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Article

# Stabilized 5′ Cap Analogue for Optochemical Activation of mRNA Translation

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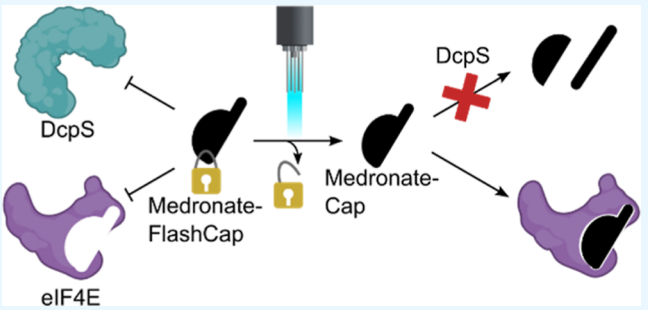
**ABSTRACT:** The 5′ cap is a distinguishing feature of transcripts made by polymerase II and characterized by an N7-methylated guanosine ($m^7G$) linked to the first transcribed nucleotide by a 5′−5′ triphosphate bridge. It stabilizes eukaryotic mRNAs and plays a crucial role in translation initiation. Its importance in mRNA processing, translation, and turnover makes the 5′ cap a privileged structure for engineering by non-natural modifications. A photocleavable group at the 5′ cap of guanosine was recently used to mute translation of exogenous mRNAs. Its removal by light enabled direct control of protein production at the posttranscriptional level. Modifications in the triphosphate bridge impede degradation by specific decapping enzymes and maintain translation. Here, we combined 5′ cap modifications at different positions and investigated how they impact 5′ cap-dependent processes in distinct manners. We synthesized 5′ cap analogues with a photocleavable group at the $N^2$-position of $m^7G$ in addition to a medronate in the triphosphate bridge to obtain a photoactivatable 5′ cap analogue featuring a methylene group between the $\beta$ and $\gamma$ phosphates. The resulting Medronate-FlashCap transiently or permanently impeded distinct crucial interactions of the 5′ cap required for translation and degradation. We show that the Medronate-FlashCap is compatible with in vitro transcription to generate muted mRNA and that light can be used to activate translation in cells. After light-induced removal of the photocleavable group, the Medronate-FlashCap remained stable against degradation by the decapping enzyme DcpS. The additional methylene group renders the 5′ cap resistant to DcpS, while maintaining the interaction with cap-binding proteins.




## 1. INTRODUCTION

Messenger RNA (mRNA) has recently become an important medical modality. Compared to DNA, it has the advantages of not being integrated into the genome and producing the product more quickly.[1] The translation of mRNA is one of the most fundamental cellular processes. mRNA therapeutics build on the observation that the translation machinery works not only for endogenous but also appropriate exogenous transcripts.[2] These need to have at least a 5′ cap and preferably a poly(A) tail for efficient translation. An enormous body of research has gone into the evaluation of suitable improvements of exogenous mRNA to increase transcript stability and the amount of protein produced.[3−6]

The approved mRNA vaccines contain natural modified nucleotides, such as $m^1\Psi$.[7−9] Several natural modifications were shown to increase the amount of protein produced by reducing immunogenicity and/or increasing stability.[10−12] The importance of the untranslated regions for the stability and translation of mRNA has long been known.[13,14] Modifications in the poly(A) have been tested to increase the stability of exogenous mRNAs and thus improve the amount of protein produced.[15−17] Along these lines, we observed that modifying the poly(A) tail with multiple fluorophores increased the amount of protein produced three-fold.[18]

Considering all of the elements of the architecture of eukaryotic mRNAs, the 5′ cap provides the best leverage. It is an N7-methylated G connected to the first transcribed nucleotide by a 5′−5′ triphosphate bridge and occurs in mRNA only a single time. It is universally found in all mRNAs, independent of their sequence. For in vitro generation of mRNA, it can be synthesized and, in many cases, added to the transcription mix. Access to the repetitive poly(A) tail requires either sequence engineering of the template or a combination of multiple molecular biology steps. Changes in the sequence (be it the open reading frame or the untranslated regions) require template engineering and must be performed for every

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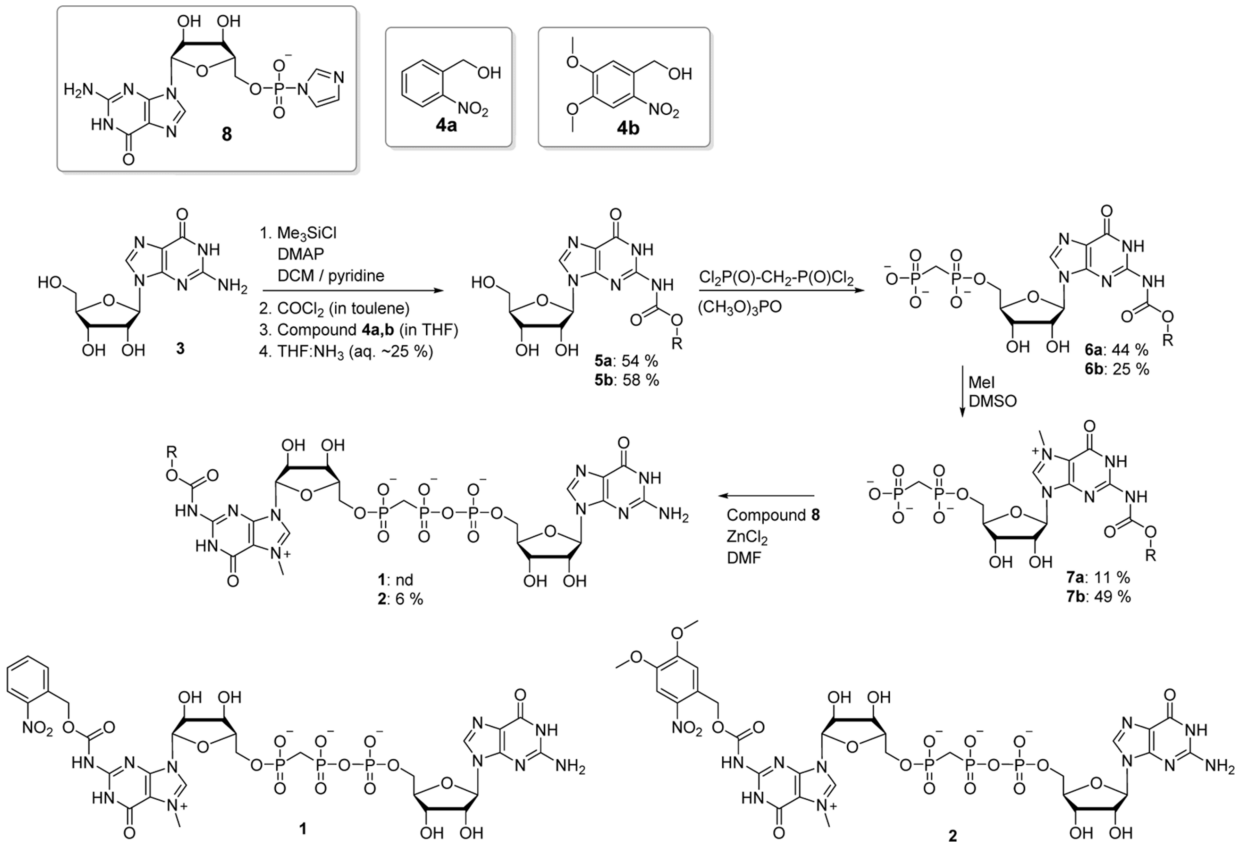
**Scheme 1. Synthesis Scheme of Medronate-FlashCaps (1,2)**[a]




[a]Compound **4a** = *ortho*-nitrobenzyl alcohol (ONB−OH), **4b** = 4,5-dimethoxy-2-nitrobenzyl alcohol (DMNB−OH), and nd = not determined.

new target mRNA. The introduction of modified nucleotides typically completely replaces a given nucleoside by a modified counterpart or requires a combination of synthetic and molecular biology approaches. The effects can vary depending on the template, requiring extensive efforts for optimization.

The 5′ cap binds directly to translation initiation factor 4E (eIF4E), which is rate limiting for the beginning of translation. Numerous modifications of this privileged structure have been tested in vitro and in cells regarding affinity to eIF4E, stability against decapping enzymes, and translation.[19−21] Modifications of the 5′ cap triphosphate bridge have been shown to increase the stability against cap hydrolyzing enzymes. The introduction of an imidophosphate $\alpha$- and $\beta$- or the $\beta$- and $\gamma$-phosphorus atoms stabilizes the 5′ cap against DcpS digestion while maintaining translation of the respective mRNA.[22] The exchange of an oxygen within the triphosphate bridge for a sulfur atom stabilizes the 5′ cap and increases the amount of protein produced. The sulfur atom can be bridging[23] or nonbridging.[24,25] Nonbridging oxygen was also successfully exchanged for boron and selenium, yielding $\beta$-boranophosphate and $\beta$-phosphoroselenoate-containing 5′ caps, respectively. These showed increased stability against enzymatic degradation and led to higher amounts of protein.[20,26]

The privileged role of the 5′ cap has recently been harnessed to bring the translation of mRNA under the control of light. By connecting a photocleavable group to the $N^2$-position of $m^7G$ in the synthetic 5′ cap, important functions of this molecule could be blocked and activated by brief irradiation with light [e.g., 30 s at 365 nm for the 4,5-dimethoxy-2-nitrobenzyl (DMNB)-moiety as photocleavable group]. These FlashCaps and CouCaps were compatible with in vitro transcription (IVT), permitting the generation of translationally muted mRNAs. We showed that translation of various exogenous mRNAs can be activated by light in vitro and in different cell types, and that up to 30-fold increase in the amount of protein produced is achieved.[27,28]

The FlashCap technology provides a valuable tool to activate exogenous mRNAs by light. As of today, different reporter mRNAs were activated in different cell types, and an up to 30-fold turn-on effect was achieved. The combination of different photocleavable groups even permitted the wavelength-selective activation of translation of two different mRNAs.[28] One limitation of activating mRNAs for translation is their limited stability in cells. Although FlashCap-mRNAs were not destabilized compared to the respective cap 0 transcripts, they will be degraded over time, limiting the effect of activation at later time points.

We therefore sought to combine the light activation of the 5′ cap with the increased stability and the maintained ability for translation. Decapping enzymes can cleave between the $\alpha$ and $\beta$ phosphate groups (Dcp1/2) or the $\beta$ and $\gamma$ phosphate group (DcpS). The introduction of a phosphonate can prevent degradation by these enzymes.[19,29] Importantly, this modified 5′ cap retained translation of mRNA vitro and in cells.[30]

Here, we report the synthesis of a FlashCap containing a methylene group between the $\beta$ and $\gamma$ phosphates. The novel Medronate-FlashCap is resistant to degradation by DcpS and showed increased stability in the cell lysate. We show that Medronate-FlashCap can be incorporated into mRNAs via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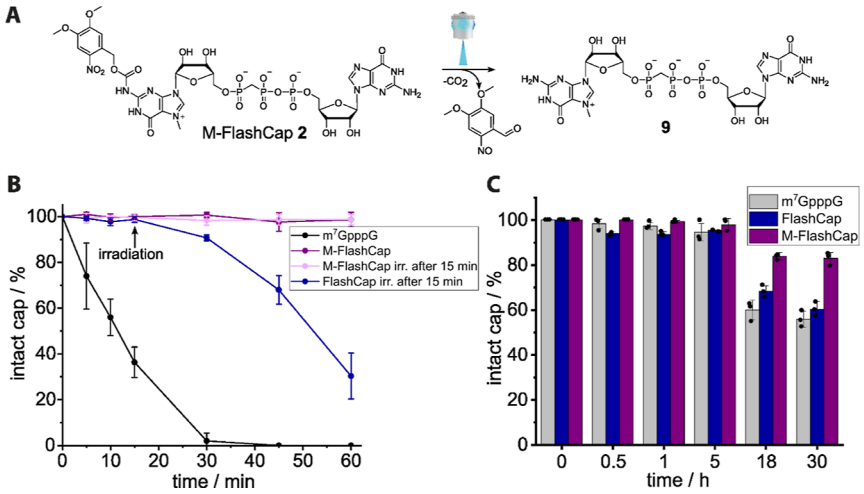


**Figure 1.** In vitro studies of Medronate-FlashCap **2** to assess the stability. (A) Photolysis reaction of **2** releasing the medronate analogue of cap 0 (**9**), carbon dioxide, and 4,5-dimethoxy-2-nitroso-benzaldehyde. (B) 5′ Cap stability against enzymatic degradation by DcpS. (C) 5′ Cap stability in lysate from HeLa cells. Data and error bars show average and standard deviation of $n = 3$ independent replicates.

IVT. The resulting mRNAs are translationally muted, and brief irradiation leads to prominent protein production in cells.

## 2. RESULTS

The medronate or methylenebis(phosphonate) was previously shown to stabilize 5′ caps against degradation by decapping enzymes and at the same time retain functionality in translation.[30] We therefore hypothesized that the methylenebis(phosphonate) modification should also stabilize FlashCaps, which are photocaged versions of the 5′ cap.

For the synthesis of **1** and **2**, we first conjugated the photocleavable protecting group, i.e., the *ortho*-nitrobenzyl-(ONB) or the DMNB group, to the $N^2$-position of guanosine (**3**) via a carbamate group, as previously described.[27] The modified guanosines **5a,b** were subsequently reacted with methylenebis(phosphonic dichloride) to form the respective 5′-bisphosphonates, **6a,b**. We observed that the reaction led to more side products than that for monophosphorylation in the case of the FlashCaps, probably due to the increased reactivity of the methylenebis(phosphonic dichloride) in comparison to phosphoryl chloride. Methyl iodide was used to methylate the N7-position of compounds **6a,b**, which were then coupled with the 5′-monophosphate *P*-imidazolide, **8** to yield the desired Medronate-FlashCaps (**1** and **2**). M-FlashCap **2** was characterized by HR-MS and $^1$H- and $^{13}$C-NMR (Scheme 1 and Supporting Information).

We used **2** for all subsequent studies because the DMNB group is expected to be removed at longer wavelengths than the ONB group, which is important for cellular applications. First, we assessed whether the Medronate-FlashCap **2** exhibits efficient photouncaging observed with the previously developed FlashCaps. We performed light-induced uncaging of **2** by irradiation with an LED at 365 nm. The decrease of **2** and the formation of the 5′ cap **9** were confirmed by LC−MS (Figures 1A and S1).

Next, we performed enzymatic assays with the decapping enzyme DcpS to assess the stability of Medronate-FlashCap **2** cap 0 ($m^7$GpppG), and FlashCap against enzymatic degradation. As expected, DcpS quickly hydrolyzed cap 0 into $m^7$GMP and GDP, as determined by HPLC (Figures 1B and S2). Within 15 min, ∼64% of cap 0 was degraded at the conditions used (Figure 1B). In contrast, Medronate-FlashCap **2** and the previously reported FlashCap showed little degradation within 15 min under identical conditions.

After 15 min, we irradiated the Medronate-FlashCap and FlashCap and continued to analyze the reaction at different time points (Figure 1C). After irradiation, the FlashCap was quickly degraded, in line with previous reports.[27] In contrast to FlashCap, Medronate-FlashCap **2** remained resistant to enzymatic cleavage, even after irradiation with light. These data indicate that irradiation of Medronate-FlashCap releases the medronate analogue of cap 0 ($m^7$GpcppG) **9**, which is resistant to DcpS (Figure 1A,B).[19]

We also compared the stability of different 5′ caps in HeLa cell lysate, where multiple degradation and turnover pathways exist. HPLC analysis revealed that 83% of the Medronate-FlashCap **2** remained intact after 30 h of incubation in cell lysate (Figures 1C and S3). The other 5′ cap analogues tested were less stable: 60% of the FlashCap and 56% of cap 0 remained after 30 h of incubation under the same conditions. The comparative analysis thus shows that Medronate-FlashCap **2** is more stable than the respective FlashCap and the cap 0. The data also suggest that the carbamate linkage is not prone to degradation.[27,28]

Next, we wanted to know if Medronate-FlashCap **2** is suitable for production of mRNAs. IVT of a DNA template is routinely performed by bacteriophage T7 RNA polymerases.[18,31] As transcription at the T7 promoter starts with a guanosine, $m^7$GpppG can be incorporated as a transcription start nucleotide. Due to the presence of GTP, a mixture of capped and uncapped RNA is obtained. The latter contains a triphosphate at the 5′ end and can be enzymatically degraded by a polyphosphatase and XRN1.[32] We used a DNA template containing a T7 promoter to produce 5′ capped mRNAs and routinely digested uncapped RNA. To our delight, IVT with Medronate-FlashCap **2** produced full-length mRNA in almost similar yield (65%) and capping efficiency (87%) compared to cap 0 (Figure 2A). We confirmed the production of long mRNAs with Medronate-FlashCap **2** for *Firefly* and *Gaussia* luciferase-mRNAs (Figures 2A and S4).

Next, we asked how Medronate-FlashCap-mRNA would impact translation. To evaluate the translational output, FLuc-mRNAs were produced with either Medronate-FlashCap **2** or cap 0 as control. The differently capped FLuc-mRNAs were


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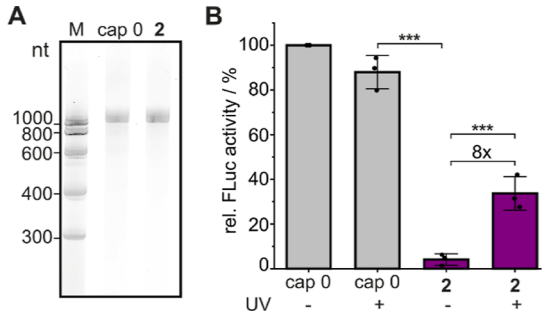


**Figure 2.** In vitro transcription (IVT) using Medronate-FlashCap **2**. (A) Gel analysis of mRNA with different 5′ caps. PAGE (7.5%) of cap 0- or Medronate-FlashCap (2) FLuc-mRNA from IVT is shown. M = RiboRuler. (B) In vitro translation of FLuc-mRNAs with the indicated 5′ caps. The mRNA (UV, +) was irradiated for 30 s with 365 nm. Values are normalized to nonirradiated cap 0-FLuc-mRNA. Data and error bars show the average and std. dev. of $n = 3$ independent replicates. Statistical significance was determined by a two-tailed $t$-test.

subsequently used in an in vitro luciferase activity assay in rabbit reticulocyte lysate. All measurements were normalized to the FLuc activity measured for cap 0-FLuc-mRNA without irradiation, and the cap-independent translation was subtracted (Figure 2B, 100%). In contrast to cap 0, the translation of Medronate-FlashCap (**2**)-mRNA was markedly reduced. Only 4% of luciferase activity compared to cap 0-mRNA was detected (Figure 2B). Irradiation with 365 nm for 30 s led to an eight-fold increase of the FLuc activity, reaching 33% of the positive control (native cap 0, no irradiation). This indicates removal of the photocleavable protecting group and the formation of 5′ cap **9**, which is functional for translation. In contrast, the in vitro translation of cap 0-mRNA was slightly reduced (to 89%) by irradiation under the same conditions (Figure 2B). Taken together, these data indicate that the translation of mRNAs with a Medronate-FlashCap is efficiently inhibited, most likely as a result of reduced eIF4E binding to the 5′ cap **2**. Its light-mediated release leads to cap **9**-mRNAs, which are translationally active.

Finally, we tested if Medronate-FlashCap-mRNA was functioning in cultured mammalian cells. We transfected HeLa and HEK293T cells with Medronate-FlashCap-GLuc-mRNA, cap 0-GLuc-mRNA, or AppppG capped GLuc-mRNA and measured translation using a *Gaussia* luciferase assay. Luciferase activity was normalized to cap-dependent translation of cap 0-GLuc-mRNA, and cap-independent translation was subtracted. As expected, the cap-dependent translation of Medronate-FlashCap-mRNA was strongly reduced. In HeLa and HEK293T cells, 4 and 2% of cap-dependent translation with respect to cap 0-GLuc-mRNA were detected (Figure 3B,C).

When the cells were irradiated, the translation of the positive control (cap 0-GLuc-mRNA) was slightly decreased, resulting in 71 and 83% yields for HeLa and HEK293T cells, respectively. Importantly, in cells transfected with Medronate-FlashCap-GLuc-mRNA, irradiation led to a significant increase of translation. A 10-fold increase was observed in HeLa (42% relative GLuc activity) as well as in HEK293T cells (21% relative GLuc activity) (Figure 3B,C). Under the irradiation conditions in vitro, we would expect complete photodeprotection and formation of cap **9**-GLuc-mRNA. These data suggest that mRNA with a 5′ cap **9** is less efficiently translated than cap 0-mRNA, in line with previous reports.[30]

Taken together, the translation assays show that light-mediated photodeprotection is even efficient in cells and confirm that uncaging generates functional mRNA.

## 3. DISCUSSION

In the accelerating field of therapeutic nucleic acids, research on mRNA is constantly growing, with a desire for proper technologies for basic and medical research. Within mRNAs, the 5′ cap is a privileged structure for engineering as it provides the best leverage to tune every given mRNA that is to be used

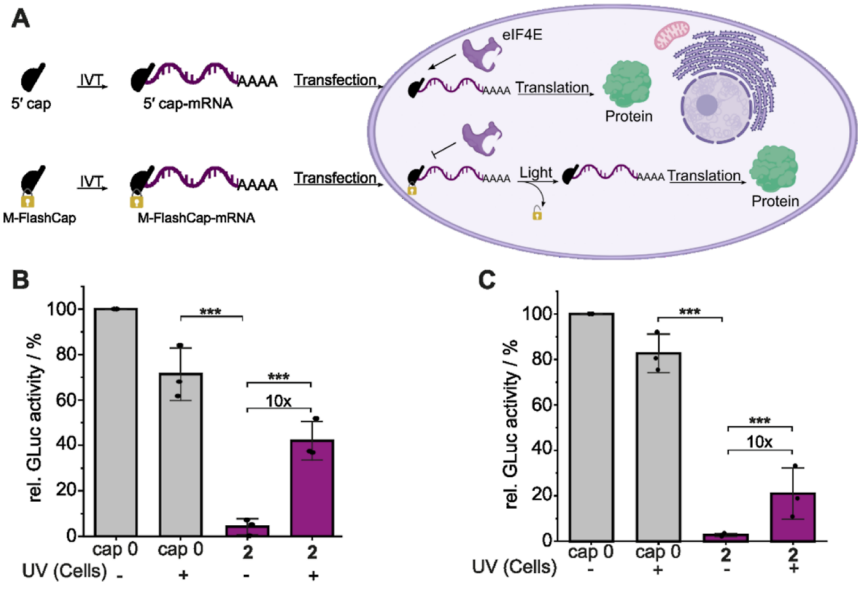


**Figure 3.** In-cell luciferase activity assay with differently capped GLuc-mRNA. (A) Concept of Medronate-FlashCap for light-induced translation in cells. (B) Translation assay in HeLa cells. (C) Translation assay in HEK293T cells. [UV (cells)]: cells were irradiated after transfection. Irradiation conditions: 30 s at 365 nm. Values are normalized to nonirradiated cap 0-GLuc-mRNA. Data and error bars show the average and std. dev. of $n = 3$ independent replicates. Statistical significance was determined by a two-tailed $t$-test.



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in cells. We recently demonstrated a novel method for efficient mRNA translation in a spatiotemporal manner.[27,28] This was the first approach to efficiently control when and where an mRNA is translated. However, mRNA has limited stability in cells, and this turnover restricts how long activation of translation is possible.

Extensive research has been performed to increase the stability of mRNA. This includes studies on stabilizing the 5′ cap against the decapping enzyme DcpS.[19] A bisphosphonate or medronate in the 5′−5′ triphosphate bridge was reported to inhibit decapping by DcpS, while maintaining the interaction with eIF4E and translation. In the present work, we combined the photoactivatable 5′ cap with the stability-enhancing methylene group between the $\beta$ and $\gamma$ phosphates of the triphosphate bridge and created the Medronate-FlashCap. The benefit of this new 5′ cap analogue is the permanent resistance to 5′ cap hydrolyzing enzymes. While the original FlashCaps were not resistant against enzymatic digestion after removal of the photocleavable protecting group, the Medronate-FlashCap remains stable.

In this work, we fully characterize the new Medronate-FlashCap and confirm that it can be used to make long mRNAs. We show in vitro and in cells that Medronate-FlashCap-mRNAs are silenced, and their translation can be triggered by light. The Medronate-FlashCap thus greatly expands our toolbox for the production of engineered mRNAs that can be activated at different time points. In future work, it could also be used as a stable and activatable eIF4E binder and potential inhibitor, which could be relevant in cancer research. For the application of the dinucleotide in cells, the cell permeability would have to be increased.

## 4. METHODS

For general experimental, instrumental, and synthetic procedures and full compound characterization, see the Supporting Information.

**4.1. Absorbance Spectra Analysis.** Photocaged guanosines were dissolved in $ddH_2O$, resulting in a final concentration of 100 $\mu$M. In a quartz cuvette (Hellma), 20 $\mu$L of the photocaged guanosine was dissolved by adding 80 $\mu$L of $ddH_2O$ (20 $\mu$M), and the absorbance was measured with an FP-8500 fluorescence spectrometer (Jasco).

**4.2. Irradiation of Samples.** To irradiate mRNA samples, photocaged guanosines, cells, and 5′ cap analogues, we used LEDs (LED Engin). The UV-A LED (maximum of 365 nm) was powered by 5 V and 600 mA.

The irradiation was carried out at 23 °C using a custom-made LED setup. Unless otherwise specified, the samples were irradiated in a PCR tube with an open lid or in a cell culture dish. Unless otherwise specified, the samples were irradiated for 30 s at 365 nm (142 mW $cm^{-2}$).

**4.3. Guanosine and Dinucleotide Irradiation Studies.** Photocaged guanosines or 5′ cap analogues were dissolved in $ddH_2O$, resulting in a final concentration of 500 $\mu$M (organic solvents were used to improve solubility if necessary). We placed 10 $\mu$L of the solution in a PCR tube and irradiated it as indicated below. Finally, photouncaging was determined by HPLC.

**4.4. HPLC Analysis.** Unless otherwise stated, all HPLC analyses were carried out on an Agilent1260 Infinity HPLC system equipped with a diode array detector (DAD) (190−640 nm) using a Nucleodur C18 Pyramid reversed-phase column (5 $\mu$m, 125 × 4 mm) from Macherey−Nagel at a flow rate of 1 mL $min^{-1}$. Elution was achieved by a linear gradient from buffer A (50 mM ammonium acetate, pH 6.0) to buffer B (1:1, buffer A: acetonitrile).

**4.5. yDcpS Hydrolysis Assay.** The enzymatic activity of yDcpS (New England Biolabs) was measured using the following experimental setup: 50 mM Tris−HCl containing 30 mM $(NH_4)_2SO_4$, 1 mM dithiothreitol (final pH 8.0), and 1 mM $Mg(AcO)_2$. The experiment was prepared on ice. An internal standard (either adenosine monophosphate or 4,5,7-trihydroxy-3-phenyl-coumarin, with a final concentration of 200 $\mu$M) was introduced along with the corresponding 5′ cap analogue. 20 U of yDcpS was added at the end, and the hydrolysis was initiated by incubation at 37 °C. Aliquots of 10 $\mu$L were taken from the reaction mixture at the designated time intervals, and the reaction was stopped by heat inactivation of the enzyme (10 min at 90 °C). After that, the samples were examined at 260 nm using analytical HPLC. Retention times were compared with standards to identify the hydrolysis products.

**4.6. Expression and Purification of yDcpS.** The enzymes yDcpS was produced and purified as previously described.[32−34]

**4.7. In Vitro Transcription.** The DNA sequences encoding *Gaussia* luciferase (GLuc) and *Firefly* luciferase (FLuc) were obtained by PCR amplification from pMRNA vectors. This dsDNA was purified (NucleoSpin Gel and PCR Clean-up, Macherey−Nagel) and used as template (200 ng) for the IVT. Alternatively, we used the runoff template for the IVT to obtain GLuc-mRNAs, which were applied in cell studies.

The plasmid DNA (3 $\mu$g) was incubated for 10 min at 37 °C in 1× FastDigest buffer (Thermo Fisher) and 3 $\mu$L of PacI FastDigest enzyme. It was then inactivated for 10 min at 65 °C. The ends were then dephosphorylated by adding 3 $\mu$L of FastAP, incubating for 15 min at 37 °C, and then inactivating for 5 min at 65 °C. The NucleoSpin Gel and PCR Clean-up kit (Macherey−Nagel) were used to purify the product. At 260 nm, the concentration was determined by using a Tecan Infinite M1000 PRO device. Of the obtained linear dsDNA, 400 ng was utilized as template.

For a 50 $\mu$L of IVT, 1× transcription buffer (Thermo Scientific), 200 ng of DNA template (PCR) (or alternatively 400 ng of runoff template), T7 RNA polymerase (50 U; Thermo Scientific), pyrophosphatase (0.1 U; Thermo Scientific), an A/C/UTP (0.5 mM) mix, guanosine-5′-triphosphate (0.25 mM), and the respective 5′ cap analogue (1 mM) were incubated for 4 h at 37 °C. Two U DNase I was added for 1 h at 37 °C to remove the DNA. The mRNA was then purified with the RNA Clean & Concentrator-5 kit (Zymo Research). Noncapped mRNAs were digested by 10 U RNA 5′-polyphosphatase (Epicenter) for 30 min at 37 °C and 0.5 U of the 5′−3′ exoribonuclease XRN1 (NEB) and $MgCl_2$ (5 mM) for 1 h at 37 °C. Finally, mRNAs were again purified with the RNA Clean & Concentrator-5 kit (Zymo Research).

**4.8. In Vitro Luminescence Assay.** The Retic Lysate IVT kit (Invitrogen), a eukaryotic cell-free protein expression method, was utilized for in vitro translation. 40 ng of the FLuc-mRNA (capped as defined), 50 $\mu$M L-methionine, and 150 mM potassium acetate were combined with 8.5 $\mu$L of the reticulocyte lysate in a total volume of 15 $\mu$L. The mixture was then incubated for 1.5 h at 30 °C. Subsequently, 2 $\mu$L of the same translation mix was utilized in an additional luminescence test. Using a luciferase assay based on the Beetlejuice


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Luciferase Assay Firefly (pjk), the translational output of the differentially capped FLuc−mRNAs was quantified. After the addition of 50 μL of freshly made substrate solution to the translation mixture, the activity of luciferase was measured. A Tecan Infinite M1000 PRO microplate reader was used to measure luminescence with a 3 s integration time. mRNAs with the indicated caps were utilized. Cap-independent translation is represented by ApppG-capped mRNA, which was removed from the other samples as background. The standard for all values was $m^7$GpppG-capped mRNA.

**4.9. Mammalian Cell Culture.** HEK293T cells (DSMZ) were cultured in Dulbecco's modified Eagle's medium (PAN) enriched with penicillin and streptomycin (1%, PAN), L-glutamine (2 mM, PAN), and fetal calf serum (FCS) (10%, PAN) under standard conditions (5% $CO_2$, 37 °C). HeLa cells (Merck) were cultured in MEM Earle's medium (PAN) enriched with penicillin and streptomycin (1%, PAN), nonessential amino acids (1%, PAN), L-glutamine (2 mM, PAN), and FCS (10%, PAN) under standard conditions (5% $CO_2$, 37 °C).

**4.10. Stability Assay of 5′ Caps in Cell Lysate.** HeLa cells ($3 \times 10^6$ cells) were placed in a Petri dish (90 mm) and lysed for 24 h. To this end, the cells were first centrifuged, and the medium was removed from the cell pellet. The cells were washed with 1× phosphate-buffered saline and then lysed with the CelLytic M reagent (1.5 mL, Sigma-Aldrich) according to the manufacturer's instructions. The mixture was centrifuged (11,000 rpm, 3 min, 4 °C), and the supernatant was used for the cell lysate stability assay. The various 5′ caps (500 μM) and an internal standard (4,5,7-trihydroxy-3-phenyl-coumarin 100 μM) were incubated in the cell lysate at 37 °C. At indicated time points, the samples were analyzed by HPLC, and the degradation was calculated from the area under the respective peaks in the chromatogram.

**4.11. In-Cell Luminescence Assay.** HEK293T or HeLa cells were seeded in a 96-well plate (30,000 cells per well) and cultured in MEM with antibiotics 1 day prior to transfection. For transfection, mRNA (100 ng) in Opti-MEM (10 μL) and Lipofectamine MessengerMAX transfection reagent (0.3 μL) in Opti-MEM (9.6 μL) were used (6 h at 37 °C in a total volume of 100 μL). The cells were irradiated at 365 nm for 30 s. Upon irradiation, the cell medium was exchanged for a fresh one, and cells were stored in an incubator overnight. The supernatant was gathered 24 h after the transfection. The *Gaussia*-Juice Luciferase Assay kit (PJK) was utilized to determine the luminescence. The cell supernatant was transferred to a 96-well plate (5 μL of supernatant per well), and 50 μL of a reaction mixture (PJK reconstruction buffer and coelenterazine) was added. Next, the luminescence was measured by using a Tecan Infinite M1000 PRO plate reader (integration time of 3 s). Cap-independent translation was subtracted from the measurements using the luminescence value of ApppG-capped mRNA. All values were normalized to $m^7$GpppG-capped mRNA.

**4.12. Statistical Analysis.** The statistical analysis was performed using the two-tailed Student's *t*-test. An additional Welch correction was used when compared with the $m^7$GpppG mRNA (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c08505.

Details about experimental methods, used materials, HPLC chromatograms, synthesis descriptions, and NMR analysis (PDF)

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### Author Contributions

F.P.W., N.K., and P.S. contributed equally. A.R. and P.S. conceived the project. P.S. and F.W. designed, performed, and analyzed chemical syntheses. N.K. designed, performed, and evaluated biochemical experiments. S.H. performed cell experiments. A.R. acquired funding and supervised the project. All authors discussed the results. A.R., N.K., and F.W. drafted and wrote the manuscript.


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### Notes

The authors declare no competing financial interest.

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