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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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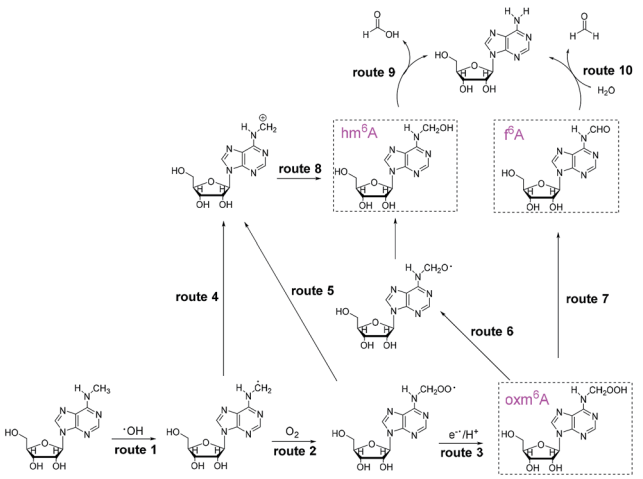

Scheme 1 Proposed mechanism of the demethylation process, and structures of oxidation products.

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 δ (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

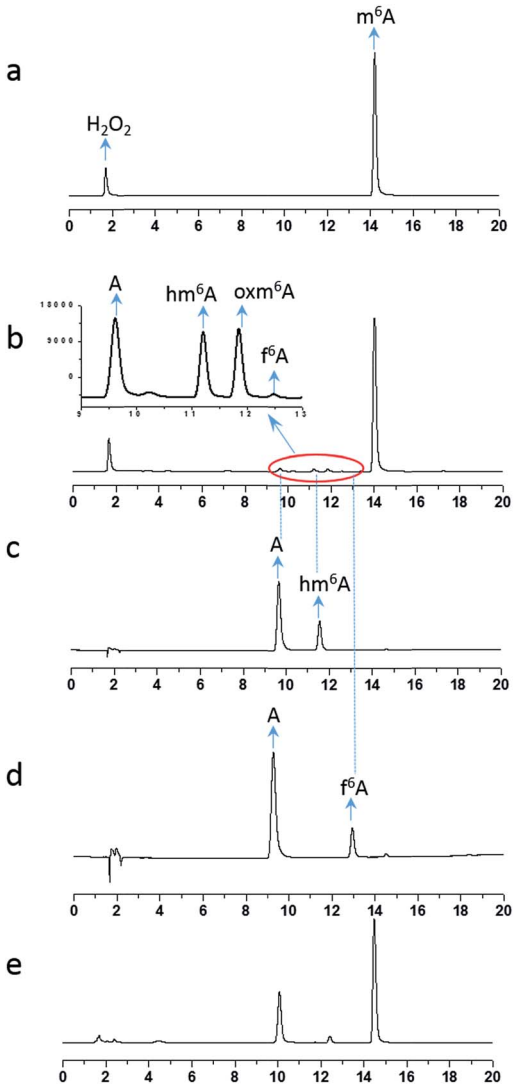

Fig. 1 HPLC chromatograph of 2 mM m^6A incubated with 200 mM H_2O_2 and 1 M NH_4HCO_3 at 37 °C for 0 h (a), 1 h (b) and 24 h (e). As shown in the HPLC profiles, when the reaction proceeded for 1 h, one major product (A) was produced, accompanied by three intermediates (hm^6A, f^6A, oxm^6A). The synthesized 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.

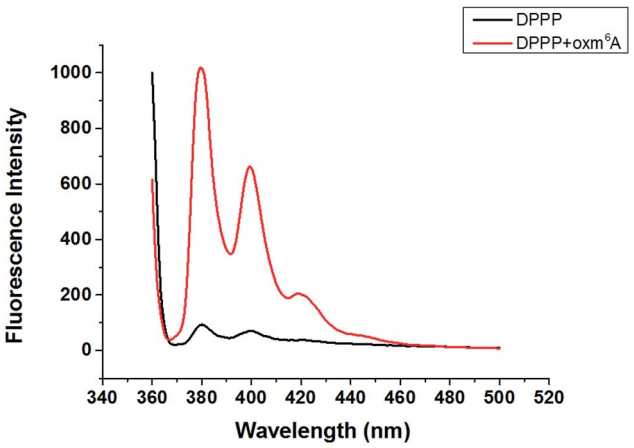

Fig. 2 Fluorescence emission spectra (λ_{ex} = 352 nm) of DPPP in the presence of (a) and in the absence of oxm^6A (b) after incubation with BHT at 37 °C for 60 min.

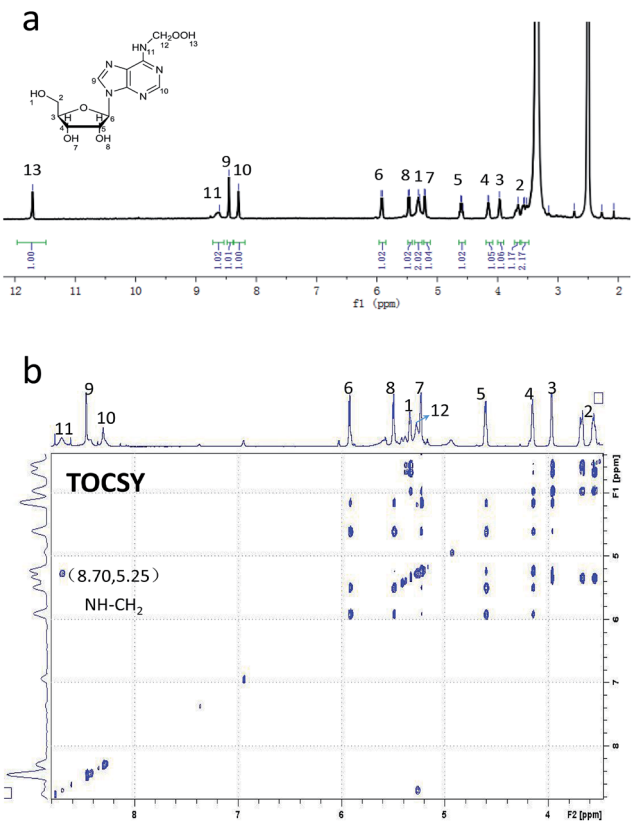

Fig. 3 ^1H NMR spectrum (a) and TOCSY spectrum (b) of oxm^6A in DMSO-d_6.

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 RRm^6ACH (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′-CUGGm^6ACUGG-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 (δ, ppm) of oxm^{6}A in DMSO-d_6 at room temperature

Number	Chemical shift (δ, ppm)	Number	Chemical shift (δ, ppm)	Number	Chemical shift (δ, 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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