Supporting Information

A Benzophenone-Based Photocaging Strategy for the N7 Position of Guanosine

Lea Anhäuser, Nils Klöcker, Fabian Muttach, Florian Mäsing, Petr Špaček, Armido Studer, and Andrea Rentmeister*

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Supporting Information
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**Experimental Procedures**

**General Information**

All chemicals were purchased from Alfa Aesar, Applichem, Fluorochem, Sigma Aldrich or TCI unless otherwise noted. The HPLC grade acetonitrile was purchased from VWR. All components were used without further purification.

G(5′)ppp(5′)A (GpppA) cap analogue and G(5′)ppp(5′)G (GpppG) cap analogue were purchased from New England Biolabs or Jena Bioscience.

The short RNA with the sequence 5′ UUGUU 3′ (purification: HPLC) was purchased from Biolegio, the short RNAs with the sequences 5′ UUUCUUUGUAUAUCUUUC 3′, 5′ UUUCUUUGUAUAUCUUUC 3′ and 5′ CACACGAACUAAGGUAGGC 3′ (all purification: HPLC) were purchased from biomers.net.

The short strand DNA from a M. TaqI recognition site, underlined with the sequence 5′CTTGGAGCCACTA CGCATCGG3′ and 3′GAACCTCGGTGATAGCTGATGCCTGTACC5′ (purification: desalination) were purchased from Biolegio.

Phusion High-Fidelity DNA Polymerase, FastAP Thermosensitive Alkaline Phosphatase, Ribolock RNA Inhibitor, T7 RNA polymerase, pyrophosphatase, DNase I were purchased from Thermo Scientific, nuclease P1 from Sigma Aldrich, Snake Venom Phosphodiesterase from MP Biomedicals, TaqI Methyltransferase (M. TaqI) and restriction enzyme TaqI (R. TaqI) were purchased from New England Biolabs, Ecm1, MTAN and LuxS were recombinantly expressed and purified as previously described.[1]

The plasmid pBR322 was purchased from New England Biolabs. NTP bundle was purchased from Jena Bioscience.

1H and 13C NMR spectra were measured at 299 K on a Bruker Avance II 300, an Agilent DD2 500 or Agilent DD2 600 spectrometer. The chemical shifts (δ) were reported in ppm relative to deuterated solvents as internal standard (δH: CDCl3 = 7.26, DMSO-δ6 = 62.50 ppm, D2O = 4.79 and δC: CDCl3 = 77.16, DMSO-δ6 = 39.52 ppm). Multiplicities are indicated as s (singlet), d (doublet), dd (doublet of doublets), ddd (doublet of doublet of doublets), dt (doublet of triplet) t (triplet), q (quartet) and m (multiplet).

MALDI spectra were recorded on a Bruker Daltonics Autoflex Speed MALDI-TOF instrument. High-resolution mass spectra were recorded on a Thermo Scientific Orbitrap LTQ XL or on a Bruker MicroTof instrument. High-resolution mass spectra of UHPLC-MS measurements were performed on a Bruker maXis II ultra-high resolution QTOF coupled to a Thermo Scientific UltiMate 3000 UHPLC using a Nucleodur® C18 Pyramid reversed-phase column (5 µm, 125 × 2 mm) from Macherey-Nagel. Elution was performed at a flow rate of 0.6 mL/min applying a linear gradient for buffer A (7 mM ammonium formiate and 12 mM formic acid, pH = 3.5) and buffer B (MeOH).

HPLC analysis and purification of cap analogues were performed on an Agilent 1260 Infinity HPLC equipped with a diode array detector (DAD) (190-640 nm) using a Nucleodur® C18 Pyramid reversed-phase column (5 µm, 125 × 4 mm) from Macherey-Nagel. Elution was performed at a flow rate of 1 mL/min applying a linear gradient for buffer A (50 mM ammonium acetate, pH = 6.0) and buffer B (1:1 buffer A : acetonitrile).

Semi-preparative HPLC purification of AdoMet analogues was performed on an Agilent 1260 Infinity HPLC using a Nucleodur® C18 Pyramid reversed-phase column (5 µm, 125 × 10 mm) from Macherey-Nagel. Elution was performed at a flow rate of 5 mL/min applying a linear gradient for buffer A (50 mM ammonium acetate, pH = 6.0) and buffer B. (acetonitrile with 0.01% trifluoroacetic acid).

Preparative HPLC purification of guanosine derivatives was performed on a Büchi PrepChrom C-700 V5 using a VarioPrep reverse-phase column (5 µm, 250 × 21 mm) from Macherey-Nagel. Elution was performed at a flow rate of 12 mL/min applying a linear gradient for buffer A (50 mM ammonium acetate, pH = 6.0) and buffer B (acetonitrile).

RNA sizing was performed using the ladder 20/100 from Integrated DNA Technologies. DNA sizing was performed using the ladder GeneRuler™ 1kb DNA Ladder from Thermo Fisher Scientific.

IR spectra were recorded on a Digilab FTS 3100 spectrometer combined with a Specac MKII Golden Gate Single Reflection ATR System. Signals were given in wavenumbers (cm⁻¹). Intensities were abbreviated with (vs) very strong, (s) strong, (m) medium, (w) weak, (vW) very weak and (br) broad.

For preparation of HeLa cell lysate HeLa cells were cultured in MEM Earle’s media (Merck) supplemented with L-glutamine (2 mM), non-essential amino acids (1%), penicillin and streptomycin (1%), and fetal calf serum (FCS, 10%) under standard conditions (5% CO₂, 37 °C). 24 h prior to cell lysis, 3 x 10⁶ cells were seeded on a Petri dish (90 mm). For cell lysis, the cell supernatant was discarded and
the cells were washed with 1× PBS, lysed with CelLytic™-M reagent (1.5 mL, Sigma Aldrich) according to the manufacturer's instructions and stored at -80 °C.

UV-A LED (3 W, λ_{max} = 365 nm) was used for the irradiation of cap analogues (see Figure S1A). The LED was purchased by LED Engin and installed in a custom made LED box (see Figure S2A). The temperature inside the box was regulated to 23°C using a fan.

UV-A LED (3 W, λ_{max} = 365 nm) was used for the irradiation of nucleoside derivatives (see Figure S1B). The LEDs were purchased from Avonec and installed in a custom made LED box (see Figure S2B). The fan regulated the temperature to no more than 9 °C above room temperature inside the box. A stirring plate installed under the LED box allowed mixing of the reaction components during irradiation. The Glorius working group (WWU Münster) provided this LED box.

An UV-transilluminator (312 nm, Intas GDS) and a polystyrene plate to remove short wavelength UV light (<290 nm) was used for the irradiation of cap analogues.
General synthesis of N7-modified guanosines (GS1)

\[ \text{1} \quad \text{DMSO} \quad 24 \text{ h, rt} \quad \text{2a-c} \quad \text{3a-c} \]

The products 3a-c were synthesized following a modified literature procedure by Pautus and coworkers.\textsuperscript{[2]}

To a 4-mL reaction vessel equipped with a magnetic stirring bar guanosine 1 (142 mg, 0.5 mmol, 1.0 equiv.) and the corresponding bromide 2a-c (0.6 mmol, 1.2 equiv.) were added. DMSO (2.5 mL) were added and the solution was vigorously stirred for 24 h at room temperature. The reaction mixture was lyophilized, and the raw product was taken up in 800-1200 µL of a mixture of ddH\textsubscript{2}O and acetonitrile and isolated by preparative HPLC. The collected product fractions were lyophilized.

2-Amino-9-((2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-7-(2-nitrobenzyl)-6-oxo-6,9-dihydro-1H-purin-7-ium (N7-ONB-guanosine, 3a)

Prepared from guanosine 1 and 1-((1-bromomethyl)-2-nitrobenzene 2a (130 mg) following the GS1 to obtain the desired product 3a (165 mg, 0.266 mmol, 53%) as white solid.

\[ \text{3a-c} \]

\textsuperscript{1}H-NMR (600 MHz, DMSO-d\textsubscript{6}, 299 K): \delta [ppm] = 9.29 (s, 1H, H-8), 8.16 (dd, 1H, H-13, \text{J}_{13,14} = 8.2 \text{ Hz}, \text{J}_{13,15} = 1.3 \text{ Hz}), 7.73 (ddd, 1H, H-15, \text{J}_{13,15} = \text{J}_{15,16} = 7.6 \text{ Hz}, \text{J}_{14,15} = 1.3 \text{ Hz}), 7.63 (ddd, 1H, H-14, \text{J}_{13,14} = \text{J}_{14,15} = 7.6 \text{ Hz}, \text{J}_{14,16} = 1.3 \text{ Hz}), 7.22 (dd, 1H, H-16, \text{J}_{13,14} = 7.8 \text{ Hz}, \text{J}_{16,17} = 1.3 \text{ Hz}), 5.97 (s, 2H, H-10), 5.85 (d, 1H, H-1\text{a}), 4.44 (dd, 1H, H-2\text{a}, \text{J}_{2,3} = 3.7 \text{ Hz}, \text{J}_{2,1} = 4.6 \text{ Hz}), 4.13 (dd, 1H, H-3\text{a}), \text{J}_{2,3} = 4.7 \text{ Hz}, \text{J}_{2,1} = 5.5 \text{ Hz}), 3.99 (dt, 1H, H-4\text{a}, \text{J}_{4,3} = 3.2 \text{ Hz}), 3.68 (dd, 1H, H-5\text{a}, \text{J}_{5,6} = 3.2 \text{ Hz}, \text{J}_{5,5a} = 12.3 \text{ Hz}), 3.57 (dd, 1H, H-5b, \text{J}_{5,5b} = 3.2 \text{ Hz}, \text{J}_{5,6b} = 12.3 \text{ Hz}), 1.78 (s, 10H, H-1\text{b}).

* Due to the preparative purification in 50 mM ammonium acetate buffer, the integral of H-17 does not correspond to 3 protons, the yield was adjusted accordingly.

\textsuperscript{13}C-NMR (151 MHz, DMSO-d\textsubscript{6}, 299 K): \delta [ppm] = 173.6 (C-18), 161.2 (C-2), 159.6 (C-6), 149.6 (C-4), 147.3 (C-12), 134.6 (C-15), 134.2 (C-8), 130.4 (C-11), 129.6 (C-14), 129.1 (C-16), 125.1 (C-13), 107.6 (C-5), 89.4 (C-1), 85.7 (C-4), 74.2 (C-2), 69.3 (C-3), 60.4 (C-5), 48.5 (C-10), 22.9 (C-17).

HR-MS (ESI): \text{m/z} calculated for [(C\textsubscript{7}H\textsubscript{30}N\textsubscript{3}O\textsubscript{7})\textsuperscript{+}]: 419.1310, found: 419.1324.

2-Amino-9-((2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-7-(4-nitrobenzyl)-6-oxo-6,9-dihydro-1H-purin-7-ium (N7-PNB-guanosine, 3b)

Prepared from guanosine 1 and 1-((1-bromomethyl)-4-nitrobenzene 2b (130 mg) following the GS1 to obtain the desired product 3b (130 mg, 0.28 mmol, 56%) as white solid.
**SUPPORTING INFORMATION**

1H-NMR (600 MHz, D$_2$O, 299 K): δ [ppm] = 8.15 (d, 1H, H-13), 3$\tilde{J}_{12,13} = 8.9$ Hz), 7.49 (d, H-12), 3$\tilde{J}_{12,13} = 9.0$ Hz), 5.96 (d, 1H, H-1', 3$\tilde{J}_{1,2} = 3.4$ Hz), 5.68 (s, 1H, H-10), 4.59 (dd, 1H, H-2', 3$\tilde{J}_{1,2} = 5.0$ Hz, 3$\tilde{J}_{1,3} = 3.5$ Hz), 4.26 (dd, 1H, H-3', 3$\tilde{J}_{1,3} = 6.0$ Hz, 3$\tilde{J}_{2,3} = 5.0$ Hz), 4.16 (dd, 1H, H-4', 3$\tilde{J}_{2,3} = 6.1$ Hz, 3$\tilde{J}_{1,2} = 3.1$ Hz, 3$\tilde{J}_{1,5} = 3.1$ Hz), 3.85 (dd, 1H, H-5'a, 3$\tilde{J}_{1,5} = 12.9$ Hz, 3$\tilde{J}_{1,9} = 2.8$ Hz), 3.72 (dd, 1H, H-5'b, 3$\tilde{J}_{1,9} = 12.9$ Hz, 3$\tilde{J}_{4,5} = 3.5$ Hz), 1.82 (s, 2H, H-15').

* Due to the preparative purification in 50 mM ammonium acetate buffer, the integral of H-15 does not correspond to 3 protons, the yield was adjusted accordingly.

13C-NMR (151 MHz, D$_2$O, 299 K): δ [ppm] = 181.3 (C-16), 157.0 (C-6'), 156.3 (C-2'), 149.81 (C-4'), 147.8 (C-14), 140.53 (C-11), 124.2 (C-12), 128.9 (C-13), 108.2 (C-5), 90.3 (C-1'), 85.3 (C-4'), 74.1 (C-2'), 69.0 (C-3'), 60.0 (C-5'), 51.6 (C-10), 23.1 (C-15).

*Signals could not be unambiguously assigned.

HR-MS (ESI): m/z calculated for [(C$_2$H$_7$N$_4$O)$_2$]+: 419.1310, found: 419.1323.

2-Amino-7-(4-benzoylbenzyl)-9-(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-6-oxo-6,9-dihydro-1H-purin-7-ium (N7-BP-guanosine, 3c)

Prepared from guanosine 1 and 4-(bromomethyl)benzophenone 2c (165 mg) following the GS1 to obtain the desired product 3c (137 mg, 0.266 mmol, 53%) as white solid.

1H-NMR (500 MHz, DMSO-d$_6$, 299 K): δ [ppm] = 9.30 (s, 1H, H-8), 7.75-7.71 (m, 4H, H-17, H-18), 7.70-7.65 (m, 1H, H-19), 7.65-7.62 (m, 2H, H-12), 7.57-7.53 (m, 2H, H-13), 5.82 (dd, 1H, H-2', 3$\tilde{J}_{1,2} = 4.6$ Hz), 5.77 (s, 2H, H-10), 4.50 (dd, 1H, H-2'), 3$\tilde{J}_{1,2} = 4.7$ Hz, 4.13 (dd, 1H, H-3', 3$\tilde{J}_{1,3} = 4.6$ Hz, 3$\tilde{J}_{1,9} = 4.6$ Hz), 4.00 (m, 1H, H-4'), 3.70 (dd, 1H, H-5'a, 3$\tilde{J}_{1,9} = 3.3$ Hz, 3$\tilde{J}_{4,5} = 12.3$ Hz), 3.58 (dd, 1H, H-5'b, 3$\tilde{J}_{4,5} = 3.3$ Hz, 3$\tilde{J}_{4,5} = 12.3$ Hz), 1.84 (s, 2H, H-20').

* Due to the preparative purification in 50 mM ammonium acetate buffer, the integral of H-20 does not correspond to 3 protons, the yield was adjusted accordingly.

13C-NMR (126 MHz, DMSO-d$_6$, 299 K): δ [ppm] = 195.3 (C-15), 173.0 (C-21), 162.6 (C-6), 161.7 (C-2), 149.5 (C-4), 140.1 (C-16), 136.9 (C-11), 136.8 (C-14), 132.8 (C-19), 132.5 (C-8), 130.6 (C-17), 129.7 (C-18), 128.6 (C-12), 128.3 (C-13), 107.9 (C-5), 89.1 (C-1'), 86.6 (C-4'), 73.7 (C-2'), 69.7 (C-3'), 60.7 (C-5'), 50.2 (C-10), 22.2 (C-20).

HR-MS (ESI): m/z calculated for [(C$_2$H$_2$N$_4$O)$_2$]+: 478.1721, found: 478.1737.
The product 7 was synthesized following a modified literature procedure by Pautus and coworkers.\[^2\]
To a 4-mL reaction vessel equipped with a magnetic stirring bar adenosine 6 (134 mg, 0.5 mmol, 1.0 equiv.) and 4-(bromomethyl)benzophenone 2c (165 mg, 0.6 mmol, 1.2 equiv.) were added. DMSO (2.5 mL) were added and the solution was vigorously stirred for 24 h at room temperature. The reaction mixture was lyophilized, and the raw product was taken up in 4 mL ddH\(_2\)O, acetonitrile and methanol and isolated by preparative HPLC. The collected product fractions were lyophilized and the desired product 7 (108 mg, 0.225 mmol, 45%) was obtained as white solid.

\[^{1}\text{H} \text{ NMR (600 MHz, DMSO-}\text{d}_6, 299 \text{ K}): [\delta \text{ ppm}] = 8.32 (s, 1H, H-2), 8.19 (s, 1H, H-8), 7.73-7.70 (m, 4H, H-17, H-18), 7.68-7.65 (m, 1H, H-19), 7.56-7.53 (m, 2H, H-13), 7.52-7.50 (m, 2H, H-12), 5.78 (d, 1H, H-1'), \text{J}_{1',2'} = 5.9 \text{ Hz}, 5.35 (s, 1H, H-10), 4.48 (dd, 1H, H-2', \text{J}_{2',3'} = 5.9 \text{ Hz}, \text{J}_{2',4'} = 5.0 \text{ Hz}), 4.12 (dd, 1H, H-3', \text{J}_{3',2'} = 5.0 \text{ Hz}, \text{J}_{3',4'} = 3.4 \text{ Hz}), 3.93 (dt, 1H, H-4', \text{J}_{4',3'} = 3.8 \text{ Hz}), 3.64 (dd, 1H, H-5'a, \text{J}_{5'a,6} = 4.0 \text{ Hz}, \text{J}_{5'a,5b} = 12.0 \text{ Hz}), 3.54 (dd, 1H, H-5'b, \text{J}_{5'b,4} = 4.0 \text{ Hz}, \text{J}_{5'b,5a} = 12.0 \text{ Hz}), 1.84 (s, 0.8H, H-20)*\]

Due to the preparative purification in 50 mM ammonium acetate buffer, the integral of H-20 does not correspond to 3 protons, the yield was adjusted accordingly.

\[^{13}\text{C} \text{ NMR (151 MHz, DMSO-}\text{d}_6, 299 \text{ K}): [\delta \text{ ppm}] = 195.4 (C-15), 172.5 (C-21), 153.6 (C-6), 148.5 (C-2), 142.3 (C-16), 141.3 (C-4), 138.0 (C-8), 137.0 (C-14), 136.0 (C-11), 132.7 (C-19), 129.9 (C-18), 129.6 (C-17), 128.6 (C-13), 127.5 (C-12), 123.0 (C-5), 87.6 (C-1'), 85.7 (C-4'), 74.0 (C-2'), 70.4 (C-3'), 61.4 (C-5'), 48.8 (C-10), 21.9 (C-20).

HR-MS (ESI): m/z calculated for [(C\(_{63}H_{98}N_{10}O_7)]^+: 462.1772, found: 462.1768.

Irradiation of N7-ONB-guanosine (3a) and isolation of photo product (4)
N7-ONB-guanosine 3a (10 mg, 16 \mu\text{mol}) was dissolved in 2 mL ddH\(_2\)O and irradiated using a LED (\(\lambda_{\text{max}} = 365 \text{ nm}\)) for 3 h under vigorously stirring. The formation of photo product 4 was followed by analytical HPLC and then the reaction mixture was lyophilized. The raw product was taken up in 800-1000 \mu\text{L} of a mixture of ddH\(_2\)O and acetonitrile and then isolated by preparative HPLC. The collected product fractions were lyophilized and the product 4 (4.0 mg, 7.3 \mu\text{mol}, 46%) was obtained as yellow-orange oil.
Irradiation of N7-PNB-guanosine (3b)

N7-PNB-guanosine 3b (5 mg, 10 μmol) was dissolved in 2 mL ddH2O, irradiated using a LED (λmax = 365 nm) for 1 h under vigorously stirring and further analyzed by HPLC.

Irradiation of N7-BP-guanosine (3c)

N7-BP-guanosine 3c (1.5 mg, 2.9 μmol) was dissolved in 200 μL acetonitrile and 800 μL aqueous solution (ddH2O, buffer (50 mM Tris-HCl (pH 8)), 200 mM NaCl, 1 mM EDTA (pH 8), 1 mM EDTA (pH 8), 200 mM NaCl or 20% glycerol) or 800 μL cell lysate, irradiated using a LED (λmax = 365 nm) for 30 min under vigorously stirring and further analyzed by HPLC.

Irradiation of N1-BP-adenosine (7)

N1-BP-adenosine 7 (1.5 mg, 3.13 μmol) was dissolved in 200 μL acetonitrile and 800 μL ddH2O, irradiated using a LED (λmax = 365 nm) for 30 min under vigorously stirring and further analyzed by HPLC.

Synthesis and purification of AdoMet analogues

AdoMet analogues 9a-d and corresponding precursors were synthesized as previously described.[3]

2-((4-Bromobenzyl)oxy)tetrahydro-2H-pyran (14)

\[
\text{Br}\text{-OH} + \text{CH}_2\text{Cl}_2, 0^\circ\text{C} \rightarrow \text{RT}, \text{4h}
\]

(4-bromomethyl)ethanol 12 (3.00 g, 16.0 mmol, 1.0 equiv.) was dissolved at 0 °C into dichloromethane (20 mL) and 3,4-dihydro-2H-pyran 13 (3.29 g, 32.0 mmol, 2.0 equiv.). After addition of p-toluenesulfonic acid (4.00 mg, 23.2 μmol, 0.15 mol%) the reaction mixture was warmed up to room temperature and then stirred for further 4 h. The reaction was stopped by adding water (30 mL). The reaction mixture was extracted with dichloromethane (3 x 20 mL), the combined organic phases dried over MgSO4 and the solvents were removed under reduced pressure. The crude product was purified by flash column chromatography using silica gel (n-pentane/methyl tert-butyl ether 10:1) to obtain the desired product 14 (4.23 g, 15.6 mmol, 98%) as colorless oil.

1H-NMR (300 MHz, CDCl3, 299 K): δ (ppm) 7.50 (d, J = 8.4 Hz, 2H), 7.27 (d, J = 8.4 Hz, 2H), 4.81-4.68 (m, 2H), 4.49 (d, J = 12.3 Hz, 1H), 3.99-3.86 (m, 1H), 3.63-3.51 (m, 1H), 1.98-1.50 (m, 6H).

13C-NMR (75 MHz, CDCl3, 299 K): δ (ppm) 137.6 (C6), 131.6 (CHaryl), 129.6 (CHaryl), 121.5 (C6), 98.0 (CH), 68.2 (CH2), 62.3 (CH2), 30.7 (CH3), 25.6 (CH2), 19.5 (CH3).

IR (ATR): ν (cm⁻¹) 2942 (m), 2870 (w), 1488 (w), 1387 (vw), 1349 (vw), 1201 (w), 1123 (m), 1069 (m), 1034 (s), 974 (w), 906 (w), 871 (w), 802 (w).

HR-MS (ESI): m/z calculated for [C12H13BrO2Na]+ 293.0148, found: 293.0152.

---

Due to the preparative purification in 50 mM ammonium acetate buffer, the integral of H-11 does not correspond to 3 protons, the yield was adjusted accordingly.

13C-NMR (151 MHz, DMSO-d6, 299 K): δ (ppm) = 172.9 (C-12), 165.2 (C-1)*, 161.3 (C-4), 154.2 (C-3)*, 152.0 (C-2)*, 148.7 (C-10), 135.8 (C-8), 130.0 (C-7), 128.2 (C-9), 128.1 (C-6), 124.3 (C-5), 86.5 (C-1*), 83.9 (C-2*), 73.9 (C-2*), 70.1 (C-3*), 60.9 (C-5*), 22.5 (C-11).

*Signals could not be unambiguously assigned.
SUPPORTING INFORMATION

2-Hydroxy-1-(4-(hydroxymethyl)phenyl)-2-methylpropan-1-one (16)

\[
\begin{align*}
\text{Br} & \quad \text{O} & \quad \text{O} & \quad \text{O} & \quad \text{O} & \quad \text{N} & \quad \text{N} \\
+ & & & & & & \\
\text{Mg, } \text{I}_2, \text{THF, } 80 \degree \text{C, 30 min} & & & & & & \\
\text{15, THF, 90 \degree \text{C, 3.5 h} \rightarrow \text{rt, 16 h} } & & & & & & \\
\text{2 M HCl, rt, 24 h} & & & & & & \\
\rightarrow & & & & & & \\
\text{HO} & \quad \text{O} & \quad \text{HO} & \quad \text{OH} & \quad \text{OH} & \quad \text{OH} \\
\end{align*}
\]

2-((Bromobenzyl)oxy)tetrahydro-2H-pyran 14 (4.00 g, 14.8 mmol, 1.3 equiv.) was dissolved in THF (10 mL) and added to magnesium (1.43 g, 59.0 mmol, 5.2 equiv.). After the addition of I₂ (some grains) the reaction mixture was heated to 80 °C for 30 min and the Grignard reagent was formed. After cooling down to room temperature, the Grignard reagent was added to a solution of 2-(bromomethyl)-2-(tetrahydro-2H-pyran-2-yloxy)propanitrile 15 (1.92 g, 11.3 mmol, 1.0 equiv.) in THF (40 mL). The reaction mixture was further treated for 3.5 h at 90 °C, cooled down to room temperature and stirred overnight. HCl (2 M in water, 30 mL) was added to the reaction mixture and then again stirred overnight. The reaction mixture was extracted with dichloromethane (3 x 20 mL), the combined organic phases dried over MgSO₄ and then again stirred over night. The reaction mixture was extracted with dichloromethane (3 x 20 mL), the organic phases were dried over MgSO₄ and the solvents were removed under reduced pressure. The crude product was purified by flash column chromatography using silica gel (n-pentane/ ethylacetate 1 : 1) to obtain the desired product 16 (720 mg, 3.71 mmol, 33%) as colorless oil.

\text{1H-NMR (300 MHz, CDCls, 299 K): } \delta \text{(ppm) 7.99 (d, } J = 8.4 \text{ Hz, 2H, } 2 \times \text{CH}_2\text{); 7.43 (d, } J = 8.4 \text{ Hz, 2H, } 2 \times \text{CH}_2\text{), 4.75 (s, 2H, CH}_2\text{), 4.11 (s, 1H, OH), 2.31 (s, 1H, OH), 1.61 (s, 6H, 2 x CH}_3\text{).}

\text{13C-NMR (75 MHz, CDCls, 299 K): } \delta \text{(ppm) 204.4 (CO), 146.3 (C_1), 133.0 (C_2), 130.1 (CH}_2\text{)), 126.6 (CH}_2\text{)), 76.5 (C_3), 64.6 (CH}_3\text{), 28.5 (CH}_3\text{).}

\text{IR (ATR): } \nu \text{ (cm}^{-1}) 3375 \text{ (br), 2979 (w), 2873 (w), 1670 (vs), 1608 (m), 1569 (vw), 1460 (w), 1413 (w), 1372 (m), 1265 (m), 1168 (vs), 958 (s), 830 (w), 755 (w).}

\text{HR-MS (ESI): } m/z \text{ calculated for [C}_{12}H_{16}O_3Na]}^+ \text{ 217.0835, found: 217.0834.}

4-(2-Hydroxy-2-methylpropanoyl)benzyl sulfonate (17)

\[
\begin{align*}
\text{HO} & \quad \text{OH} & \quad \text{Cl}_2\text{C}_2\text{O}_2\text{S, NEt}_3 & \quad \text{CH}_2\text{Cl}_2, 0 \degree \text{C, 1 h} & \quad \text{HO} & \quad \text{O} & \quad \text{S}=\text{O} \\
\rightarrow & & & & & & \\
\text{16} & & & & & & \\
\end{align*}
\]

2-Hydroxy-1-(4-(hydroxymethyl)phenyl)-2-methylpropan-1-one 16 (480 mg, 2.47 mmol, 1.0 equiv.) was dissolved in CH₂Cl₂ (10 mL) and methanesulfonyl chloride (283 mg, 2.47 mmol, 1.0 equiv.) and NEt₃ (250 mg, 2.47 mmol, 1.0 equiv.) were added at 0 °C. The reaction mixture was stirred for 1 h and the reaction was stopped by adding 20 mL water. The reaction mixture was extracted with dichloromethane (3 x 20 mL), the combined organic phases dried over MgSO₄ and the solvent was removed under reduced pressure. The crude product was purified by flash column chromatography using silica gel (n-pentane/ ethylacetate 3 : 1) to obtain the desired product 17 (346 mg, 1.27 mmol, 51%) as colorless solid.

\text{1H-NMR (300 MHz, CDCls, 299 K): } \delta \text{(ppm) 8.06 (d, } J = 8.4 \text{ Hz, 2H, 2 x CH}_2\text{), 7.50 (d, } J = 8.4 \text{ Hz, 2H, 2 x CH}_2\text{), 5.28 (s, 2H, CH}_2\text{), 3.80 (s, 1H, OH), 3.01 (s, 3H, CH}_3\text{), 1.62 (s, 6H, 2 x CH}_3\text{).}

\text{13C-NMR (75 MHz, CDCls, 299 K): } \delta \text{(ppm) 204.2 (CO), 138.4 (C_2), 134.8 (C_1), 130.3 (CH}_2\text{)), 128.3 (CH}_2\text{)), 76.7 (C_3), 70.1 (CH}_2\text{), 38.4 (CH}_3\text{), 28.5 (CH}_3\text{).}

\text{IR (ATR): } \nu \text{ (cm}^{-1}) 3513 \text{ (br), 2981 (vw), 2939 (vw), 1677 (m), 1611 (w), 1462 (vw), 1352 (s), 1262 (vw), 1172 (vs), 950 (m), 829 (m), 753 (w).}

Melting point: 58 °C

\text{HR-MS (ESI): } m/z \text{ calculated for [C}_{12}H_{16}O_3SNa]}^+ \text{ 295.0611, found: 295.0601.}
5′-[(R/S)-[3\(S\)]-3-Amino-3-carboxypropyl] 4-(2-hydroxy-2-methyl-propanoyl)-benzylsulfonio]-5′-deoxyadenosine (AdoHAK, 9e)

4-(2-Hydroxy-2-methylpropanoyl)benzylsulfonate 17 (100 mg, 0.29 mmol, 22 equiv.) was dissolved in a 1:1 mixture of formic acid (0.75 mL) and acetic acid (0.75 mL) at 0 °C. S-adenosyl-L-homocysteine 18 (5 mg, 0.013 mmol, 1 equiv.) was added and the reaction was stirred for 5 h at room temperature. Afterwards, ddH₂O (5 mL) was added and the aqueous phase was extracted with diethyl ether. The aqueous phase was lyophilized, redissolved in ddH₂O (1 mL) with 0.01 % trifluoroacetic acid and purified by semi-preparative HPLC. The collected product fractions were lyophilized, the product 9e was taken up in 40 µL ddH₂O and respective aliquots were stored at −20 °C.

HR-MS (ESI): \( m/z \) calculated for C₂₅H₃₃N₆O₇S + = 561.21 [M]+, found: 561.28 (MALDI-TOF analysis).

Enzymatic modification of the GpppA cap analog using Ecm1

For enzymatic modification, GpppA 8 (0.4 mM), AdoMet analogue 9a-e (1.2 mM), AMP (1 mM, optional addition), MTAN (4 µM), LuxS (4 µM) were incubated with Ecm1 (50-70 µM) for 3 h at 37 °C in a final volume of 25 µL. The reaction was stopped by heating to 65°C for 10 min. The denatured enzymes were removed by centrifugation for 10 min at 21130 g and 4 °C and the reaction mixture was analyzed by analytical HPLC or the modified cap analogue was isolated by analytical HPLC. The collected product fractions were lyophilized, the product 10a-d was taken up in 10 µL ddH₂O (by a turnover of 100% GpppA) and further analyzed by HPLC or UHPLC-MS.

Irradiation of N7-PC-GpppA cap analogue

When irradiating non-isolated N7-modified cap analogue, ⅔ of the reaction mixture was diluted to a final volume of 10 µL with ddH₂O for irradiation of 10a-c) or with buffer (for irradiation of 10d), irradiated using a LED (\( \lambda_{\text{max}} = 365 \) nm) and then analyzed by HPLC or UHPLC-MS. Non-isolated N7-modified cap analogue 10e (10 µL) was irradiated using the UV-transilluminator with a polystyrene filter (\( \lambda = 312 \) nm) and then analyzed by HPLC or UHPLC-MS.

When irradiating purified N7-modified cap analogue, 1.5-2.0 µL of isolated approach was diluted to a final volume of 10 µL with ddH₂O for irradiation of 10a-c) or buffer (for irradiation of 10d), irradiated using a LED (\( \lambda_{\text{max}} = 365 \) nm) and then analyzed by HPLC or UHPLC-MS. For further digestion of the photo product of irradiated N7-ONB-GpppA 10a, a complete approach of purified N7-ONB-GpppA 10a was irradiated as described above and the photo product was isolated by analytical HPLC. The collected product fractions were lyophilized, the photo product 11a was taken up in 10 µL ddH₂O and then analyzed by UHPLC-MS.

Enzymatic digestion of cap analogue
SUPPORTING INFORMATION

For enzymatic digestion, GpppA8 (0.5 mM), purified N7-ONB-GpppA 9a (10 µL) or isolated photo product 11a (10 µL) were digested in 7.5 µL digestion buffer (0.11 M Tris-HCl (pH 8.9), 0.11 M NaCl, 15 mM MgCl2) with Snake Venin phosphodiesterase (0.05 U) for 2 h at 37 °C in a final volume of 20 µL. Then, FastAP (1 U) was added and the mixture was incubated for additional 30 min at 37 °C. Enzymes were precipitated with HClO4 (200 mM) by incubation for 10 min at room temperature and further analyzed by UHPLC-MS.

Chemical modification of short RNA

For the chemical modification, short RNA (RNA1: 40 µM, RNA2: 200 µM, RNA3: 200 µM and RNA4: 20 µM) was incubated with bromide 2c (1-2.5 mM) in a ddH2O-DMSO mixture for 24 h at 37 °C in a final volume of 5 µL. Then, the sample was dialyzed for 10 min, the sample volume was adjusted with ddH2O to 20 µL and 20 µL buffer (100 mM Tris-HCl (pH 7.4) and 2 mM EDTA) was added. For irradiation, half of the sample (20 µL) was irradiated using a LED (λmax = 365 nm) for 10 min. The modified RNA was analyzed by 15% denatured PAGE. For UHPLC-MS analysis the modified RNA was digested with nuclease P1 (0.13U) and 5.1 µL 10x P1 buffer (0.2 M NH4OAc, 1 mM ZnCl2) for 1 h at 50 °C and then with FastAP (1 U) for 1 h at 37°C. Enzymes were denatured by incubation for 10 min at 65 °C.

Enzymatic plasmid DNA modification and subsequent UV irradiation

The plasmid pBR322 (0.5 µg) was incubated with AdoBP 9d (4 mM), M. TaqI (20 U), MTAN (4 µM), LuxS (4 µM) in 1x CutSmart buffer (NEB, 50 mM KOAc, 20 mM Tris-OAc, 10 mM MgOAc, 100 µg/mL BSA, pH 7.9) in a total volume of 10 µL for 2 h at 60 °C. For photolabeling, the respective samples were irradiated with UV light (λmax = 365 nm) for 10 min. Or 5 µL buffer (same as for irradiation of cap analogues) were added to the sample and irradiated as described. Afterwards, 15 µL of 1x CutSmart buffer or 10 µL of 1.5x CutSmart buffer for irradiated samples with buffer and R. TaqI (5 U) were added to the reactions and the mixture was incubated for 45 min at 65 °C. Plasmid DNA was linearized by incubation with BamHI for 45 min at 37 °C and deactivated at 50 min at 80 °C. Samples were directly loaded onto a 1% agarose gel (100 V, 1 h). The gel was stained with ethidium bromide and scanned using a Typhoon FLA9500 laser scanner (GE healthcare).

Enzymatic modification of short DNA and subsequent digestion for mass analysis

For the production of short double stranded DNA (dsDNA), the two complementary sDNAs were hybridized in a 1:1 (v/v) mixture at 95 °C for 5 min and cooled to room temperature. dsDNA (500 pmol) was incubated with AdoBP 9d (2.5 mM), M. TaqI (30 U), MTAN (4 µM) and LuxS (4 µM) in 1x CutSmart buffer (NEB) in a total volume of 20 µL for 3 h at 37 °C. Proteinase K (0.6 µU) was added to the reactions followed by incubation for 1 h at 55 °C. Then, nuclease P1 (0.33 U) and FastAP (1 U) were added to the reaction followed by incubation over night at 37 °C. Samples were directly used for UHPLC-MS measurements.

Expression of DcpS

The plasmid pET28b hDcpS H277N was transformed into the Escherichia coli strain BL21 (DE3) and used directly for the inoculation of the 30 ml LB-kanamycin culture. The culture was grown overnight at 37°C with constant shaking at 200 rpm. Five 400 ml LB-cultures were each inoculated with 5 ml overnight culture and grown at 37°C, 200 rpm until the OD600 reached 1.6. The cultures were cooled to room temperature for 30 min and then IPTG added to 1 mM and grown further at 25°C, 200 rpm for 4h. The cells were harvested by centrifugation, the cell pellets frozen in liquid nitrogen and stored at -20°C until use.

Purification of DcpS

The thawed cell pellet was resuspended in 10 ml lysis buffer (50 mM Tris-HCl pH 8, 350 mM NaCl, 20 mM imidazole, 20% sucrose and 1 mM mercaptoethanol) and PMSF added to 300 µM. The cells were lysed by sonication, 0.5 sec on, 0.5 off at 30% for 3 min (repeated 3 times). The supernatant was obtained by centrifugation at 12000 rpm for 30 min at 4°C. This was then filtered through 0.4 µm membrane and loaded on 1 ml HisTrap column. The column was washed with 5 % buffer B (50 mM Tris-HCl pH 8, 350 mM NaCl, 250 mM imidazole, 20% sucrose and 1 mM mercaptoethanol) before elution with 5-100% buffer B gradient in 10 c.v. The eluate fractions were pooled and dialyzed against dialysis buffer (10 mM Tris-HCl pH 8, 25 mM NaCl and 1 mM mercaptoethanol) overnight and then concentrated to 0.3 ml. 10 µl aliquots were frozen in liquid nitrogen and stored at -80°C. The protein concentration was determined by comparison with BSA standard on SDS-PAGE gel.

Expression of eIF4E
SUPPORTING INFORMATION

The plasmid pET16b-elf4E was transformed into the *Escherichia coli* strain BL21 (DE3) and used directly for the inoculation of the 30 ml LB-ampicillin culture. The culture was grown overnight at 37°C with constant shaking at 200 rpm. Five 400 ml LB-ampicillin cultures were each inoculated with 5 ml overnight culture and grown at 37°C, 200 rpm until the OD600 reached 0.6. IPTG added to 1 mM and the cells grown further at 37°C, 200 rpm for 3h. The cells were harvested by centrifugation, the cell pellets frozen in liquid nitrogen and stored at -20°C until use.

Purification of elf4E

The thawed cell pellet was resuspended in 10 ml lysis buffer (50 mM sodim phosphate pH 7.5, 300 mM NaCl, 10 mM imidazole) and PMSF added to 300 uM. The cells were lysed by sonication, 0.5 sec on, 0.5 off at 30% for 3 min (repeated 3 times). The supernatant was obtained by centrifugation at 12000 rpm for 30 min at 4°C. This was then filtered through 0.4 uM membrane and loaded on 1 ml HisTrap column. The column was washed with 5 % buffer B (50 mM sodium phosphate pH 7.5, 300 mM NaCl, 250 mM imidazole) before elution with 5-100% buffer B gradient in 10 c.v. The eluate fractions were pooled and dialyzed against dialysis buffer (20 mM Hepes pH 7.5, 50 mM KCl, 0.2 mM EDTA, 7 mM mercaptoethanol and 0.01% Triton X-100) overnight and then concentrated to 0.3 ml. 10 ul aliquots were frozen in liquid nitrogen and stored at -80°C. The protein concentration was determined by comparison with BSA standard on SDS-PAGE gel.

Microscale thermophoresis measurements

Microscale thermophoresis (MST) measurements were performed on a Monolith NT.115 series instrument (NanoTemper). Prior to thermophoresis measurements proteins were labeled by incubation with CyS-NHS (Lumiprobe) for 30 min at room temperature. Unreacted dye was separated from the protein using PD SpinTrap™ G-25 gel filtration columns (GE Healthcare) according to the manufacturer protocol. Serial dilutions of the cap analogues (starting from 300 µM of cap analogue) in reaction buffer pH 8 containing 0.025% Tween 20 were prepared and mixed with an equal volume of the labeled protein (~50 nM). The mixture was filled into premium coated capillaries (4 µL) and directly measured. MST power was set to 30-40%, LED power was set to 20% Red (Exc.: 625 nm, Em.: 680 nm). Thermophoresis measurements were performed with the following settings: fluorescence before (5 s), MST on (30 s), fluorescence after (3 s). The capillaries were measured three times in direct succession. MST data were normalized to baseline differences and Kd values were calculated using nonlinear regression assuming a Hill coefficient of 1.0 (GraphPad Prism).

The remethylated cap analogs were produced by irradiation of compound 10d with subsequent enzymatic modification with Ecm1 and the natural AdoMet. The MST measurements of the unmethylated GpppA cap analogue, the commercially available m^7GpppA cap analogue and the enzymatically synthesized m^7GpppA cap analogue (Figure S39) served as controls and resulted in values corresponding to the ranges of previous reported data (Table S1).[3a, 4]

Table S1. Approximate binding affinities between mRNA cap analogues and the cap-binding proteins elf4E from *S. cerevisiae* and DcpS H227N from humans.

| elf4E Saccharomyces cerevisiae | Kd [µM] | Times measured | DcpS H227N human | Kd [µM] | Times measured |
|--------------------------------|---------|----------------|------------------|---------|----------------|
| GpppA                          | No binding observed | 1               | No binding observed | 1       |                |
| m^7GpppA (commercially)        | ~ 0.60  | 1              | ~ 0.70           | 2       |                |
| m^7GpppA (enzymatically)       | ~ 0.47  | 2              | ~ 0.78           | 4       |                |
| N^7-BP-GpppA                   | No binding observed | 2               | No binding observed | 1       |                |
| remethylated m^7GpppA          | ~ 0.85  | 4              | ~ 1.98           | 2       |                |

Enzymatic synthesis of capped Firefly mRNA

For the production of the Firefly-mRNAs the respective Firefly pMRNA™ vector was used.[1] The respective DNA template was amplified in 1 x HF buffer using plasmid (70 ng), dNTP mix (0.5 mM), forward primer (0.5 µM), reverse primer (0.5 µM) and Phusion High-Fidelity DNA Polymerase (1 U). The N^7-BP-GpppG cap analogue was enzymatically prepared and purified as described above, GpppG was used instead of GpppA. Then, in vitro T7 transcription was performed in 1 x transcription buffer using DNA template (100 ng), A/C/UTP mix (0.5 mM), GTP (0.1 mM), cap analog (1 mM GpppG, ~ 0.6 mM N^7-BP-GpppG), Ribolock RNase Inhibitor (30 U), T7 RNA polymerase (50 U) and pyrophosphatase (0.1 U) for 3 h at 37°C. Remaining DNA template was digested by incubation with 2 U DNase I for 1 h at 37°C and then, mRNAs were purified using the RNA Clean & Concentrator™-5 Kit (Zymo Research). Then, the modified mRNA was irradiated in ddH2O using a LED (λmax = 365 nm) for 10 min and subsequently analyzed by 7.5% denat. PAGE.
Supplementary Figures

Figure S3. Chemical structure of bromides 1-(1-bromomethyl)-2-nitrobenzene 2a, 1-(1-bromomethyl)-4-nitrobenzene 2b and 4-bromomethylbenzophenone 2c.

Figure S4. Chemical structures of guanosine 1, N7-photocaged guanosine derivatives N7-ONB-guanosine 3a, N7-PNB-guanosine 3b and N7-BP-guanosine 3c, adenosine 6 and N1-benzophenone-modified adenosine 7.

Figure S5. Mass spectrometric analysis of purified N7-photocaged guanosine derivatives 3a-b (A-B). (A) 3a: Calculated mass of [C_{17}H_{19}N_{6}O_{7}]^{+} = 419.1310 [M]^+, found: 419.1324. (B) 3b: Calculated mass of [C_{17}H_{19}N_{6}O_{7}]^{+} = 419.1310 [M]^+, found: 419.1323.
Figure S6. Section of the three-dimensional spectrum of irradiated N7-OHB-guanosine 3a and formation of photo product 4. 3D-plot shows section from 8-16 min and \( \lambda = 200-400 \) nm.

Figure S7. MS/MS analysis of isolated photo product 4. Fragmentation patterns were obtained by low energy CID at 25 eV. Mass for the precursor ion is marked with a blue diamond. 4: Calculated mass of \([C_{15}H_9N_6O_4]^+ = 347.1462 \) [M]+, found: 347.1472.
Figure S8. Irradiation of N7-PNB-guanosine 3b. N7-PNB-guanosine 3b was irradiated in ddH₂O using a LED (λ_{max} = 365 nm) for different times. HPLC analysis showed no cleavage of the 3b.

Figure S9. Photo stability of guanosine 1. Guanosine 1 was irradiated in ddH₂O using an LED (λ_{max} = 365 nm) for different times and analyzed by HPLC.
Figure S10. Mass spectrometric analysis of purified N7-BP-guanosine 3c and of photocleavage products 1 and 5 after irradiation of 3c. (A) 3c: Calculated mass of \([\text{C}_{24}\text{H}_{24}\text{N}_5\text{O}_6]^+ = 478.1721\) [M]+, found: 478.1737. (B, C) Irradiation in buffer. (B) 1: Calculated mass of \([\text{C}_{10}\text{H}_{14}\text{N}_5\text{O}_5]^+ = 284.0989\) [M+H]+, found: 284.1001. (C) MS/MS analysis of 5. Fragmentation patterns were obtained by low energy CID at 25 eV. Mass for the precursor ion is marked with a blue diamond. 5: Calculated mass of \([\text{C}_{14}\text{H}_{13}\text{O}]^+ = 197.0961\) [M+H]+, found: 197.0968. (D, E) Irradiation in buffer used for irradiation of RNA. (D) 1: Calculated mass of \([\text{C}_{10}\text{H}_{14}\text{N}_5\text{O}_5]^+ = 284.0989\) [M+H]+, found: 284.1003. (E) MS/MS analysis of 5. Fragmentation patterns were obtained by low energy CID at 25 eV. Mass for the precursor ion is marked with a blue diamond. 5: Calculated mass of \([\text{C}_{14}\text{H}_{13}\text{O}]^+ = 197.0975\) [M+H]+, found: 197.0978.
Figure S11. Irradiation of purified N7-BP-guanosine 3c. (A) Purified N7-BP-guanosine 3c was irradiated in ddH₂O, in buffer, in aqueous solution under addition of individual buffer components or in cell lysate using a LED (λ<sub>max</sub> = 365 nm) for 30 min. HPLC analysis showed no recovery of guanosine 1 in ddH₂O, 50 mM Tris-HCl and 200 mM NaCl, however recovery of guanosine 1 in buffer, 1 mM EDTA, 20% glycerol, and cell lysate. (B, C) Mass spectrometric analysis of photocleavage products after irradiation of N7-BP-guanosine 3c. (B) 1: Calculated mass of [C₁₀H₁₄N₅O₅]+ = 284.0989 [M+H]<sup>+</sup>, found: 284.0998. (C) MS/MS analysis of 5. Fragmentation patterns were obtained by low energy CID at 25 eV. Mass for the precursor ion is marked with a blue diamond. 5: Calculated mass of [C₁₆H₁₂O₅]+ = 197.0961 [M+H]<sup>+</sup>, found: 197.0977.
Figure S12. Irradiation of purified N1-BP-adenosine 7 in H₂O or in buffer. (A) Concept scheme. (B) Purified N1-BP-adenosine 7 was irradiated in ddH₂O or in buffer using a LED (λ_max = 365 nm) for 30 min. HPLC analysis showed no recovery of adenosine 6 in ddH₂O, however recovery of adenosine 6 in buffer.
Figure S13. Mass spectrometric analysis of purified M1-BP-adenosine 7 and of photocleavage products 6 and 5 after irradiation of 7. (A) 7: Calculated mass of $[\text{C}_{24}\text{H}_{24}\text{N}_5\text{O}_5]^+ = 462.1772$ [M$^+$], found: 462.1791. (B, C) Irradiation in buffer. (B) 6: Calculated mass of $[\text{C}_{10}\text{H}_{14}\text{N}_5\text{O}_4]^+ = 268.1040$ [M+H$^+$], found: 268.1056. (C) MS/MS analysis of 5. Fragmentation patterns were obtained by low energy CID at 25 eV. Mass for the precursor ion is marked with a blue diamond. 5: Calculated mass of $[\text{C}_{14}\text{H}_{13}\text{O}]^+ = 197.0961$ [M+H$^+$], found: 197.0981.
Figure S14. Chemical structure of AdoMet analogues AdoONB 9a, AdoPNB 9b, AdoANB 9c, AdoBP 9d and AdoHAK 9e.
Figure S15. Chemical structures of G(5)ppp(5)A cap analogue 8 and N7-photocaged cap analogues N7-ONB-GpppA 10a, N7-PNB-GpppA 10b, N7-ANB-GpppA 10c, N7-BP-GpppA 10d and N7-HAK-GpppA 10e.
Figure S16. Enzymatic installation of N7-PNB-GpppA 10b and subsequent irradiation. GpppA 8 was enzymatically modified using Ecm1 and AdoPNB 9b and then the unpurified N7-PNB-GpppA 10b was irradiated in ddH2O using a LED ($\lambda_{\text{max}} = 365$ nm) for 10 min. HPLC analysis showed no cleavage of the 10b.
Figure S17. Enzymatic installation of N7-ANB-GpppA 10c and subsequent irradiation. (A) GpppA 8 was enzymatically modified using Ecm1 and AdoANB 9c and then the unpurified N7-ANB-GpppA 10c was irradiated in ddH2O using a LED (λ_{max} = 365 nm) for 10 min. HPLC analysis showed no recovery of GpppA 8 instead the formation of a new photo product 11c. (B) Purified N7-ANB-GpppA 10c was irradiated in ddH2O using a LED (λ_{max} = 365 nm) for 10 min. HPLC analysis showed no recovery of GpppA 8 instead the formation of a new photo product 11c.
Figure S16. Enzymatic modification to and irradiation of N7-BP-GpppA 10d. (A) GpppA 8 was enzymatically modified using Ecm1 and AdoBP 9d and then the unpurified N7-BP-GpppA 10d was irradiated in buffer using a LED (λ <sub>max</sub> = 365 nm) for different times. HPLC analysis showed recovery of GpppA 8 and decrease of N7-BP-GpppA 10d (Signal marked with an * refer to compounds of the buffer). (B) Decrease of 10d and recovery of 8 after irradiation (365 nm, 10 min), analyzed by HPLC. Data and error bars show averages and standard deviations of three independent experiments.
Figure S19. Mass spectrometric analysis of purified N7-photocaged GpppA cap analogues 10a-d (A-D) and photo product 11c of irradiated 10c (E). (A) 10a: Calculated mass of $[C_{27}H_{33}N_{11}O_{19}P_3]^+ = 908.1162$ [M$^+$], found: 908.1164. (B) 10b: Calculated mass of $[C_{27}H_{33}N_{11}O_{19}P_3]^+ = 908.1163$, found: 908.1163. (C) 10c: Calculated mass of $[C_{29}H_{35}N_{11}O_{20}P_3]^+ = 950.1267$ [M$^+$], found: 950.1271. (D) 10d: Calculated mass of $[C_{31}H_{38}N_{10}O_{18}P_3]^+ = 967.1573$, found: 967.1559. (E) 11c: Calculated mass of $[C_{27}H_{35}N_{11}O_{17}P_3]^+ = 878.1420$ [M$^+$], found: 878.1447.
Figure S20. Irradiation of purified N7-ONBGpppA 10a. Irradiation of 10a in ddH₂O using a LED (λ<sub>max</sub> = 365 nm) for different times showed no recovery of GpppA instead the formation of a new photo product 11a, which was analyzed by HPLC.
Figure S21. Mass spectrometric analysis of GpppA after enzymatic digestion. (A) UV chromatogram of digested sample and extracted ion count (EIC) chromatograms of adenosine and guanosine. (B) Adenosine: Calculated mass of \([\text{C}_{10}\text{H}_{14}\text{N}_{5}\text{O}_{4}]^{+}\) = 268.1040 \([\text{M}+\text{H}]^{+}\), found: 268.1050. (D) Guanosine: Calculated mass of \([\text{C}_{10}\text{H}_{14}\text{N}_{5}\text{O}_{5}]^{+}\) = 284.0989 \([\text{M}+\text{H}]^{+}\), found: 284.1006. (C-E) MS/MS analysis of adenosine and guanosine. Fragmentation patterns were obtained by low energy CID at 25 eV. Mass for the precursor ion is marked with a blue diamond.
Figure S22. Mass spectrometric analysis of N7-ONB-GppA 10a after enzymatic digestion. (A) UV chromatogram of digested sample and extracted ion count (EIC) chromatograms of adenosine and N7-ONB-guanosine. (B) Adenosine: Calculated mass of $[C_{10}H_{14}N_5O_4]^+ = 268.1040$ [M+H]$^+ $, found: 268.1048. (D) N7-ONB-guanosine: Calculated mass of $[C_{17}H_{19}N_6O_7]^+ = 419.1310$ [M]$^+ $, found: 419.1328. (C+E) MS/MS analysis of adenosine and N7-ONB-guanosine. Fragmentation patterns were obtained by low energy CID at 25 eV. Mass for the precursor ion is marked with a blue diamond.
Figure S23. Mass spectrometric analysis of photo product 11a after enzymatic digestion. (A) UV chromatogram of digested sample and extracted ion count (EIC) chromatograms of adenosine and an unknown guanosine derivative. (B) Adenosine: Calculated mass of $[C_{10}H_{14}N_5O_4]^+ = 268.1040$ [M+H]$^+$, found: 268.1050. (C) Unknown guanosine derivative: Calculated mass of $[C_{15}H_{19}N_6O_4]^+ = 347.1462$ [M]$^+$, found: 347.1473. (D+E) MS/MS analysis of adenosine and unknown guanosine derivative. Fragmentation patterns were obtained by low energy CID at 25 eV. Mass for the precursor ion is marked with a blue diamond.
Figure S24. Photo stability of GpppA 8. Cap analogue GpppA 8 was irradiated in ddH₂O using an LED ($\lambda_{\text{max}} = 365$ nm) for different times and analyzed by HPLC.

Figure S25. Irradiation of purified N7-BP-GpppA 10d in H₂O or in buffer. Purified N7-BP-GpppA 10d was irradiated in ddH₂O or in buffer using a LED ($\lambda_{\text{max}} = 365$ nm) for 15 min. HPLC analysis showed no recovery of GpppA 8 in ddH₂O, however recovery of GpppA 8 in buffer.
**Figure S26.** Mass spectrometric analysis of photocleavage products 8 and 5 after irradiation of 10d in buffer solution. (A) 8: Calculated mass of \([\text{C}_{20}\text{H}_{28}\text{N}_{10}\text{O}_{17}\text{P}_{3}]^+ = 773.0841 \text{ [M+H]}^+\), found: 773.0855. (B) MS/MS analysis of 5. Fragmentation patterns were obtained by low energy CID at 25 eV. Mass for the precursor ion is marked with a blue diamond. 5: Calculated mass of \([\text{C}_{14}\text{H}_{13}\text{O}]^+ = 197.0961 \text{ [M+H]}^+\), found: 197.0968.

**Figure S27.** HPLC trace and mass spectrometric analysis of AdoHAK 9e (A-B). (B) 9e: Calculated of \([\text{C}_{25}\text{H}_{33}\text{N}_{6}\text{O}_{7}\text{S}]^+ = 561.21 \text{ [M]}^+\), found: 561.28 (MALDI analysis).
Enzymatic modification of N7-HAK-GpppA 10e and subsequent irradiation with recovery of GpppA 8. (A) GpppA 8 was enzymatically modified using EcH1 and AdoHAK 9e and then the unpurified N7-HAK-GpppA 10e was irradiated using a UV-transilluminator with a polystyrene filter (λ<sub>max</sub> = 312 nm) for 30 min. HPLC analysis showed recovery of GpppA 8 and decrease of N7-HAK-GpppA 10e. (C) Time series for the recovery of GpppA 8.
Figure S29. Structure of recovered GpppA 8 and side-products 11ea-eb upon irradiation of N7-HAK-GpppA 10e.

Figure S30. Mass spectrometric analysis of N7-HAK-GpppA 10e and photocleavage products 8 and 11ea-11eb. (A) 10e: Calculated mass of [C_{31}H_{40}N_{10}O_{19}P_{3}]^{+} = 949.17 [M]^+$, found: 949.14 (MALDI analysis). (B) 8: Calculated mass of [C_{20}H_{28}N_{10}O_{17}P_{3}]^{+} = 773.0841 [M+H]^+$, found: 773.0843. (C) 11ea: Calculated mass of [C_{28}H_{34}N_{10}O_{19}P_{3}]^{+} = 907.1209 [M]^+$, found: 907.1219. (D) 11eb: Calculated mass of [C_{28}H_{34}N_{10}O_{18}P_{3}]^{+} = 891.1260, found: 891.1268.
Figure S31: Enzymatic modification of DNA plasmid pBR322 using MTase TaqI and subsequent photocleavage. (A) Concept for the transfer of AdoPC to the N6 position of 2′-deoxyadenosine using MTase TaqI followed by irradiation with UV light (λmax = 365 nm) (For clarity, only three of the seven M. TaqI recognition sites in pBR322 are shown). (B) Analysis of enzymatic modification and irradiation using a LED (λmax = 365 nm, 10 min) of plasmid DNA using AdoBP and M. TaqI, which was analyzed by 1% agarose gel (100 V, 1 h) and visualized by staining with ethidium bromide. (C) Structure of N6-BP-modified 2′-deoxyadenosine after enzymatic digestion of enzymatically modified short dsDNA. (D) Mass spectrometric analysis of N6-BP-modified 2′-deoxyadenosine: Calculated mass of [C24H24N5O4]+ = 446.1823 [M+H]+, found: 446.1821.

Figure S32. Irradiation of purified N7-BP-guanosine 3c in buffer used for irradiation of RNA. Purified 3c was irradiated in 1x T1 buffer using an LED (λmax = 365 nm) for 30 min. HPLC analysis showed recovery of guanosine 5.
Figure S33. Photouncaging of internal N7-BP-modified guanosines in short RNA. (A) Sequences of short RNAs used in this study. (B) Extracted ion count (EIC) chromatogram of N7-BP-guanosine ([C_{24}H_{24}N_{5}O_{6}]^{+}M)^{+} 478.1721±0.005 of chemically modified and then digested RNA1 before and after irradiation. (C) Mass analysis of N7-BP-guanosine (calculated mass of [C_{24}H_{24}N_{5}O_{6}]^{+}M = 478.1721 [M]^{+}, found 478.1726.

Figure S34. Mass spectrometric analysis of chemically modified RNA1 UUGUU before and after irradiation. (A) Extracted ion count (EIC) chromatograms of uridine, guanosine and N7-BP-guanosine before irradiation. (B) MS/MS analysis of N7-BP-guanosine. Fragmentation patterns were obtained by low energy CID at 25 eV. Mass for the precursor ion is marked with a blue diamond. Calculated mass of [C_{24}H_{24}N_{5}O_{6}]^{+}M = 478.1721 [M]^{+}, found: 478.1725. (C) Extracted ion count (EIC) chromatograms of uridine, guanosine and N7-BP-guanosine after irradiation.
Figure S35. Mass spectrometric analysis of chemically modified RNA2 UUUCUUUGUUAUCUUC before and after irradiation. (A) Extracted ion count (EIC) chromatograms of cytidine, uridine, guanosine, adenosine and N7-BP-guanosine before irradiation. (B) MS/MS analysis of N7-BP-guanosine. Fragmentation patterns were obtained by low energy CID at 25 eV. Mass for the precursor ion is marked with a blue diamond. Calculated mass of [C_{24}H_{24}N_{5}O_{6}]^{+} = 478.1721 [M]^{+}, found: 478.1727. (C) Extracted ion count (EIC) chromatograms of cytidine, uridine, guanosine, adenosine and N7-BP-guanosine after irradiation.
Figure S36. Mass spectrometric analysis of chemically modified RNA3 UUUUCUUGGUAAUUCUUC before and after irradiation. (A) Extracted ion count (EIC) chromatograms of cytidine, uridine, guanosine, adenosine and N7-BP-guanosine before irradiation. (B) MS/MS analysis of N7-BP-guanosine. Fragmentation patterns were obtained by low energy CID at 25 eV. Mass for the precursor ion is marked with a blue diamond. Calculated mass of \([\text{C}_{24}\text{H}_{24}\text{N}_5\text{O}_6]^+ = 478.1721 \text{ [M]}^+\) found: 478.1732. (C) Extracted ion count (EIC) chromatograms of cytidine, uridine, guanosine, adenosine and N7-BP-guanosine after irradiation.
Figure S37. Mass spectrometric analysis of chemically modified RNA4 CACACGAACUUAUAGGUAGGC before and after irradiation. (A) Extracted ion count (EIC) chromatograms of cytidine, uridine, guanosine, adenosine and N7-BP-guanosine before irradiation. (B) MS/MS analysis of N7-BP-guanosine. Fragmentation patterns were obtained by low energy CID at 25 eV. Mass for the precursor ion is marked with a blue diamond. Calculated mass of [C\textsubscript{24}H\textsubscript{24}N\textsubscript{5}O\textsubscript{6}]\textsuperscript{+} = 478.1721 [M]\textsuperscript{+}, found: 478.1732. (C) Extracted ion count (EIC) chromatograms of cytidine, uridine, guanosine, adenosine and N7-BP-guanosine after irradiation.

Figure S38. Chemical modification of short RNA. (A, B, C) RNA2, RNA3 or RNA4 was incubated with bromide 2c for 24 h at 37 °C and then irradiated using a LED (\(\lambda\text{max} = 365\ \text{nm}\)) for 10 min. RNAs were analyzed by PAGE (15% PAGE, 15 W, 2 h, rt) and visualized using SYBR\textsuperscript{TM} Gold (Invitrogen) staining.
Figure S3. UV chromatogram of chemically modified RNA4 CACACGAUUAUAGGUAGGC after irradiation using a LED ($\lambda_{\text{max}} = 365$ nm) for 10 min. Then RNA4 was digested and dephosphorylated to single nucleosides and analyzed by HPLC.
Figure S40. Representative microscale thermophoresis measurements to determine $K_d$ values of different RNA cap analogues and eIF4E from *S. cerevisiae* as well as human DcpS H227N. The proteins were fluorescently labelled using Cy5-NHS. Both enzymes were used in a concentration of 50 nM. (A) enzymatically
synthesized m′GpppA. (B) Commercially available m′GpppA. (C) N7-BP-GpppA showed no binding or fitting curve. (D) unmethylated GpppA showed no binding or fitting curve either. The K<sub>d</sub> values shown in Table S1 were calculated using nonlinear regression assuming a Hill coefficient of 1.0 (GraphPad Prism).

**Figure S41**: Denatured 12 % PAA-gel loaded with a prestained protein ladder, h DcpS H227N (53 kDa) and Sc eIF4E (30 kDa). Gel electrophoresis was performed with 160 V for 60 min.
NMR Spectra

Figure S42. $^1$H NMR spectrum (600 MHz, DMSO-$d_6$, 299 K) of 3a.

Figure S43. $^{13}$C NMR spectrum (151 MHz, DMSO-$d_6$, 299 K) of 3a.
Figure S44. $^1$H, $^1$H-COSY NMR spectrum (600 MHz, DMSO-d$_6$, 299 K) of 3a.

Figure S45. $^1$H, $^{13}$C-HSQC NMR spectrum (600 MHz, 151 MHz, DMSO-d$_6$, 299 K) of 3a.
**Figure S46.** $^1$H, $^{13}$C-HMBC NMR spectrum (600 MHz, 151 MHz, DMSO-$d_6$, 299 K) of 3a.

**Figure S47.** $^{13}$C-DEPT NMR spectrum (151 MHz, DMSO-$d_6$, 299 K) of 3a.
**Figure S48.** $^1$H NMR spectrum (600 MHz, D$_2$O, 299 K) of 3b.

**Figure S49.** $^{13}$C NMR spectrum (151 MHz, D$_2$O, 299 K) of 3b.
Figure S50. $^1$H, $^1$H-COSY NMR spectrum (600 MHz, D$_2$O, 299 K) of 3b.

Figure S51. $^1$H, $^{13}$C-HSQC NMR spectrum (600 MHz, 151 MHz, D$_2$O, 299 K) of 3b.
**Figure S52.** $^1$H, $^{13}$C-HMBC NMR spectrum (600 MHz, 151 MHz, D$_2$O, 299 K) of 3b.

**Figure S53.** $^{13}$C-DEPT NMR spectrum (151 MHz, D$_2$O, 299 K) of 3b.

**Figure S54.** $^1$H NMR spectrum (600 MHz, DMSO-d$_6$, 299 K) of 4.
Figure S5. $^{13}$C NMR spectrum (151 MHz, DMSO-d$_6$, 299 K) of 4.

Figure S5. $^1$H, $^1$H-COSY NMR spectrum (600 MHz, DMSO-d$_6$, 299 K) of 4.
Figure S57. $^1$H, $^{13}$C-HSQC NMR spectrum (600 MHz, 151 MHz, DMSO-d$_6$, 299 K) of 4.

Figure S58. $^1$H, $^{13}$C-HMBC NMR spectrum (600 MHz, 151 MHz, DMSO-d$_6$, 299 K) of 4.
Figure S59. $^{13}$C-DEPT NMR spectrum (126 MHz, DMSO-$d_6$, 299 K) of 4.

Figure S60. $^1$H NMR spectrum (500 MHz, DMSO-$d_6$, 299 K) of 3c.

Figure S61. $^{13}$C NMR spectrum (126 MHz, DMSO-$d_6$, 299 K) of 3c.
Figure S62. $^1$H, $^1$H-COSY NMR spectrum (500 MHz, DMSO-δ6, 299 K) of 3c.

Figure S63. $^1$H, $^{13}$C-HSQC NMR spectrum (500 MHz, 126 MHz, DMSO-δ6, 299 K) of 3c.
Figure S64. $^1$H, $^{13}$C-HMBC NMR spectrum (600 MHz, 126 MHz, DMSO-$d_6$, 299 K) of 3c.

Figure S65. $^{13}$C-DEPT NMR spectrum (126 MHz, DMSO-$d_6$, 299 K) of 3c.
Figure S66. $^1$H NMR spectrum (600 MHz, DMSO-$d_6$, 299 K) of 7.

Figure S67. $^{13}$C NMR spectrum (151 MHz, DMSO-$d_6$, 299 K) of 7.
Figure S68. $^1$H, $^1$H-COSY NMR spectrum (600 MHz, DMSO-d$_6$, 299 K) of 7.

Figure S69. $^1$H, $^{13}$C-HSQC NMR spectrum (600 MHz, 151 MHz, DMSO-d$_6$, 299 K) of 7.
Figure S70. $^1$H, $^{13}$C-HMBC NMR spectrum (600 MHz, 151 MHz, DMSO-d$_6$, 299 K) of 7.

Figure S71. $^{13}$C-DEPT NMR spectrum (151 MHz, DMSO-d$_6$, 299 K) of 7.

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