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# $N^6$-Hydroperoxymethyladenosine: a new intermediate of chemical oxidation of $N^6$-methyladenosine mediated by bicarbonate-activated hydrogen peroxide†

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$N^6$-Methyladenosine ($m^6A$) represents a relatively abundant modification in eukaryotic RNA. Because $m^6A$ has similar properties to adenosine and a low reactivity, limited research has been focused on this nucleoside. In this study, we revealed an important intermediate in the oxidation of $m^6A$ through the bicarbonate-activated peroxide system. Over the course of oxidation, we found a new mechanism in which $N^6$-hydroxymethyladenosine ($hm^6A$), $N^6$-formyladenosine ($f^6A$) and $N^6$-hydroperoxymethyladenosine ($oxm^6A$) were intermediate products, and adenosine was the final product. In this study, $oxm^6A$ was isolated using HPLC and characterized by mass spectrometry, NMR and diphenyl-1-pyrenylphosphine (DPPP) fluorescence detection. This study provides a new modified nucleoside and demonstrates oxidative demethylation of $m^6A$ by reactive oxygen species at the nucleobase level and in RNA strands.

$N^6$-Methyladenosine represents the most abundant modification in the mRNA of higher eukaryotes, present at a frequency of approximately three sites on each mRNA.[1] $m^6A$ is also present on tRNA, rRNA and lnRNA.[2] This modification plays an important role in the regulation of gene expression.[3] Since its discovery last century,[4] $m^6A$ has been the object of relatively few studies. Recently, fat mass- and obesity-associated proteins (FTO)[5] and AlkBH5 [6] were found to be $m^6A$ demethylases, indicating a novel regulatory mechanism in mammalian cells. Two new modifications, $N^6$-hydroxymethyladenosine ($hm^6A$) and $N^6$-formyladenosine ($f^6A$), have been found to participate in the FTO-mediated demethylation process, which may influence RNA–protein interactions and regulate gene expression.[7] In addition, transcriptome-wide profiling of $m^6A$ in mRNA and lnRNA has revealed new insights into the role of RNA modification.[8] These developments have renewed interest in the investigation of this particular, distinctive modification. Therefore, we aspire to use a chemical method to differentiate $m^6A$ from A.

Hydrogen peroxide is a widely used oxidant with a high content of active oxygen,[9] but its relatively slow oxidizing rate limits its usage. Bicarbonate is present in cells and serum at high concentrations, ranging from 14.7–25 mM,[10] and plays an important role in biological oxidation.[11] $H_2O_2$ and $NH_4HCO_3$ are environmentally friendly reagents; $H_2O_2$ produces only water as a by-product, and $NH_4HCO_3$ easily decomposes to $NH_3$, $CO_2$, and $H_2O$. The reaction conditions are mild at natural pH values.

Owing to its high reactivity towards secondary amines, we considered whether the oxidant could react with $m^6A$. Surprisingly, instead of producing *N*-oxides, demethylated adenosine was produced, and the presence of several intermediates in the reaction system suggested a potential mechanism in the chemical reaction (Scheme 1). These results suggest that $H_2O_2$/bicarbonate can act as a reactive oxygen species (ROS) for demethylation. In this study, we determine a key intermediate in the demethylation process, and we investigate the underlying mechanism.

To investigate the demethylation process, we used high-performance liquid chromatography (HPLC) to monitor the reaction (UV detector at 260 nm). When a 2 mM aliquot of $m^6A$ was treated with 200 mM $H_2O_2$ and 1 M $NH_4HCO_3$ at 37 °C for one hour, four products were formed: A, $hm^6A$, $oxm^6A$ and $f^6A$ (Fig. 1). The LC-MS data showed masses corresponding to A (267.9), $hm^6A$ (297.8), $oxm^6A$ (313.8) and $f^6A$ (295.9), successively in the positive-ion mode (Fig. S1†). Product A was further characterized by $^1H$ and $^{13}C$ NMR (see ESI†). To confirm the

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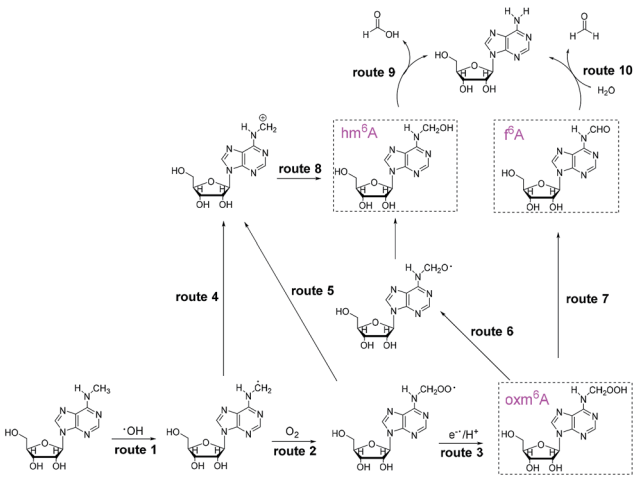


Scheme 1 Proposed mechanism of the demethylation process, and structures of oxidation products.

occurrence of $hm^6A$ and $f^6A$, these compounds were synthesized according to reported procedures.[7] An equilibrium reaction between adenosine and formaldehyde produced $hm^6A$ (Scheme 1, Route 9). Further HPLC analysis indicated that the synthesized $hm^6A$ and $f^6A$ have the same retention times as the reported $hm^6A$ and $f^6A$, respectively (Fig. 1b–d). We found that $hm^6A$ and $f^6A$ were unstable and could decompose to A (adenosine) during HPLC analysis (Fig. 1c and d). $N^6$-Hydroperoxymethyladenosine ($oxm^6A$) was found to be a new intermediate, in addition to $hm^6A$ and $f^6A$, during the demethylation of $m^6A$ (Fig. 1b). When we incubated the $m^6A$ with bicarbonate or $H_2O_2$ alone, no reaction was observed (Fig. S2 and S3†).

Diphenyl-1-pyrenylphosphine (DPPP), as a fluorescent reagent, can be used for hydroperoxide determinations.[12] When we incubated the intermediate with DPPP in the presence of butylated hydroxytoluene (BHT) at 37 °C for 1 h, the fluorescence increased, indicating the formation of a hydroperoxide intermediate (Fig. 2). Further characterization of $oxm^6A$ was achieved using high-resolution mass spectrometry, $^1H$ NMR, $^{13}C$ NMR and TOCSY (ESI, Fig. 3, S4 and S5†), with the corresponding chemical structures shown in Scheme 1. To confirm the chemical shifts of the protons in N–H and OO–H, $^1H$ NMR was performed in DMSO-$d_6$ and in $D_2O$. In the DMSO-$d_6$ solution, the chemical shifts of the protons were 8.61 ppm (–N–H) and 11.71 ppm (–OOH) (Table 1). To confirm our hypothesis, we changed the solution to $D_2O$, where deuterons can be incorporated at the N–H and O–H positions because of hydrogen–deuterium (H/D) exchange behavior. As we expected, these two protons disappeared in the $D_2O$ solution (Fig. 3). We then used total correlation spectroscopy (TOCSY) to show the H–H correlation; the TOCSY spectrum was acquired using a 600 MHz Bruker Avance II spectrometer equipped with a 5 mm triple resonance cryoprobe. The pulse sequence was DIPSI2ETGP. The relaxation delay was 1 s, with 8 acquisitions per increment, and a spectral width of 8 × 8 ppm and time domain of $2k \times 176$ were used. In the spectrum, the NH proton had a cross peak with $CH_2$ at $\delta$ (8.66, 5.26 ppm), further confirming the $oxm^6A$ structure. When we analysed the reaction mixture using LC-MS, we detected a relatively small mass signal of 311.8; this finding may indicate the generation of another intermediate, $N^6$-carboxyladenosine, in a relatively low yield (Fig. S1c†). Meanwhile, our control experiments indicated that adenosine, uridine, cytidine and guanosine were stable in the $H_2O_2$/bicarbonate solution at concentrations of 200 mM $H_2O_2$ and 1 M $NH_4HCO_3$ (Fig. S6†) after one hour.

Because our goal was to fully investigate the mechanism of $m^6A$ demethylation, we extended the reaction time to 24 hours. After 24 hours, we found that only A (primary product) and a small amount of $oxm^6A$ were present (Fig. 1e), whereas $hm^6A$ and $f^6A$ disappeared. This result suggested that $hm^6A$ and $f^6A$ were converted into A (Scheme 1, Routes 9 and 10).

To investigate the behaviour of $oxm^6A$, it was separated from the reaction mixture, incubated in HEPES buffer (50 mM, pH 7.4) at 37 °C and then analysed by HPLC every 2 h. We found that the amount of A increased at the expense of $oxm^6A$ (Fig. S7 in the ESI†), and it had a half-time of approximately 8.5 h (Fig. S8†), which was more stable than $hm^6A$ and $f^6A$ (approximately 3 h).

In the $H_2O_2$/$NH_4HCO_3$ system, the hydroxyl radical was trapped by 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) to give a signal using Electron Paramagnetic Resonance (EPR) (Fig. S9†). In the reaction system, the addition of DMSO, a hydroxyl radical scavenger, dramatically decreased the chemical demethylation level of $m^6A$ (Fig. S10†). We speculate that the reaction underwent a hydroxyl radical mechanism. A hydroxyl radical abstracted a hydrogen atom from a methyl group to yield a carbon radical, which could then bind with $O_2$ to form $oxm^6A$ (Scheme 1, Routes 2 and 3) or bind with ·OH to form $hm^6A$ (Scheme 1, Routes 4 and 8), parallel to the decomposition mechanism for 5′-hydroperoxymethyluracil and 5′-hydroperoxymethylcytosine, as proposed by Richard Wagner's group.[13] To confirm the possibility of the ·OH radical mechanism, we used Fenton-type reagents to react with $m^6A$. The formation of $hm^6A$, $oxm^6A$, $f^6A$ and A was also observed using LC-MS analysis, confirming the reaction mechanism (Fig. S18†). Under identical experimental conditions but with the addition of a small amount of $(NH_4)_2Fe(SO_4)_2$ in the $H_2O_2$/$NH_4HCO_3$ reaction mixture, the reaction rate markedly increased (Fig. S11†). As the reaction is based on the hydroxyl radical mechanism, and $Fe^{2+}$ as well as $Cu^{2+}$ have great influences on the reaction, we therefore used Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) to investigate the presence of iron(II) and copper(II) in the $H_2O_2$/bicarbonate reaction system. No signals were observed, and both the concentration of $Fe^{2+}$ and $Cu^{2+}$ were lower than 10 ng $mL^{-1}$, indicating the reaction could proceed with just a bicarbonate-activated peroxide system (the optimized operating conditions are shown in Table S1†). In the demethylation process, two pathways are shown. A hydroxyl radical attacks the methyl radical to form $hm^6A$ (Scheme 1, Routes 4 and 8) and $O_2$ attacks the methyl radical to form $oxm^6A$ (Scheme 1, Routes 2 and 3). The $oxm^6A$ and its peroxide radical can decompose to $hm^6A$ (Scheme 1, Routes 5, 8 and 6) and $f^6A$ (Scheme 1, Route 7), and

 

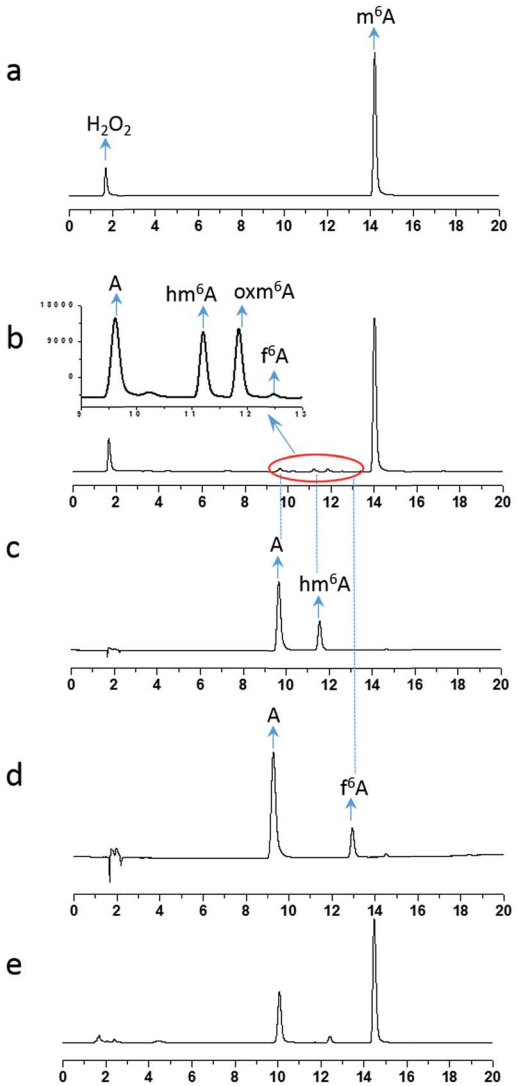


Fig. 1 HPLC chromatograph of 2 mM $m^6A$ incubated with 200 mM $H_2O_2$ and 1 M $NH_4HCO_3$ at 37 °C for 0 h (a), 1 h (b) and 24 h (e). As shown in the HPLC profiles, when the reaction proceeded for 1 h, one major product (A) was produced, accompanied by three intermediates ($hm^6A$, $f^6A$, $oxm^6A$). The synthesized $hm^6A$ (c) and $f^6A$ (d) standards have the same retention time as two of the new peaks in the reaction mixture. Because $hm^6A$ and $f^6A$ are unstable, they can coexist with A during HPLC analysis.

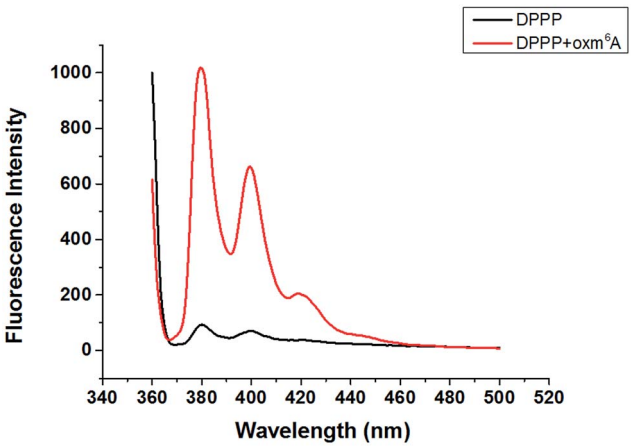


Fig. 2 Fluorescence emission spectra ($\lambda_{ex}$ = 352 nm) of DPPP in the presence of (a) and in the absence of $oxm^6A$ (b) after incubation with BHT at 37 °C for 60 min.

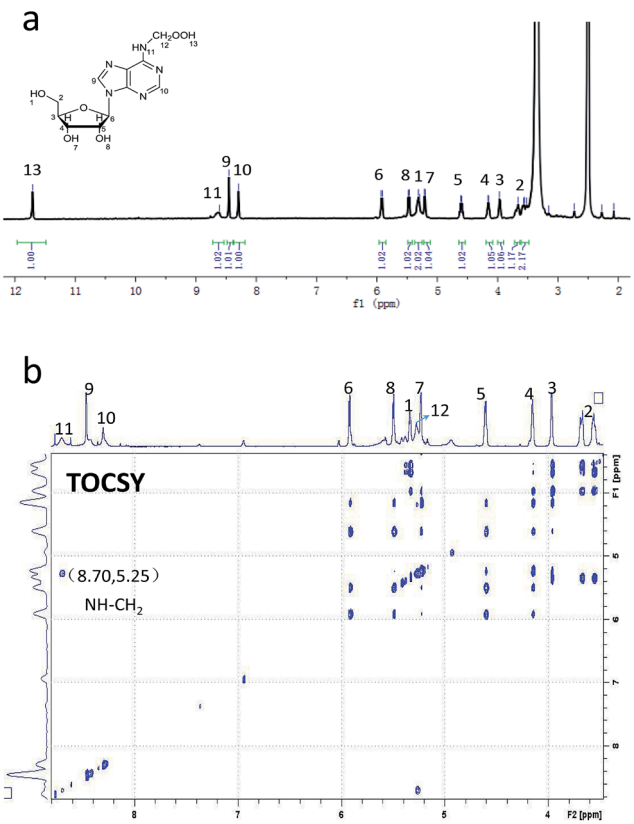


Fig. 3 $^1H$ NMR spectrum (a) and TOCSY spectrum (b) of $oxm^6A$ in DMSO-$d_6$.

we propose that the new route in the demethylation process would improve the efficiency of the demethylation reaction compared to just attacking the methyl radical by a hydroxyl radical.

Next, because $m^6A$ is preferentially present in the consensus sequence RR$m^6$ACH (R is A/G and H is A/C/U),[14] to examine whether the reaction occurs in RNA oligos, we prepared a 9-mer oligoribonucleotide (5′-CUGG$m^6$ACUGG-3′) containing one $m^6A$ site and treated it with 10 mM $H_2O_2$ and 100 mM bicarbonate at 37 °C for 48 h. Because RNA may decompose in a high concentration of $H_2O_2$, we decreased the concentration of $H_2O_2$ and $NH_4HCO_3$. After the reaction, the oligo RNA was analysed using MALDI-TOF mass spectrometry as shown in Fig. S12.† We found a $m^6A$ −14 Da peak, representing a demethylation product, as well as a +14 Da peak and a +17 Da peak, which may correspond to $N^6$-formyladenosine and $N^6$-hydroxymethyladenosine intermediates in the demethylation pathway, respectively. At natural pH levels (pH 7.4), $hm^6A$, $oxm^6A$ and $f^6A$ were relatively stable, but an alkaline phosphate digestion may accelerate their decomposition. Therefore, to verify the

Table 1 $^1$H chemical shifts ($\delta$, ppm) of oxm$^6$A in DMSO-$d_6$ at room temperature

| Number | Chemical shift ($\delta$, ppm) | Number | Chemical shift ($\delta$, ppm) | Number | Chemical shift ($\delta$, ppm) |
|---|---|---|---|---|---|
| 1 | 5.33 | 6 | 5.91 | 11 | 8.66 |
| 2 | 3.68 | 7 | 5.23 | 12 | 5.26 |
| 3 | 3.96 | 8 | 5.50 | 13 | 11.72 |
| 4 | 4.14 | 9 | 8.46 | | |
| 5 | 4.61 | 10 | 8.31 | | |

presence of hm$^6$A, oxm$^6$A and f$^6$A in the oligo RNA after the reaction, we used RNase T1 followed by nuclease P1 to digest the oligo RNA,[7] then analysed the reaction using LC-MS. In this analysis, RNase T1 can selectively digest the phosphodiester bond after G. We successfully detected the formation of A, hm$^6$A, oxm$^6$A, and f$^6$A in the digested nucleoside, similar to our proposed mechanism for a single nucleoside (Fig. S13†).

To explore the reaction kinetics of the oxidation, two micrograms of oligo RNA were incubated with 100 μM $H_2O_2$ and 300 μM $NH_4HCO_3$ at 37 °C for 30 h in six parallel experiments, followed by digestion with nuclease P1 and alkaline phosphate. The amount of A generated from m$^6$A was quantified using LC-MS every 3 hours (the calibration curve is shown in the ESI, Fig. S14†). As depicted in Fig. S15,† the A content exhibited a strong linear relationship with reaction time over a period of 30 hours. After adding $Fe^{2+}$ to the $H_2O_2$/$NH_4HCO_3$ mixture and incubating it with oligo RNA, HPLC analysis of the enzymatically digested nucleosides in RNA showed the presence of demethylated adenosine with a decreased level of m$^6$A after oxidation for 1 h (Fig. S16†).

Although FTO-mediated oxidation of m$^6$A may decrease the level of m$^6$A *in vitro*, no *in vitro* experiments have been reported in which a chemical reagent was used to demethylate m$^6$A. We explored whether m$^6$A in genomic RNA is a substrate of $H_2O_2$/$NH_4HCO_3$ *in vitro*. Total RNA was extracted from Hela cells using the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Four micrograms of genomic RNA was incubated with 100 μM $H_2O_2$ and 1 mM $NH_4HCO_3$ at 37 °C for 12 h. After digestion with nuclease P1 and alkaline phosphatase, the solution was analysed by LC-MS. The results showed a decrease in the m$^6$A/A ratio by 10% in the genomic RNA (Fig. S17†), indicating that the reagents demethylated m$^6$A *in vitro*.

## Conclusions

In conclusion, we reported a new chemical method for the oxidative demethylation of m$^6$A and determined an important intermediate in the reaction system. Three intermediates, $N^6$-hydroxymethyladenosine (hm$^6$A), $N^6$-formyladenosine (f$^6$A), and $N^6$-hydroperoxymethyladenosine (oxm$^6$A), were characterized, and the mechanism underlying the decomposition was illustrated. We also determined that the reaction could occur in oligo RNA and genomic RNA *in vitro*. $H_2O_2$ is a reactive oxygen species that is endogenously produced during normal metabolism[15] and immune responses,[16] and a high concentration of bicarbonate is found in cells and serum. Thus, this route may occur *in vivo* and play a role in cells. ROS have been proven to directly react with genomic DNA in a chemical reaction.[17] Recently, reports have shown that ROS can induce the oxidative conversion of 5mC to 5hmC in a TET dioxygenase-dependent manner,[18] indicating ROS regulate the enzymatic catalytic reaction. We propose that the oxm$^6$A was formed through direct oxidation by ROS *in vivo*, just like the nucleoside analogues formed in RNA induced by Fenton-type reagents.[17b] Further study is in progress to study the presence and biological function of oxm$^6$A *in vivo*. The discovery of the new intermediate oxm$^6$A and the chemical route for the demethylation of m$^6$A to A may offer new insight into the study of m$^6$A.

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