disturbing other vulnerable C-H bonds in other biomolecules (Scheme 1B). Such approach, without over-expression or inhibiting corresponding enzymes, would be of great potential for *in vivo/vitro* studies of

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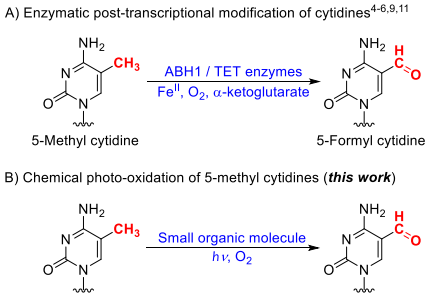
5-formylcytidine and other epigenetic cytosine variants. On the other hand, it would also facilitate the f^{5}C-containing nucleic acid oligo synthesis or NTP synthesis for enzymatic catalyzed polymerization.

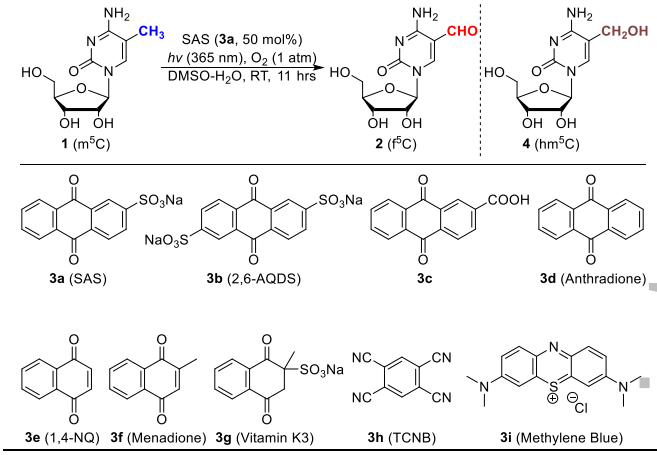


Scheme 1. A) Enzymatic post-transcriptional modification of cytidines. B) Chemical photo-oxidation of 5-methylcytidine (this work).

Results and Discussion

In the journey to develop a bioorthogonal approach on the conversion on 5-methylcytidine **1** (m^{5}C) to produce the 5-formyl functionality **2** (f^{5}C) without interfering with other oxidation-labile components, we were inspired by the biological enzymatic oxidation mediated by quinone-type cofactors[16] (Table 1). After screening diverse photocatalysts, sodium 2-anthraquinone sulfonate (SAS, **3a**), a water-soluble anthraquinone derivative, was found to be the optimal molecule for this oxidation (Table S1). Upon the irradiation with near-UV light (365 nm) under atmospheric O_2 at room temperature, the major oxidative product f^{5}C was isolated in 67% yield in an aqueous solution at room temperature (entry 1, Table 1). To the best of our knowledge, this is the so far the best result of converting the methylated cytosine directly to the formyl product under physiological conditions. The generation of **2** was not detectable without O_2 (entry 2), light (entry 3), or photocatalyst (entry 4), indicating that SOM-promoted irradiation was crucial for activating the methyl group. No improvement in yield was observed when using other quinone-type photosensitizers **3b-g** (entries 5-10). It is worthy to note that Menadione **3f** and its sulfonate adduct **3g** (Vitamin K_3), the catalysts previously used for oxidizing 5-methyl-2'-deoxycytidine,[13] showed inferior activity towards **1** (10% yield with **3f**, entry 9 and 30% with **3g**, entry 10), which was in consistence with previous work. Traditional photoactive molecules, such as TCBN **3h** and Methylene blue **3i**, were totally inactive under the standard conditions (entries 11-12). It should be mentioned that due to the solubility difficulty of m^{5}C in water, DMSO was largely used as co-solvent. Nonetheless, DMSO-PBS buffer mixture can also be utilized for this process and comparable yields were obtained (entries 13-14), indicating that this chemistry has the potential to be applied to nucleic acid oligo modification. It's also worth to mention that in most cases 5-hydroxymethylcytidine **4**, the preliminary oxidative product, was produced in trace levels and can only be observed with HPLC (*vida infra*).

Table 1. SAS-catalyzed photo-oxidation of 5-methylcytidine **1**[a]



Entry	Photosensitizer	Yield (%)
1	**3a**	67
2[b]	**3a**	NR
3[c]	**3a**	NR
4	None	NR
5[d],[e]	**3b**	34
6[d],[e]	**3c**	21
7[d],[e]	**3d**	trace
8[d],[f]	**3e**	trace
9[e],[g]	**3f**	10
10[d],[e]	**3g**	30
11[e],[g]	**3h**	NR
12[e],[g],[h]	**3i**	NR
13[i]	**3a**	62
14[j]	**3a**	64

[a] Unless otherwise specified, all reactions were carried out using **1** (5-methylcytidine, 0.1 mmol, 25 mg) and **3a** (SAS, 0.05 mmol, 16 mg) in the solvent of DMSO and H_2O (9/1, 2.5 mL total) at room temperature (37 °C) for 11 hours under 1 atm of oxygen (balloon). Isolated yield was given. NR: No reaction. [b] Oxidation conducted under N_2 instead of O_2. [c] Oxidation conducted under dark. [d] DMSO-H_2O (1/1) was used. [e] Reaction time: 8 hrs. [f] Reaction time: 10 hrs. [g] H_2O was used. [h] Photoreceptor loading: 10 mol%. [i] Oxidation conducted in DMSO-PBS Buffer (Na_2HPO_4-NaH_2PO_4, 40 mM, pH = 7.0). [j] Oxidation conducted in DMSO-PBS Buffer (10 mM, pH = 7.0).

Considering the low cellular toxicity of SAS[17] and the developed oxidation was proceeded smoothly in aqueous conditions at room temperature and physiological pH, we set out to explore the reactivity and selectivity of this promising bioorthogonal

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reaction. Thus, the chemical photo-oxidation of other epigenetic methylated nucleosides/bases was investigated (Figure 1). As expected, 5-methyl-2'-deoxycytidine furnished the corresponding 5-formyl-2'-deoxycytidine with a comparable isolated yield (65%). In contrast, natural nucleosides rA, rU, rC, rG and other epigenetic patterns, such as *N*-methyl m^6A and *O*-methyl A_m/C_m, remained largely untouched under the standard conditions according to thin-layer chromatography and high performance liquid chromatography analysis. However, due to the purification difficulties encountered with some nucleosides, reduced recovery yields were obtained in some cases. To our delight, the C-methyl pyridine nucleoside analogue, dT, was predominately recovered after the process (80%), suggesting this approach represents a particularly selective recognition and activation of the C-methyl cytidines. To explore the selectivity of this oxidation process, the standard procedure was applied to various endogenous and exogenous biomolecules, such as alkaloid (Theophylline), natural and unnatural amino acids ($_L$-Tryptophan and *N*-Bz-Alanine), and oligosaccharides ($_D$-Xylose and α-$_D$-galactopyranose). Most of these substrates largely remained intact under the opto-oxidative condition, again demonstrating the compatibility of this process. To further validate this approach can be applied to biomacromolecules, we subjected two nucleic acid oligo (5'-GGGUACAGAU-3' and 5'-ATTGTCAACAGCAGA-3') to the standard reaction conditions and no decomposition was observed (Supporting Information).

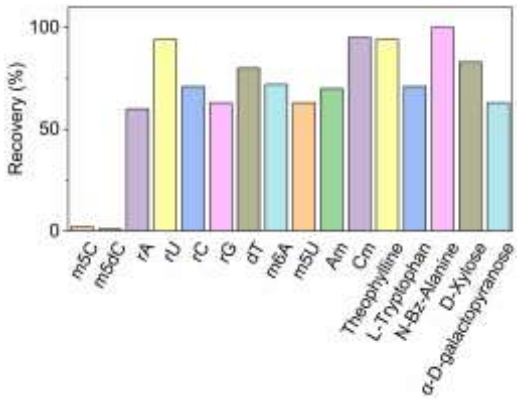

Figure 1. Recovery of nucleosides, alkaloids, amino acids and carbohydrates upon the oxidative condition (Raw data in Supporting Information).

In an effort to determine the intermediates on the oxidation of 5-methylcytidine, we further studied the time-course of the reaction by means of HPLC and LC-MS (Figure 2 and Figure S3). The enzymatic oxidation of 5-methylcytidine with TET/ABH1 afforded 5-formylcytidine as the major product, while our chemical opto-oxidative transformation in Figure 2A also suggested that this SAS-mediated photochemical oxidation proceeded in a similar manner and predominately yielded 5-formylcytidine. We also observed a trace amount of 5-hydroxymethylcytidine during the oxidative process from HPLC analysis, whose existence was confirmed with an authentic sample. Integration of the signals showed that the generation of 5-formylcytidine (ember line) was correlated with the decomposition of 5-methylcytidine (gray line), while the amount of 5-hydroxymethylcytidine adopted a bell shaped curve (blue line) (Figure 2B). It should be noted that the reason for the late-stage decrease of 5-hydroxymethylcytidine was still unclear. A control experiment with 5-hydroxymethylcytidine as the substrate under the standard oxidation condition didn't not yield 5-formylcytidine, suggesting that 5-hydroxymethylcytidine could not undergo further oxidation under this circumstances, but may decompose to other products. In addition, another intermediate 5-hydroperoxymethyl cytidine (oxm^5C)[13[c],18] was observed by LC-MS analysis (Figure 2C), indicating that peroxyl radicals may be involved in this process.

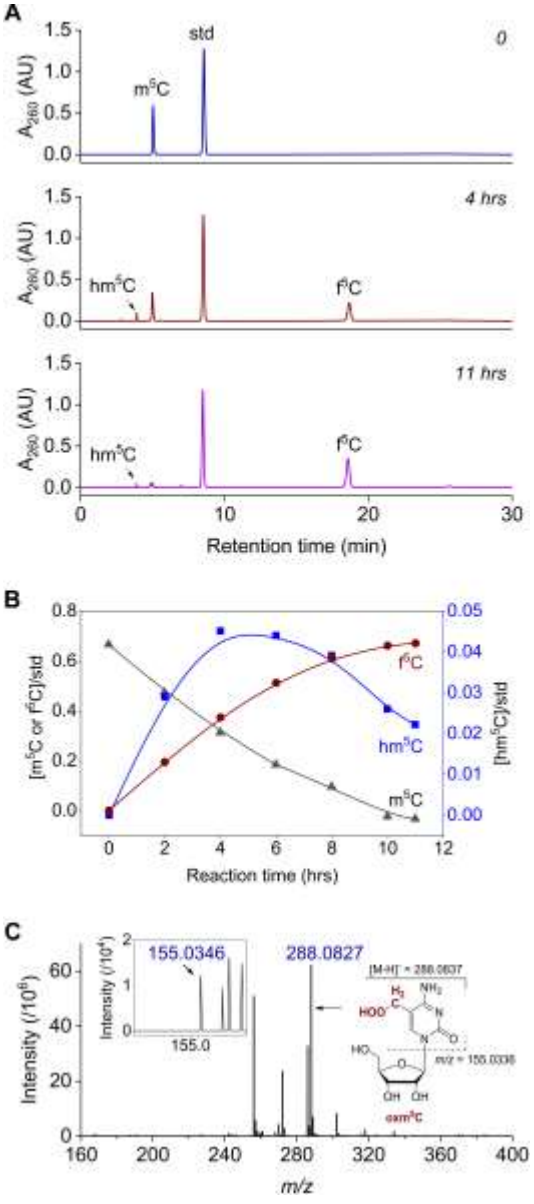

Figure 2. Mechanistic studies. A) HPLC analysis of the oxidative procedure at 0 hrs, 4 hrs and 11 hrs, respectively. The new peaks were assigned as 5-hydroxymethylcytidine and 5-formylcytidine. B) Kinetic measurement: 5-methylcytidine (0.1 mmol, 25 mg), SAS (0.05 mmol, 16 mg), U (internal standard, 0.1 mmol, 24 mg) in DMSO/H_2O (2.5 mL), irradiated with UV light of 365 nm under O_2 at room temperature. (Raw date in Supporting Information). C) Mass profile of the reaction mixtures at 4 hrs.

Based on our previous work on selective recognition of RNA[19] and in view of the above-mentioned results, it is concluded that the SAS-promoted oxidation of 5-methylcytidine may be through a radical pathway (Figure 3). The photosensitizer (PS) SAS was excited by photo absorption to a singlet/triplet state, followed by interacting with 5-methylcytidine to generate the methyl-centered radical **I**. In the subsequent step, the radical **I** reacted with oxygen leading to the formation of peroxyl radical **II**, which furnished peroxyl intermediate **III**. Dehydration of **III** gave rise to the formation of 5-formylcytidine. It should be mentioned that the formation of 5-hydroxymethylcytidine can be explained through decomposition of the unstable peroxyl radical **II** through a Russell mechanism[20] (*path a*) or from the benzyl-type cation **V** followed by trapped with water (*path b*).

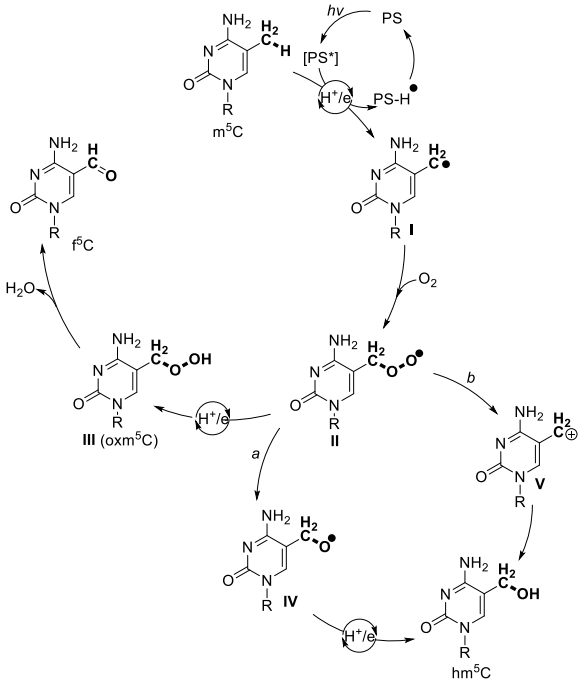

Figure 3. Proposed mechanism for the oxidation of 5-methylcytidine with SAS.

Conclusion

The oxidation of the methyl group of 5-methylcytidine and its derivatives has been previously investigated by using different chemical methods. However, those harsh reaction conditions and extreme low conversions/yields significant retarded their further application as effect bioorthogonal approaches for potential *in vivo* or *in vitro* post-modification of 5-methylcytidine. In this work, we have reported that the 365 nm irradiation of an aqueous solution of 5-methylcytidine in the presence of a small organic molecule SAS led to the formation of 5-formylcytidine as the main final oxidized product. Moreover, the detection of the preliminary oxidative product 5-hydroxymethylcytidine and the unstable intermediate 5-hydroperoxymethylcytidine provided further support for a peroxyl radical mechanism. We have found that natural biomolecules, most importantly, other epigenetic modifications did not undergo oxidation and remained relatively stable under the standard conditions, which unquestionably enables it as a potential bioorthogonal modulation of 5-methylcytidine in nucleic acids. In a future stage, attempts will be made to study the *in vivo* and *in vitro* post-modification of 5-methylcytidine at nucleic acid and live cell levels. Such artificial modulation of nucleic acid epigenetic variants and its application in the regulation of important cellular signal pathways will be reported in due courses.

Experimental Section

Procedure for the chemical photo-oxidation: To a solution of 5-methylcytosine (m^5C) (25 mg, 0.1 mmol) in a mixture of dimethyl sulfoxide and water (2.5 mL, v/v 9/1) was added sodium anthraquinone-2-sulfonate (16 mg, 0.05 mmol). The mixture was stirred for 11 hours under the irradiation of 365 nm UV light under oxygen at room temperature and then purified by reversed phase column chromatography (C_{18} column, elute: methanol/water 20/80) to afford 5-formylcytidine (f^5C) as a white solid (18 mg, yield 67%).

Acknowledgements

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