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Identification of 2-Methylthio-methylenethio- N^6 -(*cis*-4-hydroxyisopentenyl)adenosine ($msms^2i^6A_{37}$) as a Novel Modification at Adenosine 37 of tRNAs from *Salmonella typhimurium*

Marouane Libiad,^[b] Thierry Douki,^[c] Marc Fontecave,^[b] and Mohamed Atta*^[a, b]

Post-transcriptional modifications of tRNA nucleotide are important determinants in folding, structure and function. We have successfully identified and characterized a new modified base named 2-methylthio-methylenethio- N^6 -(*cis*-4-hydroxyisopentenyl)adenosine, which is present at position 37

in some tRNAs. We also showed that this new modified adenosine is derived from the known 2-methylthio-methylenethio- N^6 -(isopentenyl)adenosine nucleoside by a catalytic cycle of the tRNA-diiron monooxygenase, MiaE, present in *Salmonella typhimurium*.

Ribonucleic acids play critical roles in many biological processes, among them protein synthesis. RNAs are decorated with a variety of functional groups derived from post-transcriptional chemical modifications conserved through evolution.^[1] Incontestably, transfer RNA (tRNAs) are the most heavily modified RNA species, with the presence of specific ribonucleoside structures affecting the rate and fidelity of translation, cellular stress responses and cell growth.^[2] In addition, recent studies suggest that tRNA modification profiles (the complete pattern of modified nucleosides in a total tRNA sample) could be associated with human diseases.^[3]

To date, more than 100 different chemical modifications have been characterized on tRNAs from all three domains of life, and their number is still progressively increasing (<https://iimcb.genesilico.pl/modomics/>).^[1a] Nevertheless, approximately two-thirds of all known tRNA modifications were reported before 1980 and, quite often, modified ribonucleosides were discovered by unexpected observations of chromatographic and/or mass spectrometric features. Recent, technological progress in liquid chromatography-mass spectrometry (LC-MS) methods allowed the threshold limits in RNAs analysis to be lowered and new ways of screening for the presence of

unknown modified nucleosides to be developed.^[4] Helm and co-workers developed an untargeted LC-MS/MS method specifically designed for the detection of new modified nucleosides.^[5] In this technique, stable isotope labelling of RNA is integrated into an LC-MS/MS workflow showing that even *Escherichia coli* (with >40 known tRNA modifications) may hold a large number of still unidentified modified nucleosides. Among the new structures that have been detected, one was fully characterized to be the 2-methylthio-methylenethio- N^6 -isopentenyladenosine ($msms^2i^6A_{37}$).^[6] This thioacetal-containing adenosine is an extremely rare structure in biological compounds. Its biosynthesis was shown to derive from the known, hypermodified nucleoside, 2-methylthio- N^6 -isopentenyladenosine ($ms^2i^6A_{37}$) by a second catalytic cycle of the MiaB protein, an iron-sulfur enzyme that belongs to the Radical-SAM superfamily.^[7] Indeed, in *E. coli*, MiaB catalyzes the final step in the biosynthesis of $ms^2i^6A_{37}$ found at adenosine base at position 37, adjacent to the anticodon of specific subsets of tRNAs that decode codons that begin with uridine, except for tRNA_{Ser(GGA)}.^[8] MiaB enzymatic reaction consists in the insertion of a methylthio (-SCH₃) group at carbon C2 of i^6A_{37} -tRNAs (Scheme 1).^[9] In *Salmonella typhimurium*, $ms^2i^6A_{37}$ is further modified by hydroxylation of one methyl group of the dimethylallyl group at the position N^6 , during an oxygen-dependent reaction catalyzed by MiaE, a metalloenzyme that belongs to the superfamily of non-heme di-iron monooxygenases (Scheme 1).^[10]

The presence of both MiaB and MiaE enzymes in *S. typhimurium* raised the question of whether the $msms^2i^6A_{37}$, originally found in *E. coli*, would also be formed in this organism as well as the product of its hydroxylation, namely 2-methylthio-methylenethio- N^6 -(*cis*-4-hydroxyisopentenyl)adenosine ($msms^2i^6A_{37}$). Here, we establish that *S. typhimurium*, indeed, contains not only $msms^2i^6A_{37}$ but also $msms^2i^6A_{37}$ as a novel derivative. We also demonstrate that the *S. typhimurium* MiaE protein is responsible for the hydroxylation of $msms^2i^6A_{37}$ into $msms^2i^6A_{37}$ by complementation of an *E. coli* strain naturally lacking MiaE and thus deficient in $ms^2i^6A_{37}$ hydroxylation. Both modifications were extensively characterized by a series of

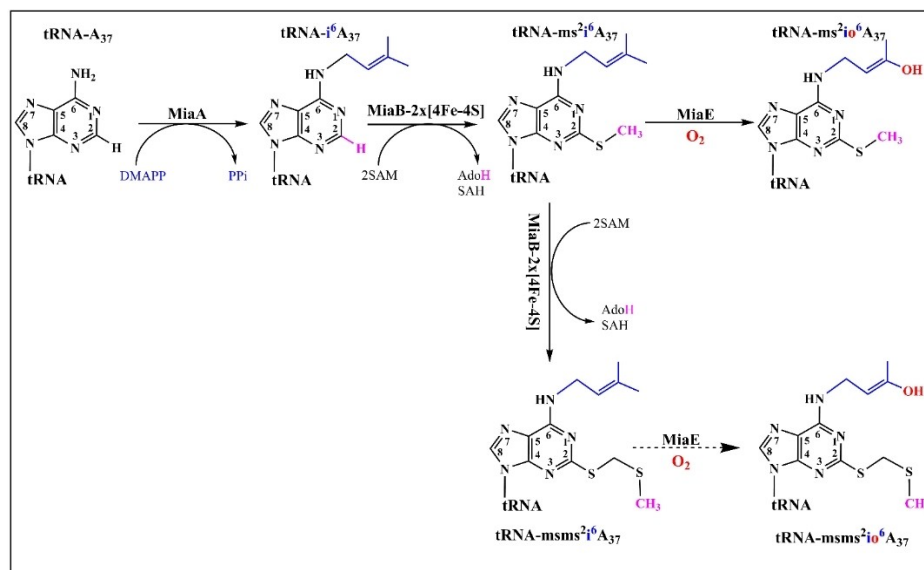
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Scheme 1. Biosynthetic pathway of $ms^2i^6A_{37}$ and its derivatives. The enzymes involved in this pathway are MiaA, MiaB, and MiaE. DMAPP: dimethylallyl diphosphate, PPI: pyrophosphate, SAM: S-adenosylmethionine, SAH: S-adenosylhomocysteine, AdoH: 5'-deoxyadenosyl.

analyses using various liquid chromatography-tandem mass spectrometry methods.

Identification of $msms^2i^6A_{37}$ and $msms^2i^6A_{37}$ in *S. typhimurium* tRNAs

To confirm the presence of both $msms^2i^6A_{37}$ and $msms^2i^6A_{37}$ modified nucleosides in tRNAs, *S. typhimurium* cells were grown at 37 °C and total tRNAs were extracted, purified and digested with nuclease P1 and phosphatase, and the released nucleosides were first analyzed by HPLC as described previously.^[11] Within the chromatogram, as expected, the peaks corresponding to $ms^2i^6A_{37}$ and $ms^2i^6A_{37}$ were observed at ~84 and ~69 min, respectively (Figure S1 in the Supporting Information).

No additional compounds were observed in amounts above the limit of detection. We then applied the ultra-high-performance liquid chromatography (UHPLC) method coupled with mass spectrometry detection in the product ion scan mode. This technique combines the detection of products with expected molecular weight and simultaneously provides structural information allowing the analytes to be identified. With the elution gradient used, the peak corresponding to $ms^2i^6A_{37}$ was detected at 10.8 min with the expected mass spectrometry features such as the loss of a ribose fragment (Figure 1a). In agreement with the HPLC-UV results, the peak at 15.7 min on the MS/MS chromatogram corresponding to $ms^2i^6A_{37}$ exhibited an intensity similar to that of $ms^2i^6A_{37}$. The product ion scan mode is more sensitive than the UV detection and allowed us

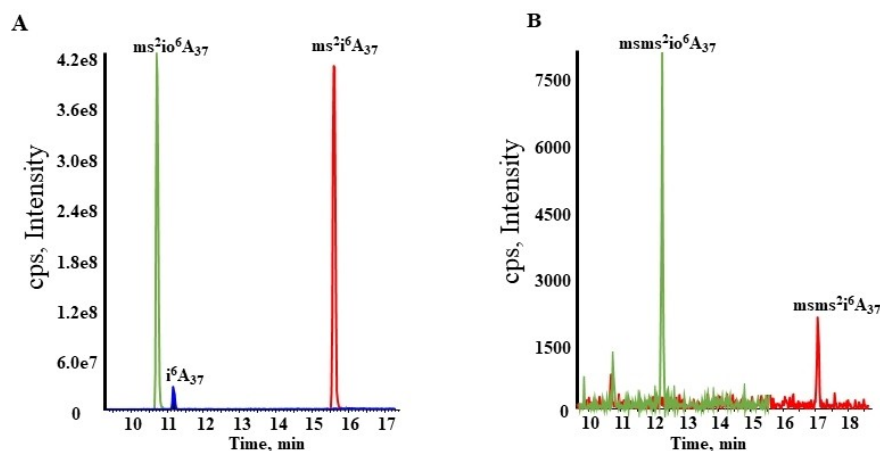


Figure 1. UHPLC-MS/MS analyses of digested total tRNA from *S. typhimurium* cells. A) Detection of the product ions of i^6A_{37} (blue), $ms^2i^6A_{37}$ (red) and $ms^2i^6A_{37}$ (green). B) Detection of $msms^2i^6A_{37}$ (red) and $msms^2i^6A_{37}$ (green) in the MRM mode with respective m/z 428→296 and 444→312 transitions.

to detect small amounts of i^6A at 11.3 min (Figure 1a) in agreement with the HPLC-UV analyses (Figure SI).

Further, the UHPLC-MS/MS analyses were then specifically conducted to assess the presence of $msms^2i^6A_{37}$ and $msms^2i^6A_{37}$ in the digested tRNA isolated from *S. typhimurium*. For this purpose, we used the highly specific and sensitive multiple reaction monitoring (MRM) mode because of the expected low amounts of these nucleosides (Figure 1b). The chosen daughter ions for the detection were at m/z $[M + H - 132]^+$ and corresponded to the loss of a ribose moiety. A peak with an MRM signal m/z 428 \rightarrow 296 and with a retention time of 17.1 min was attributed to $msms^2i^6A_{37}$ nucleoside (Figure 1b, red trace). This identification was based on a comparison of results obtained by analysis of tRNA extracted from *E. coli*^[6] as confirmed below. The structure was confirmed in product ion scan analyses that revealed the loss of both a $CH_2=S$ and a *cis*-isopentenyl group. A second compound, named N^{444} , that eluted at 12.2 min (Figure 1b, green trace) was detected with the m/z 444 \rightarrow 312 MRM transition. We assigned this compound to $msms^2i^6A_{37}$. This was first based on the observation that the UHPLC retention time of the N^{444} is \sim 5 min shorter than that of $msms^2i^6A_{37}$. This is reminiscent of the difference between $ms^2i^6A_{37}$ and $ms^2i^6A_{37}$ and is explained by the increase in polarity provided by the hydroxy group. Further structural identification was obtained from the ion trap mode analyses that is more sensitive than the triple quadrupolar approach for full spectra recording. We first obtained a MS^2 fragmentation spectrum of N^{444} (m/z 444) that confirmed the loss of 132 mass units (Figure 2a). This fragmentation is typical of RNA nucleosides as it corresponds to the loss of the ribose. We then studied the fragmentation of the base moiety by $MS/MS/MS$ analysis of the daughter ion at m/z 312 (Figure 2b). A number of fragments were observed that confirmed the presence of both a methylthio-methylenethiol and a *cis*-4-hydroxyisopentenyl group. These results allowed us to propose a fragmentation pattern for m/z 444 (N^{444}) in complete agree-

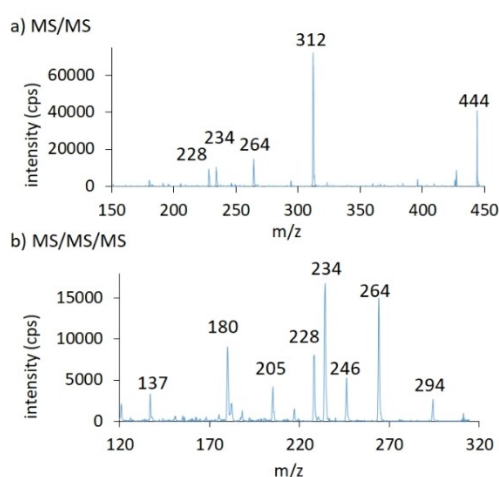


Figure 2. Ion trap mass spectrometry characterization of $msms^2i^6A$. a) MS/MS fragmentation spectrum of the pseudo-molecular ion m/z 444 and b) $MS/MS/MS$ fragmentation spectrum the ion at m/z 312 arising from the fragmentation of m/z 444.

ment with its identification as $msms^2i^6A_{37}$ (Figure 3). N^{444} is $msms^2i^6A_{37}$, which represents a new derivative in the $ms^2i^6A_{37}$ biosynthetic pathway in *S. typhimurium*, has never been detected before in any microorganism.

MiaEst catalyzes the transformation of $msms^2i^6A_{37}$ into $msms^2i^6A_{37}$ in vivo

To confirm that biosynthesis of $msms^2i^6A_{37}$ requires the catalytic activity of the MiaE protein from *S. typhimurium* (MiaEst), we conducted an in-vivo assay using $DH5\alpha$ *E. coli* strain. As all wild-type *E. coli* strains, the latter naturally lacks the *miaE* gene and thus contains $msms^2i^6A_{37}$, the potential substrate of MiaEst. This strain was transformed with pET28a-MiaEst and the cells were grown at 37°C until OD_{600} reached 1.4–1.6 and tRNAs were then isolated, purified and digested with nuclease P1 and phosphatase and their modified nucleoside content was analyzed by HPLC as described in the Experimental Section.^[11] Thanks to HPLC analyses, only the peak corresponding to $ms^2i^6A_{37}$, which elutes at 69 min was detected confirming that MiaEst has the ability to catalyze the transformation of $ms^2i^6A_{37}$ into $ms^2i^6A_{37}$ within *E. coli*, as previously reported.^[10] Interestingly, the HPLC-MS/MS method described and used above showed the presence of the N^{444} product identified in *S. typhimurium* as $msms^2i^6A_{37}$. Analyses clearly confirmed (Table 1) that no hydroxylation of the *cis*-4-

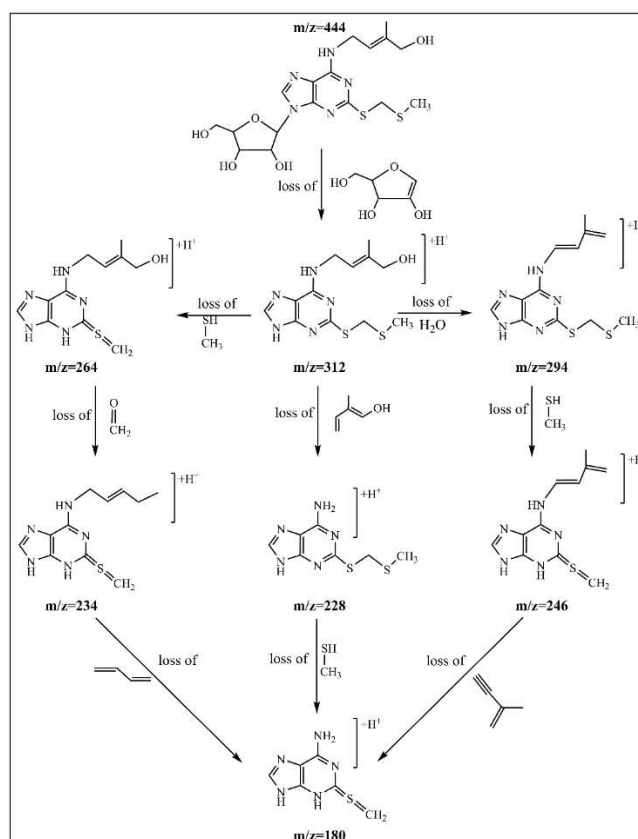


Figure 3. Fragmentation pattern of the nucleoside identified as $msms^2i^6A_{37}$.

Table 1. Formation of modified nucleosides in the tRNA extracted from *E. coli* DH5 α strain and transformed DH5 α with expressing *miaE* plasmid (DH5 α -MiaE). Reported information is the distribution [%] among the six compounds calculated from the area of the peaks of the analyses in product ion scan detection. n.d. not determined.

Strain	i ⁶ A	io ⁶ A	ms ² i ⁶ A	ms ² io ⁶ A	msms ² i ⁶ A	msms ² io ⁶ A
DH5 α	20.2	n.d.	78.9	n.d.	0.9	n.d.
DH5 α -MiaE	5.4	13.1	18	63.2	n.d.	0.3

hydroxyisopentenyl group took place in the absence of MiaE. Thus, expression of MiaE led to the formation of significant amounts of io⁶A₃₇ and ms²io⁶A₃₇ at the expense of their non-hydroxylated precursors. Similarly, detectable amounts of msms²io⁶A₃₇ were obtained at the expense of msms²i⁶A₃₇. These results unambiguously demonstrate that the tRNA-hydroxylase MiaE enzyme catalyzes the transformation of msms²i⁶A₃₇ into msms²io⁶A₃₇.

In this study, using an LC-MS/MS based-method, we unambiguously showed that tRNA isolated from *S. typhimurium* contains both modified nucleoside msms²i⁶A₃₇, previously identified in tRNA from *E. coli*,^[6] and a newly discovered modified nucleoside, msms²io⁶A₃₇. To our knowledge, this new derivative in the biosynthetic pathway of ms²io⁶A₃₇ has never been detected previously in any species. Furthermore, using in vivo complementation experiments in an *E. coli* strain, we showed that the tRNA-hydroxylase MiaE from *S. typhimurium*, catalyzes the transformation of msms²i⁶A₃₇ into msms²io⁶A₃₇. This discovery expands the large variety of modified nucleosides encountered in tRNAs; we also emphasize that the spectacular development in LC-MS methods will contribute to the identification and characterization of new modified nucleosides even in well-established metabolic pathways such the one investigated in this communication. Even though research in the field of tRNA modification is a very active subject, the physiological role of modified nucleosides is still in its infancy, and much work is needed to spotlight their potential roles as essential players in cell metabolism.

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Conflict of Interest

The authors declare no conflict of interests.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

Keywords: hydroxylation · LC-MS · tRNA modifications · tRNA-diiron monooxygenase

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