mRNA Modification

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A Biocatalytic Cascade for Versatile One-Pot Modification of mRNA Starting from Methionine Analogues

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Dedicated to Professor Ulrich Hahn on the occasion of his 65th birthday

Abstract: *Methyltransferases have proven useful to install functional groups site-specifically in different classes of biomolecules when analogues of their cosubstrate S-adenosyl-L-methionine (AdoMet) are available. Methyltransferases have been used to address different classes of RNA molecules selectively and site-specifically, which is indispensable for biophysical and mechanistic studies as well as labeling in the complex cellular environment. However, the AdoMet analogues are not cell-permeable, thus preventing implementation of this strategy in cells. We present a two-step enzymatic cascade for site-specific mRNA modification starting from stable methionine analogues. Our approach combines the enzymatic synthesis of AdoMet with modification of the 5′ cap by a specific RNA methyltransferase in one pot. We demonstrate that a substrate panel including alkene, alkyne, and azido functionalities can be used and further derivatized in different types of click reactions.*

S-Adenosyl-L-methionine (AdoMet or SAM) is the second most abundant cosubstrate used in nature after ATP, and is the major source of methyl groups in biomolecules.[1] Methylation is involved in fundamental biological processes, such as bacterial host defense, epigenetic silencing, and cellular signaling, and is found in all classes of biopolymers as well as lipids and many small molecules.[1,2] Recent studies on different methyltransferases revealed that these enzymes can show remarkable substrate promiscuity and accept synthetic AdoMet analogues.[2d,3] In several cases, the promiscuous activity was significantly enhanced by single amino acid substitutions.[4] The efficient transfer of a range of functional groups to methyltransferase substrates allows for subsequent highly selective conversion through various click reactions.[5]

However, AdoMet and its analogues are not cell-permeable, and dedicated transporters in mammals have not been described. Therefore, a major limitation of the above-mentioned approach is to deliver AdoMet analogues inside a mammalian cell. The limited stability of AdoMet in aqueous solution aggravates this problem.[6] Moreover, chemical synthesis of AdoMet analogues requires acidic conditions and results in a mixture of epimers at the sulfur atom, with only the *S*,*S* epimer being accepted as a substrate of methyltransferases.[7]

These limitations of synthetic AdoMet analogues may be addressed by switching to the biosynthetic route. In nature, AdoMet is produced from methionine and ATP, and the reaction is catalyzed by methionine adenosyltransferases (MATs).[8] MATs have been used to produce enantiomerically pure AdoMet both by the isolated enzyme and by microbial fermentation.[9] Methionine analogues have been used in MAT-catalyzed reactions to investigate quorum sensing,[10] alkyl randomization of secondary metabolites,[11] and to identify methylation sites of protein arginine methyltransferases in chromatin modification,[12] but not for nucleic acids.

Herein we report the enzymatic production of AdoMet analogues coupled to an RNA methyltransferase. By using this enzymatic cascade, we can directly and site-specifically modify eukaryotic model mRNAs at their 5′ cap starting from methionine analogues instead of synthetic AdoMet analogues, and subsequently label the cap structure by using different types of click reactions.

To realize this approach for a representative substrate panel, we required both a MAT and an RNA methyltransferase with significant activities on the respective substrate analogues. We used a variant of the RNA methyltransferase GlaTgs2 (GlaTgs-Var) from *Giardia lamblia*, which is known to transfer allyl, alkyne, azido, or benzyl moieties to the N^2-position of the mRNA 5′ cap.[4a,d,13]

The coproduct, *S*-adenosylhomocysteine (SAH), inhibits methyltransferases, but can be enzymatically degraded in the same pot.[14] To produce the required AdoMet analogues in situ, we explored the substrate promiscuity of recombinantly produced human MATIIa I117A (MAT-Var), which was reported to accept longer alkyl side chains bearing methionine derivatives (see Figure S1 in the Supporting Information).[12b] Since MAT shows strong product inhibition,[8,9b] we set up an enzyme-coupled reaction to directly consume AdoMet (or its analogues) and label the mRNA 5′ cap (Scheme 1).

We first established our coupled MAT/GlaTgs system using the natural substrate L-methionine, but also D-methionine and a racemic mixture of both, and the mRNA cap analogue m^7GpppA as methyl acceptor (Figure S2). As

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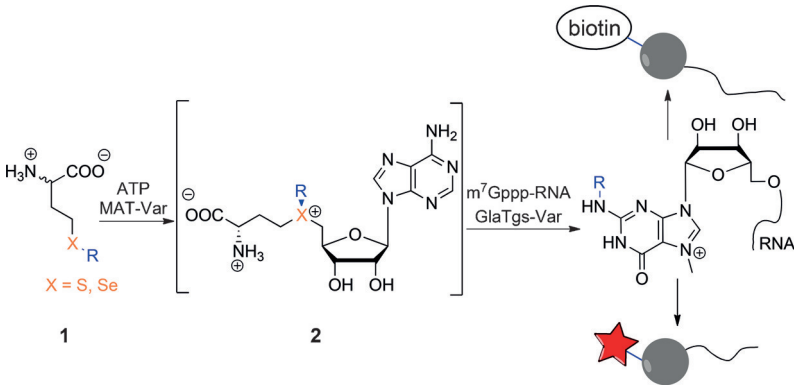


Scheme 1. Schematic overview of the enzymatic modification of eukaryotic mRNA bearing a 5′ cap starting from racemic methionine analogues. An AdoMet analogue **2** is generated in situ from **1** and ATP using MAT-Var and serves as a cosubstrate for the RNA methyltransferase GlaTgs-Var, thereby leading to modification of the 5′ cap at position N^2. The introduced groups (R) can be further treated in different types of click reactions to introduce affinity or fluorescent tags.

expected, L-methionine led to the formation of $m^{2,7}$GpppA, whereas D-methionine was not consumed but did not compromise the conversion, thereby allowing us to use racemic methionine analogues in the following experiments.[15]

Next, we used the enzymatic cascade to convert methionine analogues and modify m^7GpppA with different functional groups site-specifically at N^2. We synthesized a panel of *S*-derivatized homocysteines bearing different alkene, alkyne, and azido groups (**1a**–**f**) as well as benzylic moieties (**1g**,**h**; Table 1, and Figure S3). Furthermore, we synthesized two analogues based on selenohomocysteine because the resulting Se-AdoMet analogues are more efficient at side-chain transfer.[6b,16]

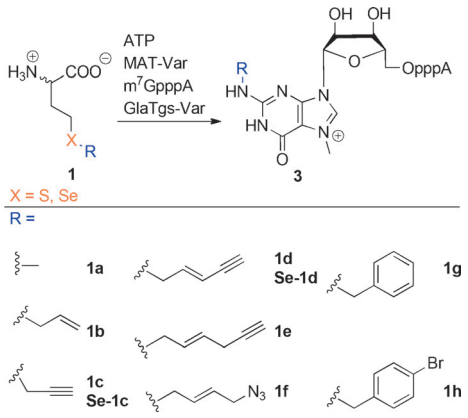
The racemic methionine analogues were directly converted in one pot containing both enzymes as well as ATP and m^7GpppA. Typically, 300 μM m^7GpppA was treated with an 8-fold excess of ATP and a 20-fold excess of racemic methionine analogues at a catalyst loading of 3–7 mol% MAT-Var (relative to m^7GpppA). The GlaTgs-Var concentration was typically three times higher than the MAT-Var concentration to ensure that in situ formed AdoMet analogues are directly converted.[4a,12a] This is important because MAT generally shows strong product inhibition by AdoMet.[9b] In comparison to synthetic AdoMet analogues, only one epimer is formed (Figure S4).

We observed the transfer of allyl, alkyne, and azido moieties with different steric demands to m^7GpppA with moderate to excellent yields (Table 1, Figure 1, and Figure S5). The small allyl group in **1b** was transferred almost quantitatively at 3 mol% MAT-Var. The chemically more interesting propargyl group of **1c**, however, resulted in only 11% conversion, even at 7 mol% MAT-Var, most likely because of the low stability of the in situ formed AdoMet analogue **2c**.[6b] This limitation was solved by switching to the selenium-based methionine analogue. **Se-1c** drastically increased the conversion, and 91–100% of N^2-propargyl-m^7GpppA could be obtained at 3–7 mol% MAT-Var. These results are in line with results using chemically synthesized **Se-2c**.[6b]

The longer pentenynyl and hexenynyl groups of **1d** and **1e**, respectively, were also transferred. The conversion could be pushed to 35% and 100% at 7 mol% catalyst, respectively. In contrast to **Se-1c**, the selenium-based amino acid **Se-1d** increased the yield of **3d** only moderately compared to its sulfur-based counterpart **1d**. Interestingly, despite its longer side chain, **1e** gave higher yields than **1d**, which has also been observed in combination with protein methyltransferases.[12b] Aromatic groups, however, turned out to be poor substrates. The benzyl-bearing methionine analogue **1g** gave only 2–5% conversion, and the *para*-substituted **1h** yielded only traces of **3h**.

After establishing this biocatalytic cascade for site-specific modification of the 5′ cap we wanted to test whether the functional groups

Table 1: One-pot enzymatic modification of m^7GpppA starting from S- and Se-based methionine analogues.[a]



Entry	Substr.	MAT [mol%]	Prod.	Conv. [%]
1	**1a**	3	**3a**	100
2	**1b**	3	**3b**	95
3	**1b**	5	**3b**	100
4	**1c**	3	**3c**	4
5	**1c**	7	**3c**	11
6	**Se-1c**	3	**3c**	91
7	**Se-1c**	5	**3c**	100
8	**Se-1c**	7	**3c**	100
9	**1d**	3	**3d**	10
10	**1d**	7	**3d**	35
11	**Se-1d**	3	**3d**	17
12	**Se-1d**	7	**3d**	47
13	**1e**	3	**3e**	38
14	**1e**	5	**3e**	77
15	**1e**	7	**3e**	100
16	**1f**	3	**3f**	12
17	**1f**	5	**3f**	72
18	**1g**	3	**3g**	2
19	**1g**	7	**3g**	5
20	**1h**	3	**3h**	traces

[a] Conversions at different catalyst loadings (relative to m^7GpppA) are shown. Reaction conditions: 6 mM D/L-**1**, 2.5 mM ATP, 300 μM m^7GpppA, MAT-Var as indicated, GlaTgs-Var 3× higher than MAT-Var, incubation for 8–9 h at 23 °C.

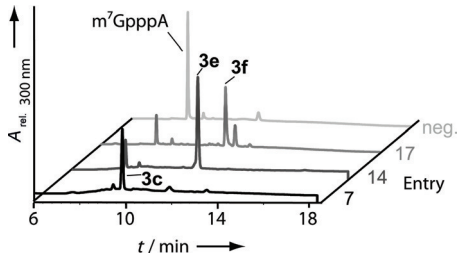


Figure 1. Representative HPLC chromatograms of selected reactions from Table 1. Neg.: negative control without **1**.

appended to the N^2-position are suitable handles for subsequent derivatization. Therefore, we treated **3c** (from conversion of **Se-1c**) and **3e** with biotin-azide in a copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC; Figure 2a)

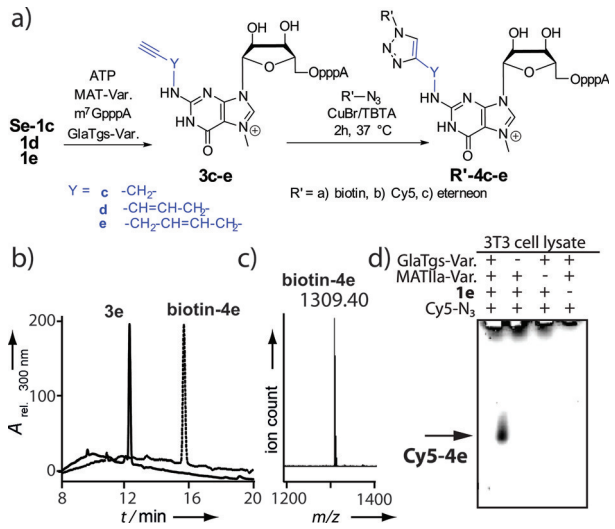


Figure 2. Postsynthetic labeling of alkyne-functionalized m^{7}GpppA. a) Enzymatic introduction of different terminal alkynes followed by CuAAC with different labels R′. b,c) HPLC and MS analysis confirming CuAAC of **3e** with biotin-azide. d) In-gel fluorescence of **Cy5-4e** after separation on a 10% denaturing PAGE (1.5 h at 25 W).

and confirmed the formation of the expected products, **biotin-4c** and **biotin-4e**, respectively, by HPLC and MALDI-TOF-MS (Figure 2b,c and Figures S10 and S11). Two fluorescent dyes, Cy5-azide and Eterneon-azide, were also treated with **3c** and **3e**, respectively. Gel analysis revealed that a fluorescent band was only obtained if both enzymes and the required amino acid (**1e** or **Se-1c**) were present (Figure S12A,B). Importantly, we could also perform the enzymatic cascade reaction in eukaryotic cell lysate, which indicates that the enzymatic cascade also works selectively in the presence of cellular components (Figure 2d, Figure S13).

To make our approach suitable for intracellular applications, we wanted to introduce a functional group that reacts in a truly bioorthogonal reaction. We devised methionine analogue **1f** to transfer an azido moiety, which can be reacted in a strain-promoted azide–alkyne cycloaddition (SPAAC). The reaction of **1f** with m^{7}GpppA resulted in a 72% yield of **3f** at 5 mol% MAT and the product was validated by MALDI-TOF-MS (Table 1, Figure 1, and Figure S14). The subsequent SPAAC reaction of **3f** with dibenzocyclooctyne-sulforhodamine B (DBCO-SRB) yielded a fluorescently labeled 5′ cap, which showed a fluorescent band after PAGE separation only if all the components were present (Figure S15). Again, enzymatic transfer was also successful in eukaryotic cell lysate (Figure S15).

To ensure that our approach is suitable for labeling eukaryotic mRNAs, we produced 24 and 106 nt long RNAs by in vitro transcription and capping with the vaccinia capping system[17] and subjected them to our biocatalytic MAT/GlaTgs cascade (Figure 3 and Figure S22). Starting from **1e**, subsequent labeling with Cy5-azide resulted in specific fluorescent modification of alkyne-modified RNA (Figure 3a, see Figure S16 for the 106 nt RNA). Biotin-azide reacted in a similar manner (Figure S17).

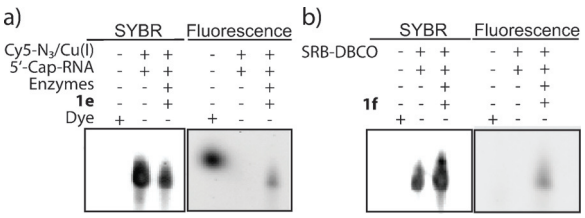


Figure 3. Labeling of a 24 nt 5′-capped model mRNA starting from methionine analogues **1e** or **1f** followed by CuAAC or SPAAC, respectively. a) Gel analysis after CuAAC by SYBR staining and fluorescence (λ_{ex} = 628 nm, λ_{em} = 716 nm). As a control, 5′-capped RNA that was not treated with **1e** is shown. b) Gel analysis of the labeled 24 nt model mRNA after SPAAC by SYBR staining and fluorescence (λ_{ex} = 520 nm, λ_{em} = 609 nm). As a control, 5′-capped RNA that was not treated with **1f** is shown. Bromophenol blue was used as a dye marker (λ_{max} = 590 nm). Resolution on a 15% denaturing PAGE (1.5 h at 25 W).

Starting from **1f**, the SPAAC reaction was also established with DBCO-SRB for the 24 nt 5′-capped RNA. The resulting SRB-labeled RNA was analyzed by in-gel fluorescence. A comparison with RNA staining by SYBR revealed that the SPAAC reaction also works with 5′-capped RNA modified in an enzymatic cascade starting from **1f** (Figure 3b). Mass analysis of unmodified 24 nt RNA and capped 24 nt RNA is shown in the Supporting Information (Figure S18).

In summary, our cascade approach allows for the site-specific transfer of a variety of chemical functionalities to the mRNA cap structure starting from stable and easy to synthesize analogues of the amino acid methionine. Alkyne-bearing side chains of different lengths and azido moieties were efficiently transferred to the 5′ cap as well as 5′-capped RNA. Subsequent click reactions gave access to biotin- and fluorophore-modified mRNAs. Since methionine analogues can be taken up by the cell[12b,18] and the enzymatic steps also work selectively in eukaryotic cell lysate, we anticipate that our approach will enable the intracellular labeling of RNA. In combination with other RNA or DNA methyltransferases, our approach should provide a general concept towards the site-specific intracellular modification of nucleic acids. In light

of the recent discoveries of reversible adenosine methylation in bacterial and eukaryotic mRNA,[19] we anticipate a broad range of applications in the near future.

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