Supporting Information

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Quantum Chemical Calculations and Experimental Validation of the Photoclick Reaction for Fluorescent Labeling of the 5’ cap of Eukaryotic mRNAs

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Supporting Information

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**Experimental Section**

**Materials**
The cap analog m<sup>7</sup>GpppA was purchased from NEB. S-adenosyl-L-homocysteine, allyl bromide, mesyl chloride, aniline, sodium nitrite, p-methoxybenzaldehyde and phenylsulfonylhydrazine were obtained from Sigma-Aldrich, (Z/E)-2-penten-4-yn-1-ol was from Alfa Aesar and methyl terephthalaldehyde was purchased from TCI. The protease inhibitor cocktail “cOmplete mini” was purchased from Roche diagnostics. MF<sup>TM</sup>-Membrane Filters (0.025 µm VSWP) were obtained from Roth and Amicon®Ultra-15 centrifugal filters (Regenerated Cellulose, MWCO 10,000) from Millipore. The enzymes were purified via HisTrap™ FF 1 columns on an ÄKTA purifier™ system (GE Healthcare). HPLC analysis of enzymatic conversions as well as purification of AdoMet-analogs were performed on an Agilent 1260 Infinity HPLC equipped with a Diode Array Detector (190–640 nm) using NUCLEODUR<sup>®</sup> C18 columns as indicated from Macherey-Nagel. Fluorescence images of PAA-gels were recorded on a VersaDoc Gel Imager (biorad). Proton nuclear magnetic resonance spectra (1<sup>1</sup>H NMR) were recorded on a Bruker 300 MHz instrument. The chemical shifts (δ) were reported in ppm relative to TMS or deuterated solvents as internal standard (δ<sub>H</sub>: CHCl<sub>3</sub> 7.26 ppm). Coupling constants are expressed in Hz. HPLC-ESI-TOF measurements were performed on Bruker maXis ESI-Q-TOF coupled with Dionex Ultimate 3000 UPLC. ESI-TOF mass spectra for analysis of tetrazoles were recorded on an Agilent 6224 instrument.

**Synthesis of 5'-[(R/S)(3S)-3-amino-3-carboxypropyl]prop-2-enylsulfonio]-5'-deoxyadenosine (AdoPropen, 2b) and 5'-[(R/S)(3S)-3-amino-3-carboxypropyl]pent-2-en-4-ynylsulfonio]-5'-deoxyadenosine ((E/Z)-AdoEnYn, 2c)**

Both AdoMet analogs were prepared as described earlier.<sup>1–3</sup> In detail, AdoPropen 2b was synthesized according to Dalhoff et al.<sup>2</sup>, (E/Z)-AdoEnYn 2c (termed AdoEnYn in the following sections) was prepared as described by Peters et al.<sup>3</sup> The AdoMet analogs were purified by reversed-phase HPLC using a NUCLEODUR<sup>®</sup> C18 Pyramid column (5 µm, 125x10 mm). Elution was performed at a flow rate of 5 mL/min using a gradient of acetonitrile containing 0.01 % (v/v) TFA from 0 to 7 % (v/v) within 15 min, using water with 0.01 % (v/v) TFA as eluent A.

**Syntheses of tetrazoles**

Syntheses of tetrazoles were performed according to Ito et al.<sup>4</sup> Purified products were characterized by ESI-MS as well as 1H-NMR.

**Expression and purification of GlaTgs2-V34A, MTAN and LuxS**

MTAN and LuxS were produced and purified as described previously.<sup>5</sup>
The open-reading frame encoding GlaTgs2 (codon optimized for E. coli) was purchased from GeneArt and cloned in pRSET-A using BamHI and XhoI. The construct pRSET-GlaTgs2 was used for recombinant production of the enzyme from E. coli Tuner DE3 pLacI cells. Cells were grown in 5 mL 2YT-medium supplemented with Ampicillin (100 µg/mL) and glucose (7.3 g/L) overnight at 37 °C. For protein production 2YT-medium, supplemented as described above, was inoculated (v/v) with 2.5% (v/v) overnight culture and cells were allowed to grow for 4 h at 37 °C at constant shaking, before they were cooled to room temperature. Expression of GlaTgs2 was induced by 0.32 mM IPTG and 2% (v/v) ethanol and performed for 20 h at 17 °C with constant shaking. Cells were harvested by centrifugation and pellets were stored at -20 °C.

The enzyme was purified according to Hausmann et al. with only slight modifications. In detail, cells of 200 mL expression culture were resuspended in lysis buffer (200 mM NaCl, 10% (v/v) glycerol, 50 mM Tris, pH 8.0) and 500 µL cOmplete mini protease inhibitor cocktail (Roche diagnostics, 1 tablet per 1.5 mL ddH2O) was added before cells were lysed by sonication. Purification was performed on an ÄKTA purifier™ system with HisTrap™ FF 1 columns (GE Healthcare) and lysis buffer containing 500 mM imidazole was used as eluent. Fractions, containing purified protein were pooled and transferred in dialysis buffer (100 mM NaCl, 10% (v/v) glycerol, 2 mM DTT, 1 mM EDTA, 50 mM Tris, pH 8.0) using Amicon® Ultra-15 centrifugal filters (Regenerated Cellulose, MWCO 10,000). Purified protein was stored at -80 °C.

Enzymatic modification of cap analog m^7GpppA

**Synthesis of P^1-adenosine(5')-P^3-[N^2-prop-2-enyl,7-methylguanosine(5')] triphosphate (3b) and P^1-adenosine(5')-P^3-[N^2-pent-2-en-4-ynyl,7-methylguanosine(5')] triphosphate (3c)**

Enzymatic conversion of m^7GpppA (1, 275 µM or 1 mM) with AdoPropen (2b, 0.5-1 mM) or AdoEnYn (2c, 360-740 µM) by GlaTgs2-V34A (5-120 µM) was performed in the presence of 4 µM MTAN and 3 µM LuxS in PBS (pH 7.4) at 37 °C for 4 hours. Reactions were stopped by heating samples for 25 min at 68 °C, followed by dialysis against PBS buffer for 20 min. Enzymatic conversions were analyzed by HPLC after protein precipitation with 1 M HClO₄ (10% (v/v)) on an analytical NUCLEODUR® C18 Pyramid column (5 µm, 125x4 mm) as described earlier.

**Photoclick reaction**

For fluorescent labeling of the mRNA-cap by photoclick reactions, 8 µL of the bioconversion (see above, containing ca. 800 µM 3b or ca. 200 µM 3c as analyzed by HPLC) were mixed with 8 µL acetonitrile and tetrazoles (final concentrations of 617 µM for tetrazole 4 and 1.23 mM for tetrazole 5) in a black 384-well plate. Samples were mixed by pipetting and irradiated for 5 min with a hand-held UV lamp at 254 nm. Afterwards samples were transferred into reaction vials and wells were washed with 2.5 µL acetonitrile, which was added to reactions. The photoclick labeling was allowed to
proceed at 4 °C for up to 20 h. Afterwards samples were loaded on 20% denaturing PAA-gels (using 2x loading buffer without tracking dyes) and separated at 10 W for 50 min. Analysis was performed by irradiating gels with a hand-held UV lamp at 365 nm and detecting in-gel fluorescence with a Canon Eos D1100 respectively an Exilim 12.5x camera.

**HPLC-ESI-TOF-MS analysis**
Mass analyses of photoclick reactions were performed by HPLC-ESI-MS on an Agilent 6224 ESI-TOF-Systems coupled to an Agilent HPLC 1200 by the MS facility of the university of Hamburg. Samples were applied on a NUCLEODUR® Hilic column (5 μm, 4x125 mm) and after an isocratic step using 10 % (v/v) 20 mM ammoniumacetate (pH 5.3) and 90 % (v/v) acetonitrile for 5 min, components were eluted by a gradient to 90 % (v/v) ammoniumacetate in 13 min at a flow rate of 0.6 mL/min.

**KS-DFT calculations**
Total energies, energy gradients and molecular orbitals were calculated within a Kohn-Sham density functional theory (KS-DFT) framework[8] using the Turbomole quantum chemistry program package[9,10]
Molecular structures were optimized using a BP86 exchange-correlation functional[11,12] in combination with the resolution-of-the-identity approach[13] and Ahlrichs's triple-zeta split-valence basis set with polarization functions on all atoms, def-TZVP[14]
The convergence criteria for the self-consistent-field algorithm were set to a maximum change in total energy of 10^{-7} a.u., and for the molecular structure optimization to a maximum change of the energy gradient of 10^{-4} a.u. Additional single-point calculations were carried out using the B3LYP functional[15,16] instead of BP86. Molecular orbitals were plotted with an isosurface value of 0.06 using Molden[17,18] in combination with Povray[19].
Supplementary Figures and Tables

Supplementary Figure 1 Structural formulas of substrates used for KS-DFT-calculations. 4a) Nitrile imine generated from tetrazole 4. 5a) Nitrile imine generated from tetrazole 5. 8) Structure of \( [N^2\text{-prop-2-eny}-7\text{-methylguanosine}(5')] \) triphosphate (\( N^2\text{-allyl-m}\text{'GTP} \)) deprotonated at α-phosphate. 12) Structure of \( [N^2\text{-pent-2-en-4-yny}-7\text{-methylguanosine}(5')] \) triphosphate (\( N^2\text{-pentenyl-m}\text{'GTP} \)) deprotonated at α-phosphate.

Supplementary Figure 2 Fluorescence spectra of pyrazoline cycloadducts formed by reaction of tetrazoles 4 or 5 with acrylamide. A) Tetrazoles 4 and 5 were irradiated at 254 nm for 10 min in presence (Rct) or absence (NC) of acrylamide. Fluorescence emission spectra of samples were recorded at an excitation wavelength of 365 nm. B) Photography of samples upon irradiation with hand-held UV lamp at 365 nm. C) Structural formulas of expected pyrazolines 3-(4-methoxyphenyl)-1-phenyl-4,5-dihydro-1H-pyrazol-5-yl)carboxamid 10 and methyl-4-(5-aminocarbonyl-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)benzoate 11.
Supplementary Figure 3 Mass analysis of photoclick reaction using tetrazole 4 and $N^2$-allyl-m$^7$GpppA 3b as substrates. The cap-analog m$^7$GpppA 1 was converted to $N^2$-allyl-m$^7$GpppA 3b by the GlaTgs2-variant using AdoPropen 2b as cosubstrate. Allyl-modified cap 3b was subsequently used as substrate in photoclick reaction with tetrazole 4. The reaction was analyzed by HPLC-ESI-MS and masses corresponding to m$^7$GpppA 1 (expected [M]$^+ =$ 787.10 m/z; found [M]$^+ =$ 787.10 m/z), $N^2$-allyl-m$^7$GpppA 3b (expected [M]$^+ =$ 827.13 m/z; found [M]$^+ =$ 827.13 m/z), as well as $P^3$-adenosine(5')-$P^3$-[N$^2$-(3-(4-methoxyphenyl)-1-phenyl-4,5-dihydro-1H-pyrazol-5-yl)-methyl],7-methylguanosine-(5')triphosphate 6 (expected [M]$^+ =$ 1051.23 m/z; found [M]$^+ =$ 1051.23 m/z), were detected.
Supplementary Figure 4  Analysis of photoclick reaction of tetrazole 4 with \( N^2\)-allyl-m\(^7\)GpppA 3b including a control (lane 5) showing 4 irradiated in the absence of a dipolarophile. The photoclick reaction of 4 using 3b as dipolarophile was performed (lane 1) and two fluorescing bands were observable after PAGE (20% denat. gel) upon irradiation with UV light (365 nm). The lower band was also be detected in controls (lanes 2-4) as well as in the control containing only tetrazole (lane 5).

Supplementary Figure 5  Photoclick reaction of \( N^2\)-allyl-m\(^7\)GpppA 3b with tetrazole 5. A) Structural formula of expected photoclick product \( P^1\)-adenosine*(5')*-\( P^3\)-([\( N^2\)-3-(4-methoxycarbonylphenyl)-1-phenyl-4,5-dihydro-1H-pyrazol-5-yl]-methyl][7-methylguanosine*(5')]-triphosphate 7 (R\(_1\)= pppA). B) The alkenylated cap \( N^2\)-allyl-m\(^7\)GpppA 3b was used as substrate in photoclick reactions with tetrazole 5. Samples were analyzed after PAGE (20% denat. gel) for fluorescing products upon irradiating with UV light (365 nm). Nucleotides were detected by UV shadowing (254 nm). The reaction containing all components (lane 1) shows an additional green fluorescent band (arrow), which is absent in negative controls and probably corresponds to the expected product 7. CS is cosubstrate (2b).

Supplementary Figure 6  Determining detection limit of photoclick product and fluorescent labeling of alkenylated cap. In the bioconversion of m\(^7\)GpppA 1 to \( N^2\)-allyl-m\(^7\)GpppA 3b by the GlaTgs2-variant with 2b as cosubstrate, 1 was used in concentrations as indicated and the bioconversions were subsequently used for photoclick reactions. A fluorescent signal is observable down to a concentration of 0.25 mM 1 after PAGE (20% denat. gel) and illumination at 365 nm. UV Shadowing was performed to validate decreasing concentration of 1 (254 nm).
Characterization of 4 (5-(4-Methoxyphenyl)-2-phenyl-2H-tetrazole)

$^1$H-NMR (300 MHz, CDCl$_3$) $\delta$ 8.20 (d, $J=9.0$ Hz, 4H), 7.63-7.50 (m, 3H), 7.08-7.04 (d, $J=9.0$ Hz, 2H), 3.91 (s, 3H); ESI-MS calcd for C$_{16}$H$_{13}$N$_4$O$^+$ 253.11 [M+H]$^+$, found 253.11.

Characterization of 5 (Methyl 4-(2-phenyl-2H-tetrazol-5-yl)benzoate)

$^1$H-NMR (300 MHz, CDCl$_3$) $\delta$ 8.36 (d, $J=8.3$ Hz, 2H), 8.26-8.19 (m, 4 H), 7.65-7.51 (m, 3 H), 3.98 (s, 3H); ESI-MS calcd for C$_{16}$H$_{13}$N$_4$O$_2^+$ 281.10 [M+H]$^+$, found 281.10.

**Supplementary Table 1.** Calculated B3LYP MO energies of photoclick educts. B3LYP MO energies were obtained from single-point electronic structure calculations carried out on top of the BP86-optimized structures.

|       | $E$(HOMO)/eV | $E$(LUMO)/eV |
|-------|--------------|--------------|
| 4a    | -5.00        | -1.48        |
| 5a    | -5.44        | -2.39        |
| 8     | -8.72 (HOMO-12) | -1.51 (LUMO+2) |
| acrylamide | -7.82 (HOMO-2) | -1.33        |
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Competing financial interests
A. R. and D. S. filed a German patent application (Aktenzeichen 10 2012 222 675.3) entitled “Mittel und Verfahren zur Modifizierung der 5’-Kappe von RNA”.