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A benzophenone-based photocaging strategy for the *N7* position of guanosine

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

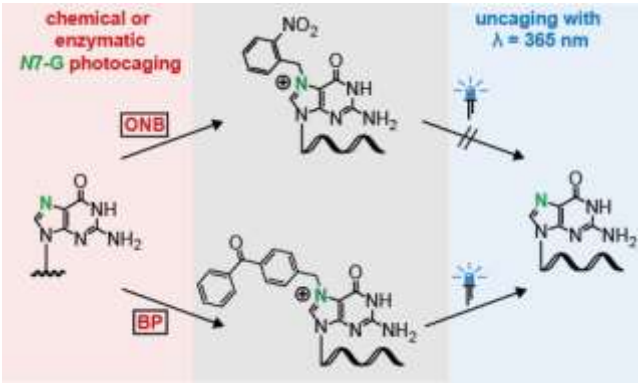
Abstract: The selective modification of nucleobases with photolabile caging groups provides one of the most versatile strategies to study and control the processes and interactions of nucleic acids. Numerous exocyclic and ring positions of nucleobases have been targeted, but all are formally substituting a hydrogen atom with a photo-caging group. Nature, however, also explicitly uses ring nitrogen methylation, such as m^7G and m^1A, to change the electronic structure and properties of RNA and control biomolecular interactions essential for translation and turnover. We report that aryl ketones such as benzophenone and *α*-hydroxyalkyl ketone are photolabile caging groups if installed at the *N*7 position of guanosine or the *N*1 position of adenosine. Common photo-caging groups derived from the *ortho*-nitrobenzyl moiety were not suitable for these positions. Both chemical and enzymatic methods for site-specific modification of *N*7G in nucleosides, dinucleotides and RNA were developed, opening the door to studying the molecular interactions of m^7G and m^1A with spatio-temporal control.

Light is a versatile regulatory element because it is fully orthogonal to most cellular components, noninvasive, controllable in timing and localization to tissues, cells and even subcellular compartments.[1] To gain optical control over nucleic acids and the respective biomolecular interactions, irreversible photo(de)activation ("uncaging") has been applied to nucleosides, nucleotides and oligonucleotides.[1-2] Various photolabile protecting groups have been placed either in the oligomer backbone or on the nucleobases and their removal by light of a defined wavelength ≥365 nm was proven to be compatible with living cells and organisms.[3] The *ortho*-nitrobenzyl (ONB) group is the most widely used photocaging (PC) group, but numerous alternative scaffolds, derived from coumarins, quinolines, dibenzofuran or the piperonyl group have been explored.[1-2]

Typically, these PC groups are installed at exocyclic heteroatoms, such as the O^4 in thymidine or the N^4 of cytidine.[4] In addition, the *N*3 position of thymidine was successfully used in optochemical biology.[3b] In purines, the exocyclic O^6 of guanosine and N^6 of adenosine are preferred sites for installation of photocaging groups.[5] In the case of guanosine, other positions like the *N*1, C8 or the exocyclic N^2 position have been explored albeit to a much lower extent.[6] For chemo-enzymatic approaches, where photo-caged NTPs are synthesized and enzymatically introduced into DNA or RNA by polymerases, the 5 position of pyrimidines is particularly favorable.[7] Recently, also the post-enzymatic photocaging of DNA was achieved using methyltransferase (MTase)-catalyzed transfer of PC groups to the N^6 position of adenosines.[8] In all of these cases, the PC group replaces a hydrogen atom and does not change the charge distribution of the nucleoside.

However, unlike the chemical modifications with PC groups developed to date, nature does not only substitute hydrogen atoms with modifications, but also explicitly uses methylation to change the electronic structure of the molecule. In naturally occurring m^7G, m^1A and m^3C, the modifications confer a positive charge to the nucleobase. Out of these, m^7G deserves particular attention because it is (*i*) one of the most conserved modified nucleosides, (*ii*) installed by numerous MTases in different organisms and (*iii*) found in rRNA, tRNA, snRNA as well as part of the 5′ cap in mRNA.[9] In the latter, m^7G is crucial for coordinating mRNA translation and turnover by several interactions, such as binding to the translation initiation factor eIF4E or decapping scavenger enzymes DcpS.[10] Due to its importance in biology, we chose the *N*7 position of guanosine for modification with functional groups with the goal to remove them upon irradiation with light and recover the free guanosine. In the 5′ cap of mRNAs, the G becomes remethylated in cells.[11] To generate photocaged guanosines in different contexts, i.e. from single nucleosides to long mRNAs, we devised both a chemical and an enzymatic preparative route (Scheme 1). PC groups for nucleosides that do not substitute a hydrogen atom but instead introduce a positive charge have not been reported to date to the best of our knowledge.

First, we explored the chemical photocaging of guanosine **1** using the well-known *ortho*-nitrobenzyl (ONB) group as well as *para*-nitrobenzyl (PNB) as negative control. The *N*7 of guanosine is the most potent nucleophile in DNA and RNA.[12] However, although *N*7G methylation was used in Maxam Gilbert sequencing,[13] the chemical modification of *N*7 has not been exploited to manipulate and control interactions by orthogonal



Scheme 1. Concept for chemical and enzymatic photocaging of the *N*7 of guanosine using classical *ortho*-nitrobenzyl (ONB) group or aryl ketones such as benzophenone (BP) to generate the respective nucleoside, 5′ cap or RNA. *N*7-BP-modified guanosine is uncaged by subsequent irradiation with light (λ_{max} = 365 nm).

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triggers such as light. Building on the good nucleophilicity of the *N*7 position of guanosine, we reacted guanosine with the respective PC-bromides **2a-b** and obtained the expected *N*7-*ortho*-nitrobenzyl- (*N*7-ONB) and *N*7-*para*-nitrobenzyl-(*N*7-PNB) guanosines **3a-b** (Figure 1A, Figure S3-S5) in good yields (**3a**: 53% and **3b**: 56%).[14] Subsequent irradiation of **3a**, however, did not yield the desired photocleavage to release guanosine **1** but instead a different main product according to HPLC (Figure 1B, Figure S6). Mass analysis revealed a mass of m/z = 347.1475 indicating a mass loss of m/z = 71.98 from **3a**, which would correspond to loss of CO and CO_2 (Figure 1C). After isolating the product, we could identify the structure of **4** containing a guanidine moiety based on NMR and UHPLC-MS/MS analyses (Figure S7, S50-55). Importantly, the controls **3b** as well as unmodified **1** were stable when irradiated under the same conditions (Figure S8-S9). These data indicate that the *N7* position of guanosine can be readily derivatized with the well-known ONB group, however the photocleavage leads to an unusual product instead of guanosine, limiting its application in photocaging approaches.

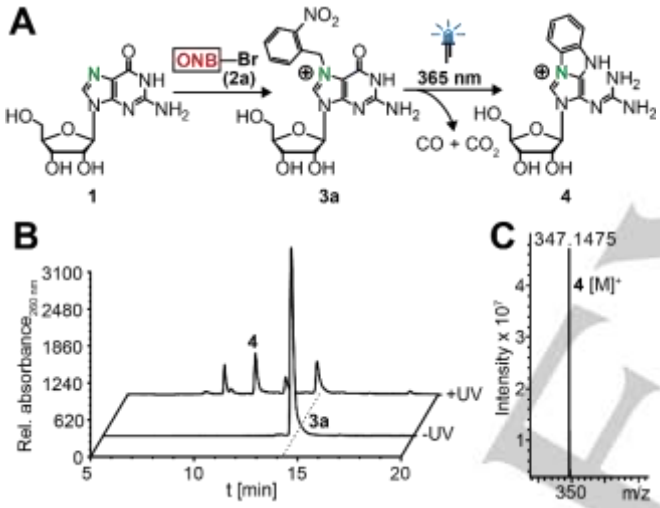

Figure 1. Chemical modification of the *N*7 position of guanosine derivatives with the common ONB group and subsequent irradiation. (A) Concept. (B) HPLC analysis before and after irradiation of **3a** at 365 nm. (C) Mass analysis of **4** (calculated mass of $[C_{15}H_{19}N_6O_4]^+$ = 347.1462 $[M]^+$, found: 347.1475).

We therefore turned away from the classical PC groups and contemplated alternative light-sensitive groups as potential position-specific PC groups. Photoactivatable aryl ketone derivatives such as benzophenone (BP) are widely used biochemical probes.[15] BP can be manipulated in ambient light and activated at λ ~ 360 nm. It is chemically stable and reacts preferentially with unreactive C-H bonds, which is widely used to study protein-protein but also protein-RNA interactions.[16] The substituents on BP can affect the photochemistry significantly and electron-withdrawing groups increase the efficiency of H-abstraction.[15] We therefore reasoned that the positive charge at the *N*7 position might lead the radical to a different reaction pathway and therefore considered aryl ketone derivatives, such as BP or a 2-hydroxy-2-methyl-1-phenylpropan-1-one group (HAK), as a potential PC groups for the *N*7 position of guanosine. We chemically synthesized *N*7-BP-guanosine **3c** (Figure S3-S4) and tested its uncaging properties (Figure 2A)**.** After irradiation of **3c** with light (λ_{max} = 365 nm) in buffer containing EDTA and glycerol, we found free guanosine **1** and 4-methylbenzophenone **5** as cleavage products, indicating that BP can be used as photocaging group for the *N*7 position (Figure S11, Figure S10A-C). To learn more about the cleavage mechanism, we tested the components of the reaction buffer in more detail. We found that the cleavage reaction did not occur in plain water but when EDTA, glycerol or especially cell lysate were present, suggesting that hydrogen abstraction occurs from these buffer components or in the case of cell lysate other components (Figure 2B, Figure S11).

A possible cleavage mechanism is shown in Figure 2C. Upon irradiation with λ_{max} = 365 nm, benzophenone generates a triplet ketone, which abstracts a hydrogen atom from a C–H bond of EDTA or glycerol.[16] The ketyl radical thus formed does not engage in a C–C cross link, but the positive charge at the *N*7 position favors fragmentation to recover guanosine **1** along with the radical cation **A**. Single electron reduction of **A** by the EDTA or glycerol derived radical and tautomerization eventually leads to **5**.

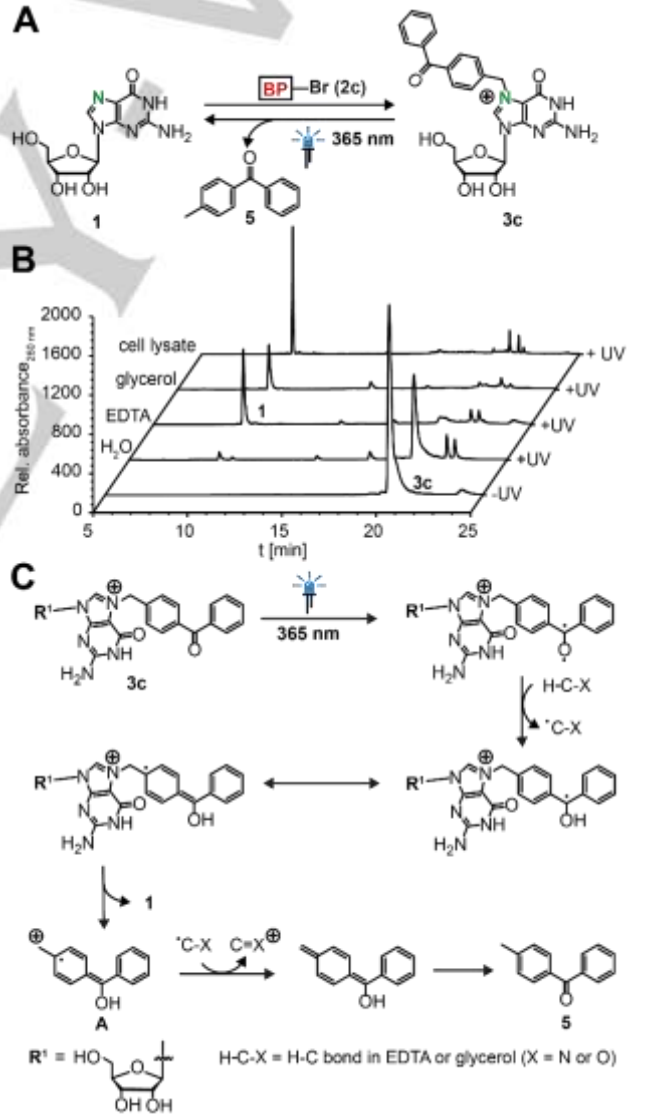

Figure 2. Chemical preparation of *N*7-BP-guanosine and subsequent irradiation. (A) Concept. (B) HPLC analysis before and after irradiation of **3c** at 365 nm in aqueous solution with different additives. (C) Postulated mechanism for the photocleavage.

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To test whether this strategy can be applied to the other purine nucleosides, we synthesized *N*1 benzophenone-modified adenosine (**7**) (Figure S4).[17] The corresponding methylated nucleoside m^1A has recently been identified in mRNA and its functional role is still investigated.[18] This compound also has a positive charge, suggesting that photouncaging of aryl ketone derivatives might be possible in an analogous way. Indeed, we observed that irradiation of **7** with light of 365 nm led to complete formation of adenosine **6** and release of **5** similar to *N*7-photocaged guanosine (Figure S12-S13).

To examine whether our strategy can be extended to more complex molecules, we chose the dinucleotide GpppA **8**, which is the hallmark structure of the 5′ cap found in eukaryotic mRNAs. Since site-specific chemical modification is not possible in this case, we devised an enzymatic approach, exploiting the site-specificity of the cap *N*7-methyltransferase Ecm1 together with its cosubstrate promiscuity.[19] To this end, we synthesized analogs of the natural cosubstrate *S*-adenosyl-L-methionine (AdoMet), suitable for transfer of the classical ONB-based and novel BP-based photocaging groups, i.e. AdoONB **9a**, AdoPNB **9b** and AdoANB **9c** as well as AdoBP **9d** (Figure S14).[8, 19c] These AdoMet analogues are well accepted by the highly promiscous Ecm1 resulting in efficient formation of the desired products *N*7-PC-GpppA **10a-d** (Figure 3A-B, Figure S15), according to HPLC and UHPLC-MS analysis (Figure 3C, Figures S16-S19). In line with the results obtained for the nucleoside, irradiation of *N*7-ONB-GpppA **10a** did not yield the uncaged **8** but instead a product **11a** with a mass loss of m/z = 71.98 (Figures 1C-D, Figure S20). Similarly, irradiation of *N*7-ANB-GpppA **10c** revealed a new product **11c** with a mass loss of m/z = 71.98 (Figure S17,S19E). More detailed analyses of **10a** and **11a** by enzymatic digestion into single nucleosides using Snake Venom phosphodiesterase revealed that the mass loss occurred exclusively at the *N*7-ONB-guanosine moiety of the dinucleotide (Figure S21-S23). The controls, PNB-caged GpppA **10b** as well as uncaged GpppA **8**, remained stable when irradiated under these conditions, in line with our results from the guanosine (Figure S16,S24).

The benzophenone group, however, was again suitable for photouncaging at the *N*7G of the 5′ cap. Specifically, **10d** was almost completely reacted (79% decrease) when irradiated in buffer, and GpppA **8** was formed as main product (Figure 3E, Figure S18A-B). As in the case of the nucleoside, addition of buffer was required to yield **8** and release 4-methylbenzophenone **5** (Figure S25-S26). Importantly, the uncaged product **8** could be re-modified enzymatically using the BP-group, confirming that the free and functional GpppA **8** had been formed after photocleavage of the BP group (Figure S18C).

To test whether other aryl ketone derivatives could also be suitable for photocaging, we used the *α*-hydroxyalkyl ketone (HAK) substituent, which is known to normally react according to Norrish type I to give the acylradical and ketylradical.[20] We enzymatically produced *N*7-HAK-GpppA **10e** from GpppA **8** using Ecm1 and AdoHAK **9e** (Figure S14-S15,S27). Irradiation of **10e** with light of 312 nm in the reaction mixture led to recovery of almost 60% of **5** (Figure S28). UHPLC-MS analysis verified the photocleavage to GpppA **8** and small amounts of side-products, namely the *N*7-benzoic acid-modified cap analogue **11e,a** and the corresponding *N*7-benzaldehyde-modified one **11e,b** (Figure S29-S230). These data show that the HAK group is also a photocaging group for the *N*7 position of guanosine nucleotides, suggesting that our approach can be extended to other aryl ketones.

Furthermore, we enzymatically modified plasmid DNA at the N^6 position of deoxyadenosine using MTase TaqI and AdoBP **9d**, which worked efficiently (Figure S31). As expected, no uncaging and thus no plasmid degradation were observed after irradiation with light of 365 nm in buffer (Figure S31B). This showed that the positive charge at the *N*7 position is required for the photocleavage of aryl ketone derivatives such as BP.

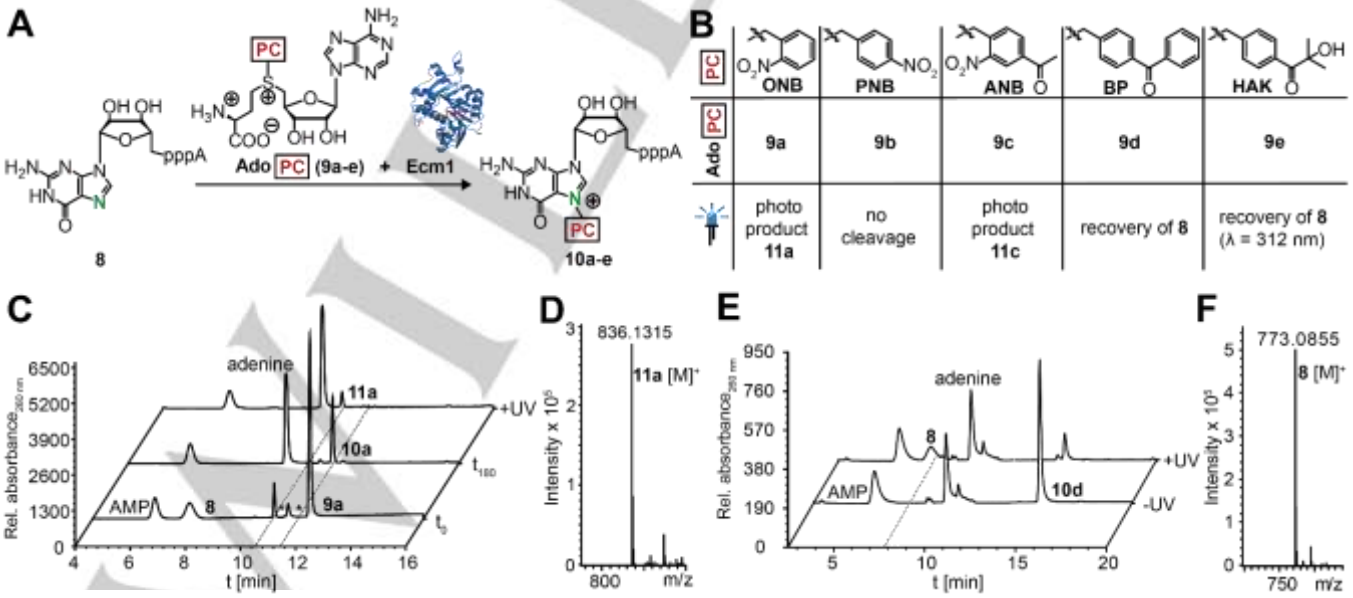

Figure 3: Enzymatic modification of *N*7 position of 5′ cap structure GpppA. (A) Concept. (B) Panel of PC groups tested and summary of irradiation results. (B) HPLC analysis of enzymatic reaction of **8** to **10a** and subsequent irradiation at 365 nm. (D) Mass analysis of **11a** (calculated mass of $[C_{25}H_{33}N_{11}O_{16}P_3]^+$ = 836.1314, found: 836.1315). (E) HPLC analysis of enzymatic reaction of **8** to **10d** before and after irradiation at 365 nm in buffer. (F) Mass analysis after irradiation, **8**: Calculated mass of $[C_{20}H_{28}N_{10}O_{17}P_3]^+$ = 773.0841 $[M+H]^+$, found: 773.0855. (* = impurities of **9a**)

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To study whether our strategy is applicable to photocaging of oligonucleotides, we used a panel of short 5–21 nucleotide long RNAs with internal guanosine sites (Figure S33A). These RNA oligonucleotides were incubated with BP-bromide **2c**, resulting in *N*7G but not *N*1A modification according to UHPLC-MS analysis after digestion and dephosphorylation to single nucleosides. The modified RNAs were then irradiated with light of 365 nm in buffer containing EDTA and single nucleosides were analyzed by UHPLC-MS. The data showed that BP was removed from *N*7-BP-guanosine after irradiation, suggesting that photocaging and uncaging is possible also in the context of RNA oligonucleotides (Figures S10D-E,S32-S39). Neither the reaction with **2c** nor the irradiation at λ_{max} = 356 nm led to RNA degradation as shown in denaturing PAGE analysis (Figure S38). Furthermore, the *N7*-modified caps were successfully used for in vitro transcription to produce long reporter mRNAs (>1000 nt) and these also remained intact after irradiation under the same conditions (Figure 4B).

Finally, we tested whether photocaging the *N7* position of guanosine can be used to block a biological function (Figure 4A). The 5′ cap is involved in several interactions, most notably eIF4E for translation and decapping enzymes, such as DcpS, for RNA turnover.[10] These interactions require *N7* methylation and unmodified caps have been show to become remethylated in the cytoplasm.[11] We measured the K_d values of recombinantly produced eIF4E and the inactive variant DcpS (H277N) for the native and modified cap and found that these proteins are not binding to *N*7-BP-modified-GpppA, whereas m^7GpppA is bound, showing a K_d value in the sub-micromolar range—in line with reports in the literature (Figures S40-S41, Table S1).[10d, 21] After irradiation and enzymatic remethylation mimicking the cellular remethylation, the binding to both proteins was fully restored (Figure 4C-D). These data show that benzophenone can be used to block and release biologically relevant functions.

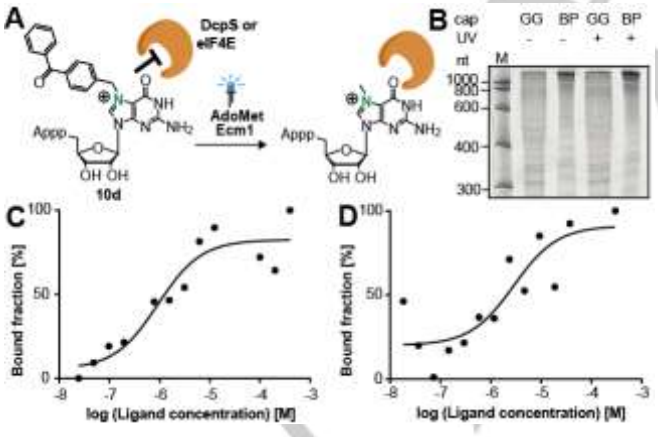

Figure 4. Photocaging of *N*7 of guanosines blocks cap binding proteins and can be used to generate long RNAs. (A) Binding assay of *N*7-BP-GpppA was performed with DcpS (H277N) and eIF4E before and after photouncaging and remethylation. (B) *N*7-BP-modified cap was used to produce long mRNAs. These were stable under irradiation, if no H-donor was added. (C, D) Binding of **10d** to eIF4E and DcpS (H277N) is restored by irradiation and remethylation.

In summary, we have developed a strategy for photocaging purine nucleosides via formation of purine iminium ions. We present aryl ketone derivatives as new class of photolabile groups for these purine imine positions as exemplified for *N*7G and *N*1A and show that the common ONB group is not suitable for *N*7G. We developed both a chemical and an enzymatic strategy to photocage and release *N*7G, presenting the first photocaged nucleoside that strictly affecting the Hoogsteen recognition site of G to the best of our knowledge. Hoogsteen interactions are important in RNA biology, e.g. in riboswitches, ribozymes or formation of G quadruplexes. Furthermore, due to the biological importance of m^7G in the 5′ cap and the enzymatic remethylation process in nature, our approach significantly expands the chemical biology toolbox and opens the door to control the functions of the 5′ cap with spatio-temporal control in the biological context. The photocaging of *N*7G and *N*1A can be further improved by testing additional aryl ketone derivatives that are excited at longer wavelengths and exploiting the two-photon excitation properties of BP in the future.[22]

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Keywords: *N*7G • photocaging • RNA modification • benzophenone • RNA

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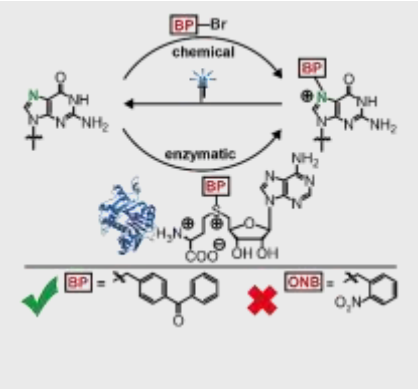
Layout 1:

COMMUNICATION

A strategy was developed to chemically or enzymatically photocage the *N*7 position of nucleosides, dinucleotides and RNA, which could be removed upon irradiation with λ_{max} = 365 nm. The well-known *ortho*-nitrobenzyl photocaging groups were unsuitable, however aryl-ketone derivatives as the benzophenone group were identified as potential photocaging group for purine imine positions.



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A benzophenone-based photocaging strategy for the *N7* position of guanosine