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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.

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$N^6$ -Methyladenosine represents the most abundant modification in the mRNA of higher eukaryotes, present at a frequency of approximately three sites on each mRNA.<sup>1</sup>  $m^6A$  is also present on tRNA, rRNA and lncRNA.<sup>2</sup> This modification plays an important role in the regulation of gene expression.<sup>3</sup> Since its discovery last century,<sup>4</sup>  $m^6A$  has been the object of relatively few studies. Recently, fat mass- and obesity-associated proteins (FTO)<sup>5</sup> and AlkBH5<sup>6</sup> 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.<sup>7</sup> In addition, transcriptome-wide profiling of  $m^6A$  in mRNA and lncRNA has revealed new insights into the role of RNA modification.<sup>8</sup> 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,<sup>9</sup> 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,<sup>10</sup> and plays an important role in biological oxidation.<sup>11</sup>  $H_2O_2$  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.

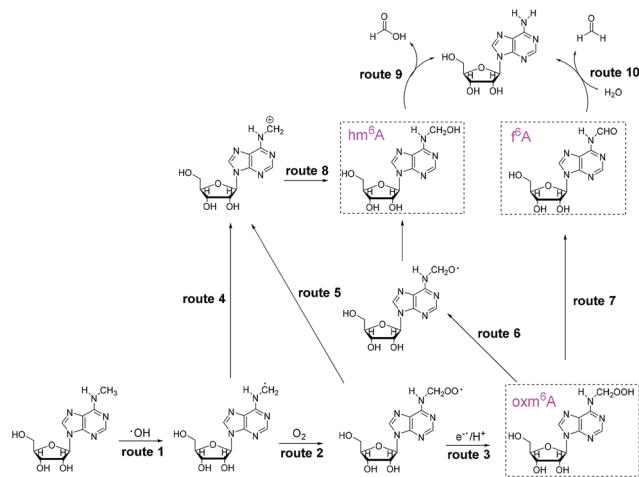
To investigate the demethylation process, we used high-performance liquid chromatography (HPLC) to monitor the reaction (UV detector at 260 nm). When a 2 mM aliquot of  $m^6A$  was treated with 200 mM  $H_2O_2$  and 1 M  $NH_4HCO_3$  at 37 °C for one hour, four products were formed: A,  $hm^6A$ ,  $oxm^6A$  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  $\text{hm}^6\text{A}$  and  $\text{f}^6\text{A}$ , these compounds were synthesized according to reported procedures.<sup>7</sup> An equilibrium reaction between adenosine and formaldehyde produced  $\text{hm}^6\text{A}$  (Scheme 1, Route 9). Further HPLC analysis indicated that the synthesized  $\text{hm}^6\text{A}$  and  $\text{f}^6\text{A}$  have the same retention times as the reported  $\text{hm}^6\text{A}$  and  $\text{f}^6\text{A}$ , respectively (Fig. 1b–d). We found that  $\text{hm}^6\text{A}$  and  $\text{f}^6\text{A}$  were unstable and could decompose to A (adenosine) during HPLC analysis (Fig. 1c and d).  $\text{N}^6$ -Hydroperoxymethyladenosine ( $\text{oxm}^6\text{A}$ ) was found to be a new intermediate, in addition to  $\text{hm}^6\text{A}$  and  $\text{f}^6\text{A}$ , during the demethylation of  $\text{m}^6\text{A}$  (Fig. 1b). When we incubated the  $\text{m}^6\text{A}$  with bicarbonate or  $\text{H}_2\text{O}_2$  alone, no reaction was observed (Fig. S2 and S3†).

Diphenyl-1-pyrenylphosphine (DPPP), as a fluorescent reagent, can be used for hydroperoxide determinations.<sup>12</sup> 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  $\text{oxm}^6\text{A}$  was achieved using high-resolution mass spectrometry,  $^1\text{H}$  NMR,  $^{13}\text{C}$  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 O–H,  $^1\text{H}$  NMR was performed in  $\text{DMSO}-d_6$  and in  $\text{D}_2\text{O}$ . In the  $\text{DMSO}-d_6$  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  $\text{D}_2\text{O}$ , 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  $\text{D}_2\text{O}$  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  $8 \times 8$  ppm and time domain of  $2k \times 176$  were used. In the spectrum, the NH proton had a cross peak with  $\text{CH}_2$

at  $\delta$  (8.66, 5.26 ppm), further confirming the  $\text{oxm}^6\text{A}$  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,  $\text{N}^6$ -carboxyladenosine, in a relatively low yield (Fig. S1c†). Meanwhile, our control experiments indicated that adenosine, uridine, cytidine and guanosine were stable in the  $\text{H}_2\text{O}_2$ /bicarbonate solution at concentrations of 200 mM  $\text{H}_2\text{O}_2$  and 1 M  $\text{NH}_4\text{HCO}_3$  (Fig. S6†) after one hour.

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

To investigate the behaviour of  $\text{oxm}^6\text{A}$ , 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  $\text{oxm}^6\text{A}$  (Fig. S7 in the ESI†), and it had a half-time of approximately 8.5 h (Fig. S8†), which was more stable than  $\text{hm}^6\text{A}$  and  $\text{f}^6\text{A}$  (approximately 3 h).

In the  $\text{H}_2\text{O}_2/\text{NH}_4\text{HCO}_3$  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  $\text{m}^6\text{A}$  (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  $\text{O}_2$  to form  $\text{oxm}^6\text{A}$  (Scheme 1, Routes 2 and 3) or bind with  $\cdot\text{OH}$  to form  $\text{hm}^6\text{A}$  (Scheme 1, Routes 4 and 8), parallel to the decomposition mechanism for 5'-hydroperoxymethyluracil and 5'-hydroperoxymethylcytosine, as proposed by Richard Wagner's group.<sup>13</sup> To confirm the possibility of the  $\cdot\text{OH}$  radical mechanism, we used Fenton-type reagents to react with  $\text{m}^6\text{A}$ . The formation of  $\text{hm}^6\text{A}$ ,  $\text{oxm}^6\text{A}$ ,  $\text{f}^6\text{A}$  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  $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$  in the  $\text{H}_2\text{O}_2/\text{NH}_4\text{HCO}_3$  reaction mixture, the reaction rate markedly increased (Fig. S11†). As the reaction is based on the hydroxyl radical mechanism, and  $\text{Fe}^{2+}$  as well as  $\text{Cu}^{2+}$  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  $\text{H}_2\text{O}_2$ /bicarbonate reaction system. No signals were observed, and both the concentration of  $\text{Fe}^{2+}$  and  $\text{Cu}^{2+}$  were lower than  $10 \text{ ng mL}^{-1}$ , 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  $\text{hm}^6\text{A}$  (Scheme 1, Routes 4 and 8) and  $\text{O}_2$  attacks the methyl radical to form  $\text{oxm}^6\text{A}$  (Scheme 1, Routes 2 and 3). The  $\text{oxm}^6\text{A}$  and its peroxide radical can decompose to  $\text{hm}^6\text{A}$  (Scheme 1, Routes 5, 8 and 6) and  $\text{f}^6\text{A}$  (Scheme 1, Route 7), and



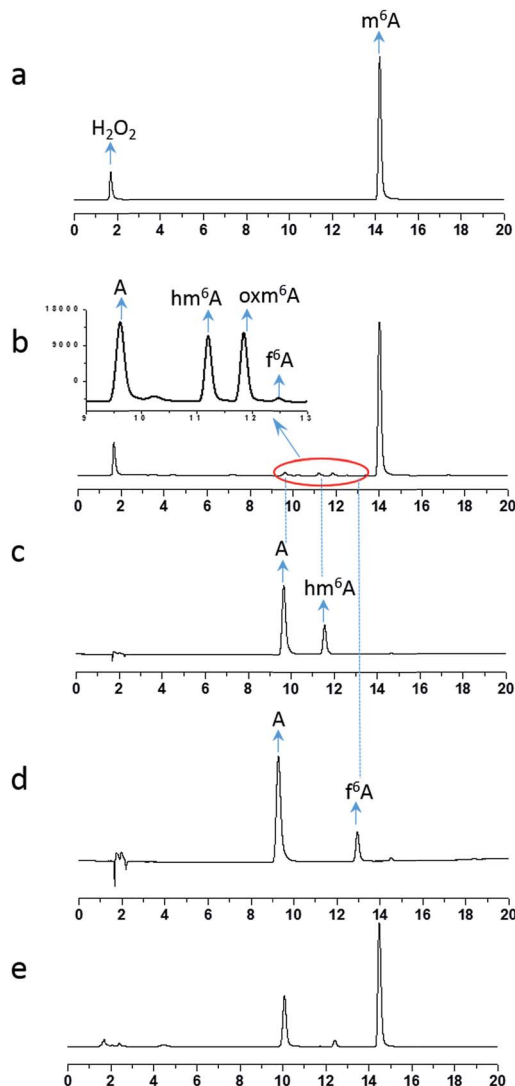


Fig. 1 HPLC chromatograph of 2 mM  $m^6A$  incubated with 200 mM  $H_2O_2$  and 1 M  $NH_4HCO_3$  at 37 °C for 0 h (a), 1 h (b) and 24 h (e). As shown in the HPLC profiles, when the reaction proceeded for 1 h, one major product (A) was produced, accompanied by three intermediates ( $hm^6A$ ,  $f^6A$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

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),<sup>14</sup> to examine whether the reaction occurs in RNA oligos, we prepared a 9-mer oligoribonucleotide (5'-CUGGm<sup>6</sup>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

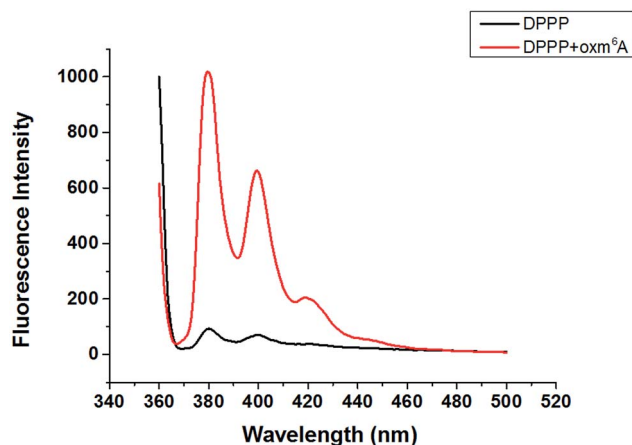


Fig. 2 Fluorescence emission spectra ( $\lambda_{ex}$  = 352 nm) of DPPP in the presence of (a) and in the absence of oxm<sup>6</sup>A (b) after incubation with BHT at 37 °C for 60 min.

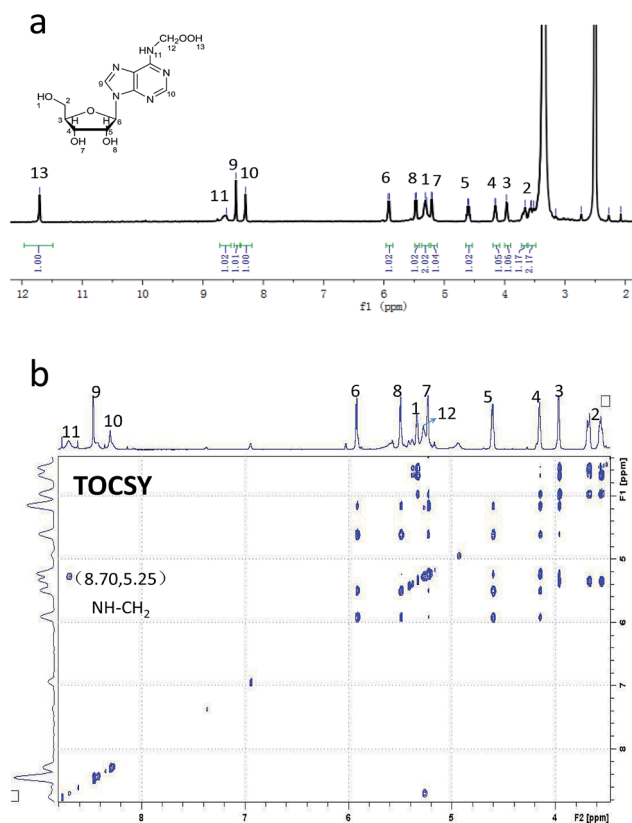


Fig. 3 <sup>1</sup>H NMR spectrum (a) and TOCSY spectrum (b) of oxm<sup>6</sup>A in DMSO-d<sub>6</sub>.

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  $^1\text{H}$  chemical shifts ( $\delta$ , ppm) of  $\text{oxm}^6\text{A}$  in  $\text{DMSO}-d_6$  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  $\text{hm}^6\text{A}$ ,  $\text{oxm}^6\text{A}$  and  $\text{f}^6\text{A}$  in the oligo RNA after the reaction, we used RNase T1 followed by nuclease P1 to digest the oligo RNA,<sup>7</sup> 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,  $\text{hm}^6\text{A}$ ,  $\text{oxm}^6\text{A}$ , and  $\text{f}^6\text{A}$  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  $100\ \mu\text{M}$   $\text{H}_2\text{O}_2$  and  $300\ \mu\text{M}$   $\text{NH}_4\text{HCO}_3$  at  $37\ ^\circ\text{C}$  for 30 h in six parallel experiments, followed by digestion with nuclease P1 and alkaline phosphate. The amount of A generated from  $\text{m}^6\text{A}$  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  $\text{Fe}^{2+}$  to the  $\text{H}_2\text{O}_2/\text{NH}_4\text{HCO}_3$  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  $\text{m}^6\text{A}$  after oxidation for 1 h (Fig. S16†).

Although FTO-mediated oxidation of  $\text{m}^6\text{A}$  may decrease the level of  $\text{m}^6\text{A}$  *in vitro*, no *in vitro* experiments have been reported in which a chemical reagent was used to demethylate  $\text{m}^6\text{A}$ . We explored whether  $\text{m}^6\text{A}$  in genomic RNA is a substrate of  $\text{H}_2\text{O}_2/\text{NH}_4\text{HCO}_3$  *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  $100\ \mu\text{M}$   $\text{H}_2\text{O}_2$  and  $1\ \text{mM}$   $\text{NH}_4\text{HCO}_3$  at  $37\ ^\circ\text{C}$  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  $\text{m}^6\text{A}/\text{A}$  ratio by 10% in the genomic RNA (Fig. S17†), indicating that the reagents demethylated  $\text{m}^6\text{A}$  *in vitro*.

## Conclusions

In conclusion, we reported a new chemical method for the oxidative demethylation of  $\text{m}^6\text{A}$  and determined an important intermediate in the reaction system. Three intermediates,  $N^6$ -hydroxymethyladenosine ( $\text{hm}^6\text{A}$ ),  $N^6$ -formyladenosine ( $\text{f}^6\text{A}$ ), and  $N^6$ -hydroperoxymethyladenosine ( $\text{oxm}^6\text{A}$ ), 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*.  $\text{H}_2\text{O}_2$  is a reactive oxygen species that is endogenously produced during normal metabolism<sup>15</sup> and immune responses,<sup>16</sup> 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.<sup>17</sup> Recently, reports have shown that ROS can induce the oxidative conversion of  $5\text{mC}$  to  $5\text{hmC}$  in a TET dioxygenase-dependent manner,<sup>18</sup> indicating ROS regulate the enzymatic catalytic reaction. We propose that the  $\text{oxm}^6\text{A}$  was formed through direct oxidation by ROS *in vivo*, just like the nucleoside analogues formed in RNA induced by Fenton-type reagents.<sup>17b</sup> Further study is in progress to study the presence and biological function of  $\text{oxm}^6\text{A}$  *in vivo*. The discovery of the new intermediate  $\text{oxm}^6\text{A}$  and the chemical route for the demethylation of  $\text{m}^6\text{A}$  to A may offer new insight into the study of  $\text{m}^6\text{A}$ .

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