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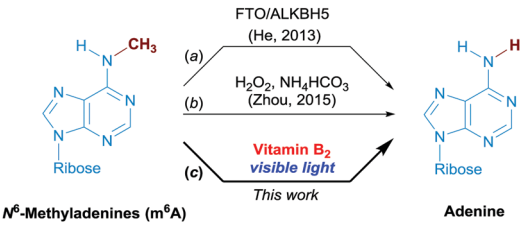
Visible-light-mediated oxidative demethylation of N^6-methyl adenines†

Li-Jun Xie,[ac] Rui-Li Wang,[ac] Dong Wang,[a] Li Liu[ac] and Liang Cheng *[abc]

We report a simple protocol that affords oxidative demethylation of N^6-methyl groups in N^6-methyl adenines (m^6A). The biologically compatible photocatalyst riboflavin prompts a highly selective C–H abstraction from N^6-methyl in adenines under the irradiation of a visible blue LED light, affording a novel and highly selective biomimetic demethylation of m^6A and related *N*-methyl adenine analogues.

N^6-Methyladenosine (m^6A) is the most abundant internal modification in eukaryotic mRNA, although the significant biological roles of m^6A methylation have remained largely unclear.[1] Recent studies on the distributions and mechanisms of m^6A modification have suggested that this methylation functionally modulates the eukaryotic transcriptome to influence mRNA transcription, splicing, nuclear export, localization, translation, stability, and other physiological processes unknown to us.[2] Fat mass-/obesity-associated proteins (FTO) and AlkB homologue 5 (AlkBH5) are two recently discovered RNA demethylases ("eraser") that remove the m^6A modification in mRNA in the presence of $Fe^{(II)}$, α-KG, and dioxygen[3] (Scheme 1, path (a)). In this oxidative demethylation mechanism, a non-heme iron$^{(II)}$ and α-KG activate oxygen to form a highly reactive $Fe^{(IV)}$-oxo species, which then inserts the oxygen into the C–H bond of m^6A *via* a radical process, followed by tandem oxidation of the intermediates hm^6A (N^6-hydroxymethyl adenosine) and/or f^6A (N^6-formyl adenosine) to furnish the demethylation.[4] However, owing to the necessity of formation of unique structures that are responsible for m^6A recognition for these proteins, the oxidative demethylation of the C–H bond in m^6A is difficult to achieve with external chemical reagents and is always lack of generality.[5] Recently, Zhou *et al.* reported an oxidation of m^6A through the bicarbonate activated peroxide system (Scheme 1, path (b)).[5a] 39% of the demethylated product – adenosine (A) – was isolated after incubating m^6A with excess H_2O_2 for 24 hours. This finding opens up a fascinating new way for the direct removal of methyl groups from m^6A; however, the comparably inferior conversion retards it further application in biological processes. Thus the quest for new approaches to a selective biocatalytic oxidation continues.



Scheme 1 Comparison of oxidative demethylation of N^6-methyladenines through biological demethylases (a), the hydroperoxide system (b) and the novel simplified light-driven pathway (c).

While it is well known that photocatalysis is a very common principle in nature that all plants and animals depend on sunlight and use it by means of photoreceptors,[6] we intentionally incorporate such photomodulation into the chemical demethylation. In contrast to the traditional photocontrol by ultraviolet light as the trigger, which is not well compatible with live cells due to its cytotoxicity and by causing irreversible nucleic acid mutations,[7] we strongly expect that, by carefully choosing diverse biologically photoreceptors, the target nucleoside will be specifically activated by longer wavelength lights, in which case the reaction would be more selective, predicable and easier to control.[8] Contrary to the relatively unspecific functionalization of nucleosides by C–H activation,[9] this kind of activation would allow a direct C–H abstraction from the inert N^6-methyl groups of m^6A to form a nucleoside radical.[10] Indeed, we have recently demonstrated that by cautiously selecting the radical initiator, the unreactive C–H bonds in

[a] *Beijing National Laboratory for Molecular Sciences (BNLMS), CAS Key Laboratory of Molecular Recognition and Function, CAS Research/Education Center for Excellence in Molecular Sciences, Institute of Chemistry, Chinese Academy of Sciences, Beijing 100190, China. E-mail: chengl@iccas.ac.cn*
[b] *Key Lab of Functional Molecular Engineering of Guangdong Province (South China University of Technology), Guangzhou 510640, China*
[c] *University of Chinese Academy of Sciences, Beijing 100049, China*

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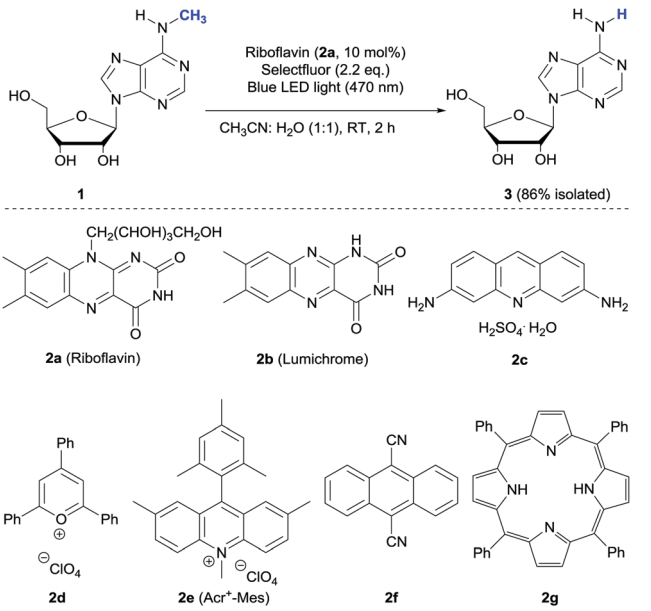
heterocycles can be selectively activated.[11] Herein, we focus on the development of a visible light-driven oxidative demethylation of m^6A with riboflavin (vitamin B_2) (Scheme 1, path (c)), which is a biological redox cofactor[12] that is essential for human and animal health because of its crucial role in many natural processes, such as DNA repair by photolyase.[13] To the best of our knowledge, this is the first example of chemical modulation of RNA epigenetics by implementing an energy-efficient and green process through a photo-induced oxidative demethylation.

As a starting point, the demethylation of m^6A N^6-methyl adenosine **1** was investigated (Table 1). Before we initiated this research, this transformation was only achieved with modest conversions using stoichiometric loadings of metal or inorganic oxidants by using protected nucleosides.[5b–d] A screening of photocatalysts (**2a–2g**), oxidants, solvents and light wavelengths quickly identified 10 mol% of riboflavin with selectfluor in aqueous acetonitrile to be the optimal conditions (entries 1–8; see the ESI† for details). The oxidative demethylation of N^6-methyl adenosine **1** proceeded quickly within 2 hours under blue LED light irradiation. A high yield (86%) of the demethylated product was achieved by simple chromatography, and upon analysis the product was revealed to be identical with authentic adenine. It should be noted that traditional photo-oxidative *N*-demethylation methods that were applicable for heterocycles, such as alkaloids,[14] are not applicable in this situation and no demethylated product was observed in all cases (entries 7 and 8). Selectfluor was chosen owing to its superior safety profile and lower cost, while other oxidants such as TBHP (entry 10) or H_2O_2 (entry 11) were proven to be ineffective for this transformation.[15] Water and light were identified as having a crucial impact on the demethylation as no product was observed when the photoreaction was conducted in dry acetonitrile or under darkness (entries 12 and 13).

By extension, the chemical demethylation of other methylated nucleosides/bases was also explored under the optimized reaction conditions (Table 2). Again, $N^{6,6}$-dimethyl adenosine ($m^{6,6}A$, **4**), deoxy-N^6-methyl adenosine (dm^6A, **5**) and N^6-methyl adenine (**6**) furnished the excepted demethylated nucleosides/bases with high yields. In contrast, other RNA epigenetic patterns, such as the methyl group at the 1-nitrogen of the adenine ring (m^1A, **7**), 5-carbon of the cytosine ring (m^5C, **8**) or at the 2′-OH of the ribose (A_m, **9**) remained inert or partially decomposed, suggesting a particularly selective recognition and activation of the N^6-methyl C–H bond with this riboflavin-based photocatalysis. To explore the potential of this demethylation with other biological substrates, the standard procedure was applied to various endogenous and exogenous biomolecules, such as alkaloid (sparteine), natural and unnatural amino acids (L-histidine, L-tryptophan, L-phenylalanine and L-phenylglycine), nucleosides (guanosine, inosine and deoxy-thymidine) and oligosaccharides (ribose and xylose). All of these substrates remained intact under the same

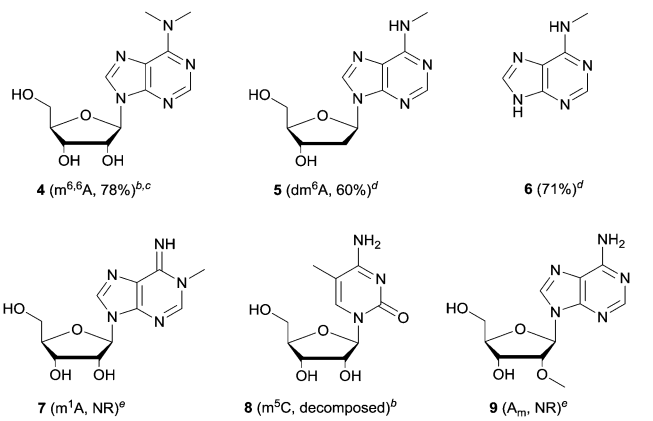
Table 1 Riboflavin-catalyzed demethylation of N^6-methyl adenosine **1**[a]



Entry	Deviation from above	Yield (%)
1	None	86
2	No photoreceptor	NR[b]
3	**2b**	79
4	**2c**	45
5	**2d**	62
6	**2e**	52
7	**2f**	NR[b]
8	**2g**	NR[b]
9	No selectfluor	NR[b]
10	TBHP	NR[b]
11	H_2O_2	NR[b]
12	CH_3CN (anhydrous)	NR[b]
13	No light	NR[b]

[a] Unless otherwise specified, all reactions were carried out using **1** (0.1 mmol, 28 mg), **2a** (0.01 mmol, 3.8 mg) and selectfluor (0.22 mmol, 78 mg) in the solvent of CH_3CN and H_2O (1 : 1, 2 mL total) at RT (37 °C) for 2 hours under 1 atm of argon (balloon). Isolated yield is given. [b] NR: no reaction.

Table 2 Riboflavin-catalyzed demethylation of other epigenetic nucleosides[a]



[a] Unless otherwise specified, all reactions were carried out using nucleoside/base (0.1 mmol), riboflavin **2a** (0.01 mmol, 3.8 mg) and selectfluor (0.22 mmol, 78 mg) in the solvent of acetonitrile and water (1 : 1, 2 mL total) at RT (37 °C) under 1 atm of argon (balloon). Isolated yield is given. [b] Reaction time: 7 hours. [c] 17% of mono-demethylated product m^6A **1** was isolated. [d] Reaction time: 3 hours. [e] Reaction time: 24 hours. NR: no reaction.

conditions, again demonstrating the bimolecular compatibility of this selective demethylation process.

To investigate the oxidative process and the demethylation mechanism, we used high-performance liquid chromatography (HPLC) to monitor the demethylation reaction (Fig. 1). When a 50 mM aliquot of m^6A (Fig. 2a) in a mixture of acetonitrile and water (1 : 1, 2 mL total) was treated with riboflavin (10 mol%) and selectfluor (2.2 equiv.) at RT for 30 minutes, three new peak were observed, one of which was identical with that of authentic adenosine (Fig. 1(a)). Liquid chromatography–mass spectrometry (LC-MS) was used to characterize the other two new peaks, and it turned out that these two initially generated products were hm^6A and f^6A, respectively (Fig. 1(b)). Substantial conversion of hm^6A was observed in 30 minutes (Fig. 2e), and f^6A to A in 60 minutes (Fig. 1(a)). To our surprise, another intermediate

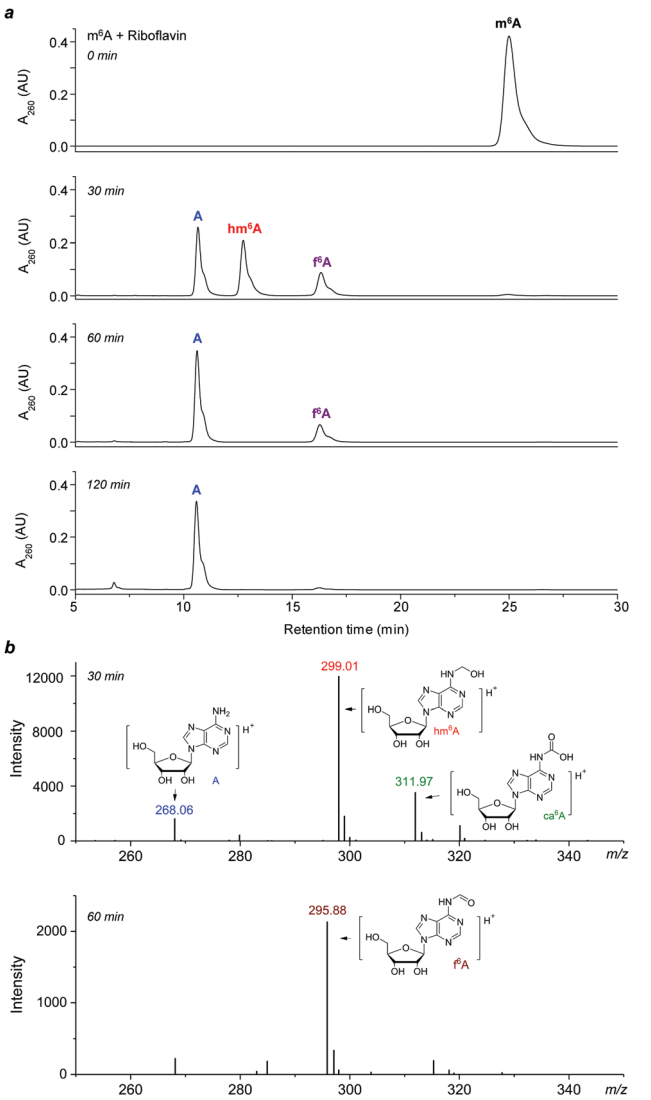


Fig. 1 Analysis of riboflavin catalysed photochemical demethylation of m^6A. (a) HPLC analysis of the demethylation process at 0 min, 30 min, 60 min and 120 min, respectively. The new peaks were assigned as A, hm^6A and f^6A, respectively. (b) Mass profile of the reaction mixtures at 30 min and 60 min, respectively. ca^6A was observed as a potential intermediate.

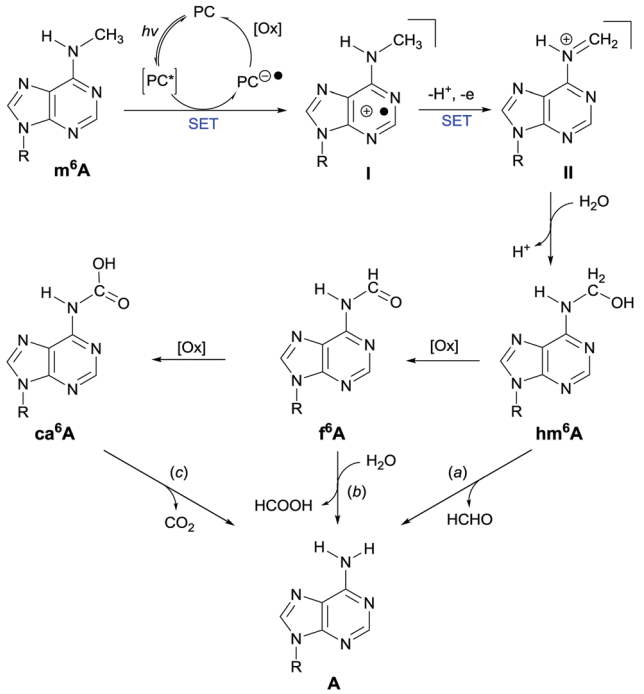


Fig. 2 Proposed mechanism for the riboflavin catalysed oxidative demethylation of N^6-methyl adenines.

ca^6A (N^6-carboxyl adenosine)[16] was also observed from LC-MS (Fig. 1(b)), which was suspected to be originated from further oxidation of f^6A. However, carbamic acid was chemically unstable under the oxidative conditions and was believed to undergo a rapid decarboxylation to afford the demethylated product A.

Based on previous reports and these experimental results, a putative mechanism (Fig. 2) is outlined to illustrate the reaction route. Initially, the irradiation of riboflavin (PC = photocatalyst) with 470 nm visible light leads to the formation of the electronically excited photocatalyst (*PC), which then activated m^6A **1** by selectively abstracting the C–H bond from the N^6-methyl group in m^6A **1** *via* an initial single electron transfer (SET) process to form a radical cationic nucleoside **I**[10] in a similar manner as enzymes FTO or AlkBH5. Deprotonation followed by further SET oxidation affords an iminium salt **II**, primed for nucleophilic attack by water to generate the intermediate hm^6A. The unstable hemiaminal would undergo decomposition in an aqueous solution to yield adenosine A and formaldehyde (Fig. 2, path (a)), which was proven by an HPLC assay for the determination of formaldehyde in the oxidation mixture (see the ESI† for details). Alternatively, a subsequent second-round oxidation of the hemiaminal hm^6A to a relatively stable formamide f^6A followed by water-assisted decomposition afforded formic acid and the demethylated nucleoside A (path (b)). A third-round oxidation product ca^6A, which seems unstable under these physiological conditions, might also be involved in this process. The major demethylation pathway of m^6A by the ''eraser'' FTO was believed to proceed *via* the decomposition of hm^6A, while the subsequent hydrolysis of f^6A was a minor and

inefficient demethylation pathway due to the slow reorganization step of the enzyme with the nucleic acid strand.[17] However, the fact that the substantial generation and hydrolysis of f^6A was observable from HPLC clearly proved the second mechanism proceeding by two rounds of oxidation followed by amide hydrolysis (path (c)).

In conclusion, we have developed a novel light-driven system for the effective oxidative demethylation of m^6A and its N^6-methyl analogues. Riboflavin was selected as a biologically compatible photoreceptor to selectively abstract hydrogen from the N^6-methyl group in adenine. Photo-induced SET and hydrogen abstraction between the m^6A and excited riboflavin afforded an iminium intermediate, which later underwent hydration and oxidation to afford the intermediates hm^6A, f^6A and ca^6A, followed by decomposition to give the demethylated product. This new platform replying on riboflavin achieves an inert C–H bond functionalization in epigenetic nucleosides, which is not amenable to direct derivation. Further extension of this biorthogonal chemistry for diverse functionalization of nucleosides and oligonucleic acids is currently investigated in our lab and will be reported in due course.

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Conflicts of interest

There are no conflicts to declare.

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