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Novel AdoMet analogues as tools for enzymatic transfer of photo-crosslinkers and capturing RNA-protein interactions

Fabian Muttach,[a] Florian Mäsing,[b] Armido Studer[b] and Andrea Rentmeister*[a,c]

Abstract: Elucidation of biomolecular interactions is of utmost importance in biochemistry. Photo-crosslinking offers the possibility to precisely determine RNA-protein interactions. However, despite the inherent specificity of enzymes, approaches for site-specific introduction of photo-crosslinking moieties into nucleic acids are scarce. Methyltransferases in combination with synthetic analogues of their natural cosubstrate *S*-adenosyl-L-methionine (AdoMet) allow for the post-synthetic site-specific modification of biomolecules. We report on three novel AdoMet analogues bearing the most widespread photo-crosslinking moieties (aryl azide, diazirine and benzophenone). We show that these photo-crosslinkers can be enzymatically transferred to the methyltransferase target, i.e. the mRNA cap, with high efficiency. Photo-crosslinking of the resulting modified mRNAs with the cap interacting protein eIF4E was successful with aryl azide and diazirine but not benzophenone, reflecting the affinity of the modified 5' caps.

Photo-crosslinking has become an enabling technique for mapping interactions of biomolecules – even if they are weak or transient – and for clarifying structures of bigger complexes. In the field of proteins, site-specific incorporation of amino acids with photo-crosslinking moieties advanced the field significantly because pinpointing the sites on a protein in the vicinity of the interacting protein became possible.[1] In the field of nucleic acids, irradiation with short-wave UV-light has long been used for crosslinking and immunoprecipitation (CLIP) methods, even though it damages nucleic acids at the required wavelengths (~254 nm).[2] Advanced CLIP based techniques therefore rely on incorporation of 4-thiouridine or 6-thioguanosine[3] compatible with longer wavelengths (~365 nm). Importantly, CLIP methods allow for transcriptome-wide identification of RNA-binding sites and are compatible with living cells[3] and could be coupled with high-resolution mass-spectrometry.[4]

Akin to the protein-centred approaches, site-specific instead of randomly distributed installation of photo-crosslinkers into nucleic acids helps to precisely assign interactions. The most widespread photo-crosslinking moieties available to date are aryl azides, benzophenones and diazirines.[5] Additionally, furan-moieties allowed for the mild red-light dependent nucleic acid interstrand[6] and nucleic acid-protein crosslinking.[7] DNA with diazirines at specific positions is accessible by solid-phase synthesis and was used for interstrand photo-crosslinking[8] as well as for crosslinking to DNA-binding proteins.[9] DNA was also modified with four different photo-reactive moieties including benzophenone and crosslinked to yeast RNA polymerase III.[10] RNA with photo-crosslinking moieties at defined positions could be synthesized as well. Diazirines were incorporated into a chemically synthesized microRNA and used for photo-crosslinking with RNA-interacting proteins.[11] Aryl azides were employed for crosslinking in an RNA duplex[12] and for probing of a Diels-Alderase ribozyme.[13]

However, only few enzymatic approaches for site-specific introduction of photo-crosslinkers have been reported. A 5' cap analogue containing 6-thioguanosine was chemically synthesized and enzymatically incorporated at the 5' end of RNA during *in vitro* transcription. Upon irradiation at 365 nm, intrastrand photo-crosslinking of RNA was observed.[14] Site-specific enzymatic incorporation of a photo-crosslinking moiety at an internal position was achieved in a different study using an unnatural base-pairing system.[15]

We thought that enzymatic conversions should be ideal for site-specific introduction of photo-crosslinking moieties if an appropriate biocatalyst is available. In particular, methyltransferases (MTases) have been successfully used for specific functionalization of biomolecules. Instead of transferring a methyl group from their natural cosubstrate *S*-adenosyl-L-methionine (AdoMet) to their target molecule, several MTases can be used or engineered to transfer other functional moieties from synthetic analogues.[16] During the past few years, AdoMet analogues with extended side-chains bearing different alkene, alkyne, amino, azido or 4-vinyl benzyl functionalities have been developed and used in combination with highly promiscuous MTases.[17] The transferred moieties were used to label the MTase targets[18], to alter their biological functions[19] or for pull-down studies.[20] In a completely different approach, *S*-adenosylhomocysteine (SAH) analogues with crosslinking moieties have been developed. These molecules are known MTase inhibitors and were used for binding and profiling MTases.[21] However, AdoMet analogues containing photo-crosslinkers have not been described to the best of our knowledge, nor has the post-synthetic enzymatic transfer of photo-crosslinkers to biomolecules been reported. We aimed to address this shortcoming by synthesizing AdoMet analogues suitable for transfer of the most widespread photo-crosslinking groups, namely aryl azides, diazirines and benzophenones. We then wanted to test whether such bulky moieties are still suitable for enzymatic transfer and if they would thus allow for site-specific post-synthetic modification of biomolecules. Lastly, we wanted to see whether the installed moieties would be functional for photo-crosslinking based on a known interaction partner.

Results and Discussion

For MTase-based enzymatic transfer of photo-crosslinking groups, suitable AdoMet analogues and a promiscuous MTase are required. For efficient transfer of the AdoMet analogue side-

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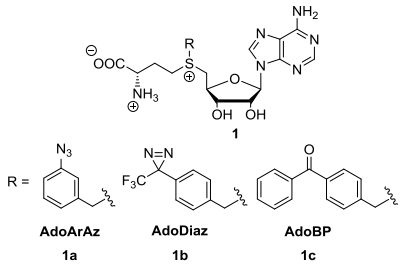
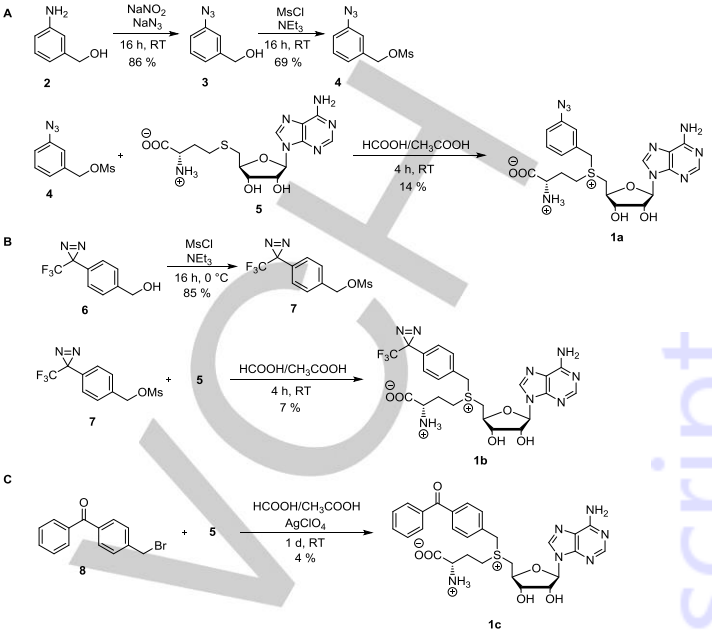
chain an unsaturated bond is required in *ß*-position of the sulfonium center, most likely due to a stabilizing conjugation of the p orbital with the antibonding π orbital in the transition state.[17a] It is known that alkyl chains other than methyl (e.g. ethyl or propyl) are barely transferred by methyltransferases.[17a] Based on these considerations we designed three novel AdoMet analogues bearing the most widespread photo-crosslinking moieties in the side-chain (Scheme 1).



Scheme 1. Chemical structure of novel AdoMet analogues with photo-crosslinking side-chains.

Since these AdoMet analogues are very bulky, they will not be accepted by every MTase but require a highly promiscuous enzyme for transfer. We recently reported on the very high substrate promiscuity of cap *N7* methyltransferase Ecm1, which accepted alkynes, azides, and even aromatic moieties with uncompromised activity.[19] Ecm1 was therefore chosen as the MTase for further studies.

Scheme 2 shows the synthesis of the three novel AdoMet analogues. To obtain the aryl-azide bearing AdoMet analogue **1a**, we started from 3-aminobenzyl alcohol (**2**) and converted it to the respective azide **3** (Scheme 2A, experimental details in ESI). Compound **3** was then converted to the mesylate **4** and reacted with *S*-adenosyl-L-homocysteine **5** to **1a**. Preparation of diazirine-containing AdoMet analogue **1b** required synthesis of 3-[(4-hydroxymethyl)phenyl]-3-trifluoromethyl-3*H*-diazirine (**6**) in seven steps according to a published procedure.[11] The benzyl alcohol **6** was then converted to the respective mesylate **7** and reacted with **5** (Scheme 2B). For the synthesis of benzophenone-containing AdoMet analogue **1c** we simply reacted commercially available 4-(bromomethyl)benzophenone (**8**) with **5** (Scheme 2C). In this case, $AgClO_4$ was added to precipitate AgBr to increase the conversion. While in the case of mesylates, reaction times of 4 h were sufficient, reactions were performed overnight if a bromide was used. AdoMet analogues **1a-c** were purified via RP-HPLC and characterized by HPLC, MS, and ^{1}H-NMR (ESI Figure S3, S4, S16-19). These data were backed up by collision induced decay (CID)-MS/MS and the fragment spectra could be assigned to the respective AdoMet analogues (ESI Figure S5). The concentration of AdoMet analogues was determined by weighing of the compounds. It is important to point out that all AdoMet analogues were isolated as equal mixtures of the *S*- and *R*-epimers, meaning that the biologically effective concentration is actually reduced to half. We always indicate the total concentrations used in the respective conversions.



Scheme 2. Synthesis of the novel AdoMet analogues AdoArAz (**1a**) (A), AdoDiaz (**1b**) (B) and AdoBP (**1c**) (C), bearing photo-crosslinking moieties in their side-chain.

To elucidate whether our new AdoMet analogues will be suitable for enzymatic conversions, we tested their stability under typical reaction conditions and analyzed degradation pathways. To this end, **1a-c** were incubated in a buffer consisting of 200 mM NaCl/1mM EDTA/10 % glycerol at 37 °C and pH = 8 and analyzed at different time points by HPLC. Quantification of remaining **1a-c** over time revealed half-lives of ~2.3 h for **1a**, ~2 h for **1b**, and ~1.5 h for **1c**, respectively (ESI Figure S6). These half-lives are in the range of previously reported AdoMet analogues and should thus be sufficient for methyltransferase-based conversions.[22] Using LC/MS, we were also able to identify the decay products of **1a-c**. In line with previous reports, we observed formation of **5**, but also adenine resulting from cleavage of the glycosidic bond, releasing adenine (ESI Figure S7 and S8).[22]

Enzymatic transfer of photo-crosslinking moieties

Next, we tested whether **1a-c** are suitable cosubstrates for enzymatic transfer of photo-crosslinking moieties. To this end, we used the methyltransferase Ecm1, which shows remarkable cosubstrate promiscuity.[19] Ecm1 naturally transfers methyl groups to the *N7* position of the 5' cap of mRNA but also acts on a minimal dinucleotide cap GpppA **9**.[23] Typically, 300 µM GpppA were reacted with 1-2 mM **1a-c** and 20 mol% Ecm1. The enzymes 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase (MTAN) and LuxS were added to all reactions in order to enzymatically degrade the by-product *S*-adenosylhomocysteine which is a strong inhibitor of methyltransferases.

For all three AdoMet analogues tested, efficient transfer of the respective photo-crosslinking group to **9** was observed (Figure 1A). The product peaks detected in HPLC analysis were also analyzed by MS to validate formation of **10a-c**, respectively (see

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Figure 1B and C). Specifically, quantitative conversions could be achieved under the conditions described above and using 2 mM of the AdoMet analogue. To further characterize the enzymatic transfer reaction, we performed time course experiments under the conditions mentioned above and using 1 mM of the respective AdoMet analogue. These measurements revealed that Ecm1-catalyzed transfer reactions are surprisingly fast and efficient considering the bulkiness of **1a-c**. For **1a-b**, transfer was complete after 60-90 minutes based on the consumption of GpppA and appearance of a new peak which could be assigned to the modified cap analogue (see Figure 1D). For **1c**, however, only 60 % modification product was observed under these conditions suggesting that the benzophenone moiety partially impedes transfer, most likely due to its extreme sterical demand.

Furthermore, we found the enzymatic transfer using Ecm1 to be highly regiospecific. When the cap analogue m^7GpppA (*N7* position not accessible) was used in combination with Ecm1 no enzymatic transfer of the photo-crosslinking moiety was observed (ESI Figure S20).

Taken together, these data suggest that the new AdoMet analogues **1a-c** are sufficiently stable and suitable for enzymatic conversions resulting in postsynthetic modification of the 5' cap with photo-crosslinking moieties. Since multiple other MTases with similar architecture (i.e. an open binding site) and high cosubstrate promiscuity are known, we anticipate that **1a-c** will also be suitable for enzymatic modification of different classes of target molecules.[22, 24]

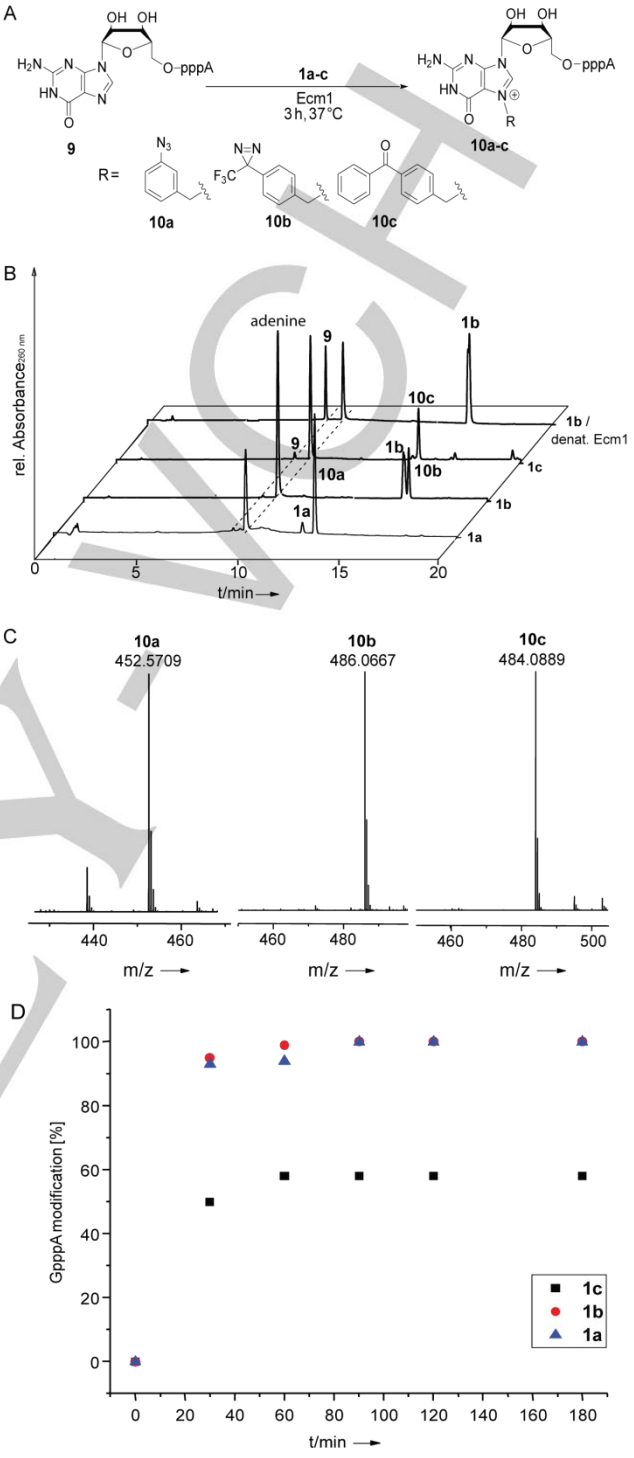

Figure 1. (A) Enzymatic transfer of photo-crosslinking moieties to the cap analogue GpppA (300 µM) using the respective AdoMet analogue (2 mM) and the methyltransferase Ecm1 (60 µM). (**B**) HPLC analysis of enzymatic transfer reactions using the photo-crosslinking AdoMet analogues **1a-c**. (**C**) Mass spectrometric analysis (LC-ESI/MS) of the enzymatic transfer reactions. Expected mass for $C_{27}H_{34}N_{13}O_{17}P_3^{2+}$ = 452.5699 [M^++H^+], found: 452.5709 (**1a**); expected mass for $C_{29}H_{34}F_3N_{12}O_{17}P_3^{2+}$ = 486.0859 [M^++H^+], found: 486.0667 (**1b**); expected mass for $C_{34}H_{39}N_{10}O_{18}P_3^{2+}$ = 484.0823 [M^++H^+], found: 484.0889 (**1c**); (**D**) Time course experiments for enzymatic transfer of the novel AdoMet analogues **1a-c** (1mM) using Ecm1 and the cap analogue GpppA. All reactions were performed in duplicates and average values are depicted.

Labelling with functional probes using aromatic azide 1a

Aromatic azides are not only suitable for photo-crosslinking purposes but also for bioorthogonal reaction in a strain-promoted azide-alkyne cycloaddition (SPAAC reaction) using a strained alkyne. To evaluate the reactivity of the aromatic azide we transferred the aromatic azide of **1a** to GpppA **9** followed by direct reaction of the azido-modified cap analogue **10a** with the cyclooctyne MegaStokes dye 608 (Sigma). Separation of the reaction mixture by PAGE and in-gel fluorescence analysis yielded a fluorescent band only if all components were present (ESI Figure S21A). Control reactions omitting either Ecm1 or the AdoMet analogue in the enzymatic reaction did not give a fluorescent band. Additionally, we reacted the azido-modified cap **10a** with DBCO-biotin for 1 h at 37 °C and confirmed product formation by LC-MS analysis (expected mass for $C_{69}H_{90}N_{19}O_{25}P_3S_3^{2+}$ = 886.7360 [M^++H^+], found: 886.7321; see ESI Figure S22). We also extended our labelling strategy to the modification of a 24 nt model RNA. To this end we enzymatically capped the RNA, subjected it to enzymatic modification and SPAAC reaction and after PAGE separation and SYBR-Gold staining analyzed the RNA. Again, a fluorescent band was only observed if all components were present (ESI Figure S21B). It should however be noted that aryl azides are susceptible to reduction as observed in an LC-MS analysis (ESI Figure S9). Reduction of the azido moiety could be circumvented by switching to a DTT-free reaction buffer.

Crosslinking

To assess whether the photo-crosslinking groups are functional and the reactive species is produced, we tested the photo-reactivity of the novel AdoMet analogues. To this end, **1a-c** were continuously irradiated at their respective wavelengths (312 nm for **1a**, 365 nm for **1b** and both wavelengths for **1c**). At different time points, samples were taken and their absorption spectra measured (Figure 2 and ESI Figure S10). In each case, changes in absorption spectra were observed indicating photo-reactivity of the AdoMet analogues.

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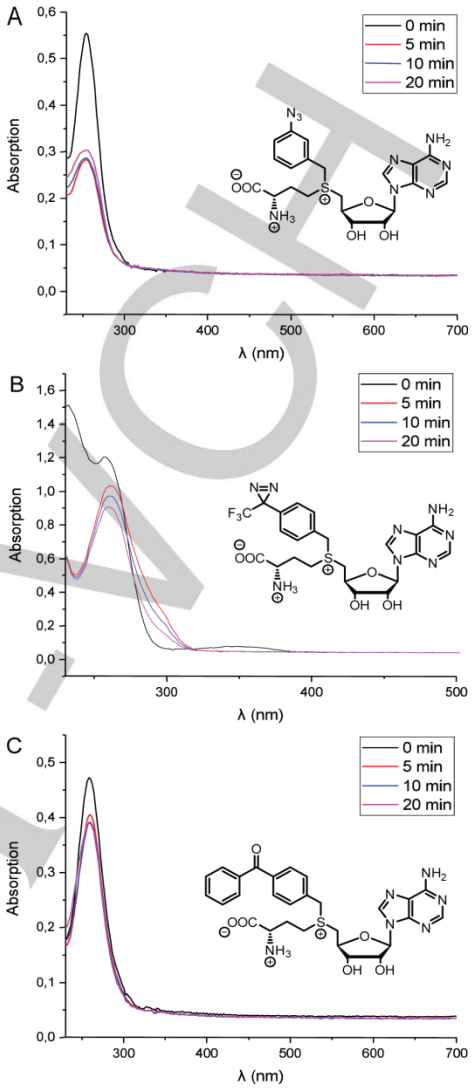

Figure 2. Representative absorption spectra of AdoMet analogues **1a-c** before and after photo-irradiation for 20 min at either 312 nm (**1a**) or 365 nm (**1b** and **1c**). After indicated time-points samples were taken and absorption spectra were recorded. Changes in the absorption spectra indicate photo-reactivity of the respective AdoMet analogues (A) **1a** with irradiation at 312 nm (poly-styrol filtered light) (B) **1b** with irradiation at 365 nm (C) **1c** with irradiation at 365 nm.

To test whether the photo-crosslinking moieties are still functional after transfer to the MTase target (i.e. the modified 5' cap), we prepared 24 nt model RNAs with different 5' caps. The RNAs were produced enzymatically (T7 transcription) and capped using the Vaccinia capping system. To produce RNAs with modified caps, the AdoMet was omitted at this step. Uncapped RNAs were enzymatically degraded using a combination of 5'-polyphosphatase (Epicentre) and terminator exoribonuclease XRN-1 (NEB) (ESI Figure S24 and S25). The remaining RNA was then enzymatically modified using Ecm1 and **1a-c**, respectively using conditions that should yield complete conversion.

These capped 24 nt RNAs (termed **10a-c**-RNAs) were then tested in binding and crosslinking studies with a known cap interacting protein eIF4E (Figure 3A). The eIF4E from *S.*

cerevisiae was recombinantly expressed in *E. coli* and purified. Identity and purity of eIF4E (≥75 %) was confirmed by SDS-PAGE and LC-ESI-MS (ESI Figure S2). *S. cerevisiae* eIF4E was incubated with the **10a-c**-RNAs or m^{7}GpppG-RNA as control and irradiated. Specifically, the **10a**-RNA mixture was irradiated for 20 min at 312 nm using an UV-table. The **10b-c**-RNAs were irradiated for 30 min at 365 nm using an LED (1000 mW). Separation on a 10 % denat. PAA gel revealed an additional band with lower electrophoretic mobility for **10a**- and **10b**-RNAs in the presence of eIF4E and after irradiation, whereas for **10c**-RNA an additional band could not be unambiguously detected (Figure 3B). The additional band does not occur if the mixture of **10a-b**-RNA and eIF4E (at identical concentrations) was not irradiated, indicating that photo-crosslinking had occurred. Interestingly, for **10b**-RNA a shifted band was also observed when irradiation was performed in the absence of the cap binding protein eIF4E, suggesting that **10b**-RNA might react with itself in the absence of a suitable binding partner.[11] This potential self-crosslinking band however showed a different length from the RNA-protein crosslink band and did not occur in the presence of eIF4E.

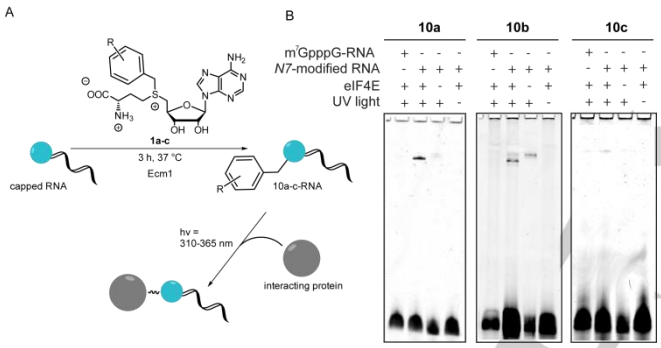

Figure 3. Reactions of differently photo-crosslinker modified 5' RNA caps. (**A**) Concept of methyltransferase-based transfer of photo-crosslinking moieties and subsequent photo-crosslinking to an interacting protein. (**B**) Photo-crosslinking of differently cap-modified 24 nt RNAs to eIF4E. Aryl-azide-, diazirine- or benzophenone-modified 24 nt RNA (**10a-c**-RNA) was subjected to photo-crosslinking with the yeast cap binding protein eIF4E (~100 ng RNA and appr. 4 equiv. eIF4E). The RNA was resolved on a 15 % denat. PAGE (run for appr. 2 h, 15 W, 1×TBE), stained with SYBR-gold and scanned on a Typhoon FLA9500 laser scanner (λ_{ex} = 473 nm; λ_{em} = 510 nm LP).

The lack of a photo-crosslinking band for **10c**-RNA prompted us to test whether the modified caps were still binding to eIF4E. In general, photo-crosslinking based on benzophenone would be expected to give higher crosslinking yields, because benzophenone in the excited state may return to the ground state in case hydrogen abstraction on the electron-deficient ketyl oxygen does not take place.[5] However, the benzophenone-group is also the sterically most demanding moiety in our set. Although this had only little effect on the enzymatic transfer from **1c** to the 5' cap, the binding of **10c** or **10c**-RNA could be abolished and thus prevent crosslinking.

To benchmark the effect of the photo-crosslinking groups at the *N7* position of the 5' cap on binding to eIF4E, we performed microscale thermophoresis measurements, which require low amounts of sample and fluorescent labelling of one binding partner.[25] To this end we prepared **10a-c** as described above. Purified eIF4E was labelled with a Cy5-NHS dye and titrated to different concentrations of m^{7}GTP, m^{7}GpppA, **9**, or **10a-c**, respectively. From these data, we could determine K_d-values using the integrated software.

However, we observed that in different sets of experiments the K_d-values varied depending on details of the protein labelling procedure and the measuring mode. This limits the accuracy of the absolute K_d-values obtained by microscale thermophoresis, as recently documented in detail.[26] Nevertheless, a reliable comparison of binding affinities of different cap analogues is possible, although the absolute numbers obtained for the K_d-values should not be over interpreted. Representative thermophoresis measurements are given in the supplementary information (ESI Table S1 and Figure S23).

In a comparative evaluation of all modified cap analogues used in this study, binding tests of eIF4E with m^{7}GTP and m^{7}GpppA clearly showed the lowest K_d-values, as would be expected for the natural binding partners. The dissociation constant of m^{7}GTP (~ 1 µM) was lower than for m^{7}GpppA (~ 5 µM), confirming previous reports based on different methods.[27] For **10a-b**, the K_d-values for binding to eIF4E were markedly increased, namely in the range of ~50 µM (Figure 4). For **9** and **10c**, however, no binding to eIF4E could be measured (ESI Figure S23). For **9**, this is in accordance with the literature (reported values for m^{7}GpppG range from 0.2 to 2.5 µM)[28] and for **10c** this explains why no crosslinking of **10c**-RNA with eIF4E could be seen. Measurements were also performed using eIF4E from *D. melanogaster*, giving comparable K_d-values and thereby confirming our thermophoresis experiments obtained with *S. cerevisiae* eIF4E (data not shown). Based on these data, we conclude that the modifications in **10a-RNA** and **10b-RNA** impair binding to eIF4E, while in **10c-RNA** binding is abolished. At high concentrations of eIF4E there seems to be enough **10a-b**-RNA bound eIF4E to obtain crosslinking, indicating that the crosslinking groups per se are functional.

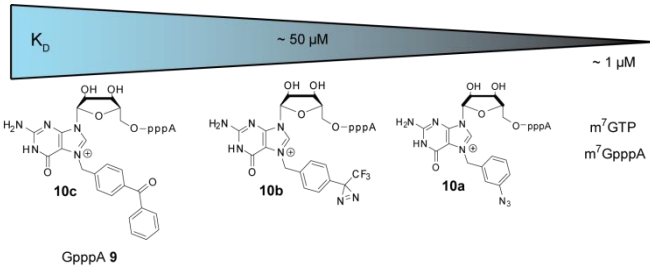

Figure 4. Trend of dissociation constants for the interaction of eIF4E with different *N7*-modified cap analogues determined by microscale thermophoresis. As controls, binding of eIF4E to m^{7}GpppA, m^{7}GTP (positive controls), and GpppA (negative control) was determined.

Conclusions

We herein described the chemical synthesis of three novel AdoMet analogues with photo-crosslinking properties. The photo-crosslinking moieties were enzymatically transferred to the *N7* position of the mRNA cap with high efficiencies. Diazirine- and aryl azide-modified cap analogues **10a-b** retained

the ability to bind to the cap binding protein eIF4E, whereas **10c** was not bound. We also produced 24 nt long model mRNAs with the differently modified caps and performed photo-crosslinking studies to eIF4E. Herein, crosslinking of **10a-b**-RNAs with eIF4E was observed, whereas **10c**-RNA showed no crosslinking, in line with the binding constants. Since the wavelength required for photo-crosslinking is longer and thus less damaging for the diazirine compared to the aryl azide, the AdoMet analogue **1b** is currently the best choice for enzymatic transfer and photo-crosslinking to a directly interacting protein.

Of course, the direct modification of a protein binding site (i.e. the *N7* position of the cap for eIF4E used in this study) is not an ideal system for photo-crosslinking studies but should rather be regarded as a proof of concept. We anticipate that our photo-crosslinking approach will be particularly useful if sites adjacent to the protein binding-site of interest can be enzymatically modified. This can be achieved by smart choices of MTase and target site or by developing new AdoMet analogues causing less steric hindrance.

Experimental Section

Experimental details can be found in the supplementary information.

Acknowledgements

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Keywords: AdoMet analogue • RNA labelling • photo-crosslinking • RNA-protein interactions

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