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Photocaged 5-(Hydroxymethyl)pyrimidine Nucleoside Phosphoramidites for Specific Photoactivatable Epigenetic Labeling of DNA

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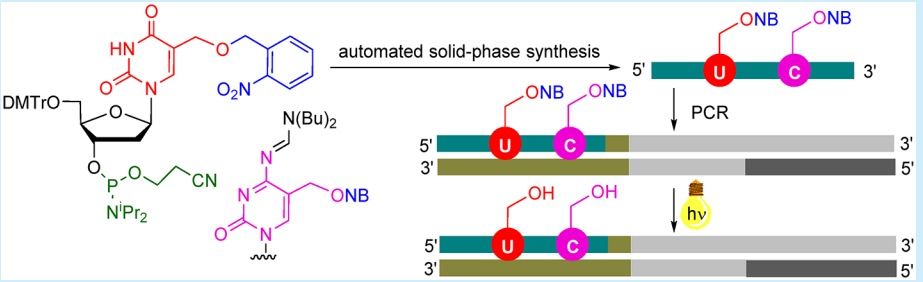

ABSTRACT: 5-Hydroxymethylcytosine and uracil are epigenetic nucleobases, but their biological roles are still unclear. We present the synthesis of 2-nitrobenzyl photocaged 5-hydroxymethyl-2′-deoxycytidine and uridine 3′-*O*-phosphoramidites and their use in automated solid-phase synthesis of oligonucleotides (ONs) modified at specific positions. The ONs were used as primers for PCR to construct DNA templates modified in the promoter region that allowed switching of transcription through photochemical uncaging.

Oxidized congeners of 5-methylcytosine, i.e., 5-hydroxymethylcytosine (5hmC) or 5-formylcytosine (5fC), are not only intermediates in active demethylation of DNA, but they are also epigenetic modifications of DNA,[1,2] which regulate gene expression by influencing binding of transcription factors and RNA polymerases (RNAP) to DNA[3] and modulating chromatin properties.[4] To the contrary, the biological role of another minor DNA base, 5-hydroxymethyluracil (5hmU), is not yet fully understood,[5] although it was found in stem cells[6] or in some types of cancer.[7] 5hmU has also been found in certain bacteriophages[8] or parasites.[9] We have recently reported[10] a transcription study with bacterial RNA polymerases (RNAP) of DNA templates containing 5hmU or 5hmC. The study revealed that the presence of these epigenetic modifications can significantly enhance transcription (by up to 3.5 times), depending on the promoter. We found that it is the nontemplate strand of the promoter region where the presence of these modifications has the strongest effect.[10] Subsequently, we also developed[11] an artificial switch for transcription. Enzymatic incorporation of photocaged (2-nitrobenzyl, NB) derivatives of 5hmU or 5hmC 2′-deoxyribonucleoside triphosphates (dNTPs) resulted in caged DNA templates that were not compatible with transcription, because of the presence of the bulky nitrobenzyl groups in the major groove. However, their irradiation by UV or visible light (up to 400 nm) removed the photocaging groups and released the hmU/hmC-modified DNA, which became transcriptionally active.[11] Unfortunately, the enzymatic synthesis of DNA from base-modified dNTPs[12] is difficult to be used for site-specific incorporation of one single or several modification(s).[13] Such site-specifically modified DNAs would be useful for deeper structural and functional studies of the effect of these epigenetic modifications. Several protected 5hmC[14,15] and 5hmU[14,16] 2′-deoxyribonucleoside phosphoramidites suitable for automated solid-phase synthesis of oligonucleotides[17] are known in the literature, but their photocaged analogues have not been described yet. Considering the general importance of DNA photocaging[18] and the specific need of site-specific photocaging of a single nucleobase in the major groove of DNA, we set out to design and synthesize photocaged 5hmU and 5hmC 2′-deoxyribonucleoside phosphoramidites and to study their use in solid-phase synthesis of photocaged oligonucleotides.

The synthesis of the photocaged phosphoramidites started from thymidine, which was converted to *tert*-butyldimethylsilyl (TBDMS) protected 5-[(2-nitrobenzyl)oxymethyl]-2′-deoxy-

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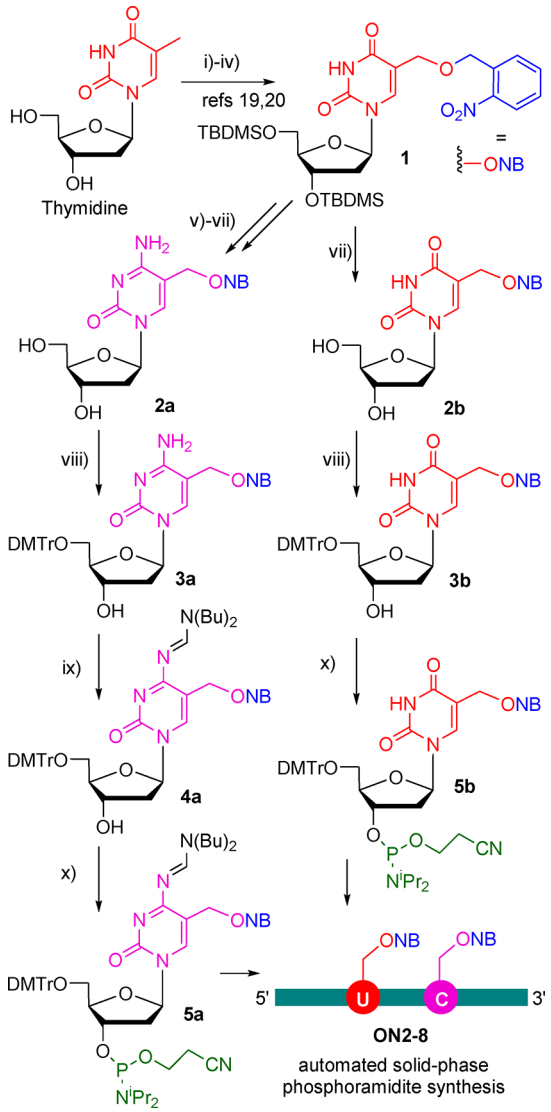
uridine (**1**) via a published four-step sequence (Scheme 1).[19,20] Intermediate **1** was then transformed to photocaged hmC (**2a**)

Scheme 1. Synthesis of the Nitrobenzyl-Photocaged Nucleoside Phosphoramidites[a]



[a]Reagents and conditions: (i) TBDMSCl, imidazole, DMF, 95%; (ii) NBS, azobisisobutyronitrile (AIBN), benzene; (iii) diisopropylethylamine (DIPEA), H_2O, DMF, 27%; (iv) 2-nitrobenzyl bromide, AgOTf, 2,6-di-*tert*-butylpyridine, DCM, 40%; (v) TIPSCl, DMAP, Et_3N, DCM; (vi) NH_3 (g), dioxane, 80%; (vii) $Et_3N{\cdot}3HF$, THF, 40%–50%; (viii) DMTrCl, DMAP, pyridine; 50%–70%; (ix) DBF-DMA, DMF, 76%; and (x) CEOP(Cl)$NiPr_2$, DIPEA, DCM, 70%–85%.

and hmU 2′-deoxyribonucleosides (**2b**) also, using known procedures.[20,21] Subsequent dimethoxytritylation of **2a** and **2b** gave the 5′-*O*-4,4′-dimethoxytrityl (DMTr) protected intermediates **3a** and **3b** in 68% and 50% yields, respectively. Various protecting group strategies were then attempted for the transient protection of the amino group of hmC in compound **3a** prior to converting it to the corresponding phosphoramidite. First, we tried to introduce benzoyl or acetyl groups, but we obtained low yields and/or side acylation at 3′-OH. Therefore, the dibutylformamidine protecting group was attached through the reaction of **3a** with dibutylformamide-dimethylacetal (DBF-DMA) in anhydrous dimethylformamide (DMF).[22] This gave the corresponding amidine derivative **4a** in good yield, which was further converted to the phosphoramidite **5a** (85% yield) by treatment with 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (CEOP-(Cl)$NiPr_2$), in analogy to the literature protocol.[17] In the same way, the photocaged hmU nucleoside **3b** was transformed to the desired phosphoramidite **5b** in 74% yield.

The photocaged building blocks **5a** and **5b** were then used in the synthesis of oligo-2′-deoxyribonucleotides (ONs) aimed for the construction of specifically modified DNA templates for the study of bacterial transcription. In previous works, we used a 339 mer DNA template containing the 38 bp-long P*veg* promoter for the study of the influence of major-groove modifications on transcription with RNAP from *E. coli*.[10,11,23] Those fully modified templates were constructed using PCR with modified dNTPs. For specific single-point modifications in the nontemplate strand of the promoter region, we previously designed the synthesis through PCR using modified forward primer.[11] In order to access the important regions of the promoter, we decided to truncate the template to a 222mer DNA containing only a core promoter flanked by only two nucleotides at the 5′-end (Figure 1A). This shortened 222mer template has retained ∼90% of the activity of the original 339 mer construct. Based on a structural study[24] and on our previous experience,[25] we identified specific sites of the core promoter, mainly in the −35 region of the nontemplate strand, as important for the interaction with the RNAP. Therefore, we designed photocaged ONs (**ON2**−**ON5**) containing one or several photocaged hmU or hmC bases in the −35 region (Figure 1B) and, for comparison, ONs containing these modifications outside of that region (**ON6**−**ON8**). ONs **ON2**−**ON8** were synthesized on an automated DNA synthesizer using phosphoramidite building blocks **5a** and/or **5b**. The synthesis proceeded under standard conditions, giving the desired 20- or 21-nt photocaged ONs with efficient incorporation of the modified phosphoramidites, compared to their natural counterparts.

The photocaged ONs (**ON2**−**ON8**), along with the natural **ON1**, were then used as forward primers for the PCR reaction on the P*veg* plasmid, along with a natural reverse primer (for the 222mer template) and natural dNTPs (Figure 1C). In all cases, full-length 222mer DNA amplicons (**222DNA1** and **NB-222DNA2**−**NB-222DNA8**) modified in the promoter region were obtained efficiently and after isolation were used as templates for in vitro transcription experiments with *E. coli* RNAP. The photocaged DNA templates **NB-222DNA2**−**NB-222DNA8** then were irradiated by a 3-W 400-nm photodiode for 10 or 30 min in the presence of additives DTT and sodium azide (in analogy to previous works[11,20,21]) to release the uncaged DNA containing unprotected bases hmU and/or hmC (**hm-222DNA2**−**hm-222DNA8**; see Figure 1D) and the uncaged templates were also used for the transcription experiments, which were performed as reported previously.[10,11,23]

The results of the in vitro transcription experiments are summarized in Figure 1E. The natural DNA template (**222DNA1**) yielded about the same transcription before and after irradiation. DNA templates containing the photocaged bases in the −35 region (**NB-222DNA2**−**NB-222DNA5**), which is critical for interaction with RNAP, displayed significantly lower transcription (14%–78%), whereas transcription of templates modified outside the −35 region (**NB-**

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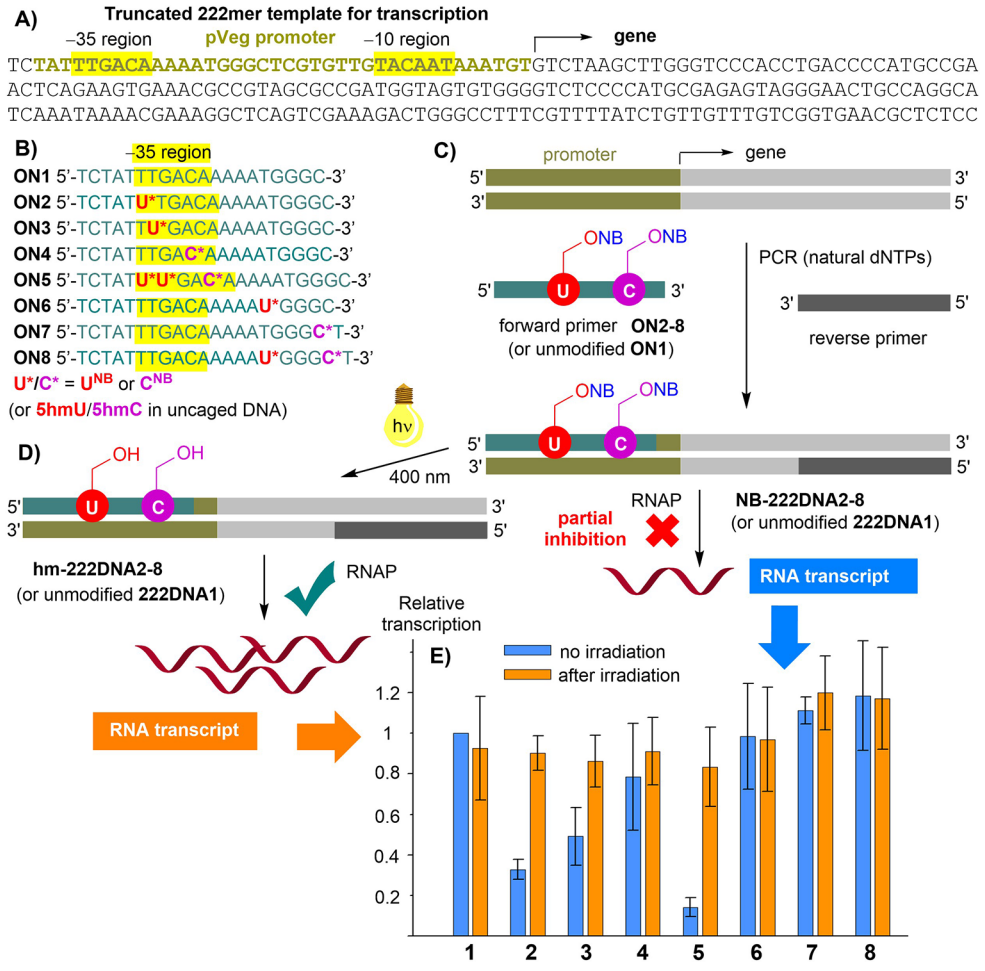

Figure 1. (A) Sequence of the DNA template. (B) Sequences of the modified photocaged ONs. (C) PCR construction of DNA templates containing photocaged pyrimidines in the promoter region. (D) Photochemical uncaging of the templates. (E) Relative yield of transcription with *E. coli* RNAP from either photocaged (blue) or uncaged (orange) DNA templates. The bars show averages from at least five independent experiments, the error bars show the standard deviation (±SD). Transcription from the nonirradiated nonmodified template was set as 1. The numbers below the graph correspond to construct numbers as shown in panel (B).

222DNA6–**NB-222DNA8**) was comparable to the unmodified control. The lowest transcription was observed from the template containing NB-U at the −35 position (**NB-222DNA2**, 33%) and the template with a combination of three photocaged pyrimidines (**NB-222DNA5**, 14%). After irradiation and uncaging of the hydroxymethylpyrimidine bases, the transcription of the out-of-region-modified DNA templates was unchanged (**hm-222DNA6**–**hm-222DNA8**), but the transcription of the in-the-region-modified uncaged templates (**hm-222DNA2**–**hm-222DNA5**) was restored to the level of the unmodified control. In other words, the transcription was switched on.

In conclusion, we have developed the synthesis of 2-nitrobenzyl-photocaged 2′-deoxyribonucleoside 3′-phosphoramidite derivatives from 5hmU and 5hmC nucleobases. These phosphoramidite building blocks were used for automated synthesis of oligonucleotides containing the photocaged pyrimidine bases bearing epigenetic modifications at specific positions. We used this approach for the synthesis of photocaged primers to obtain DNA templates modified in the promoter region. We synthesized several templates containing one or more photocaged pyrimidines within and outside the −35 region of the P*veg* promoter and tested them in in vitro transcription with RNAP from *E. coli*. As expected, the presence of photocaged pyrimidines within the −35 region significantly (though not completely) inhibited the transcription, whereas their presence outside of this region did not affect the transcription. After irradiation with visible light (400 nm), the release of hmU or hmC bases was achieved and the transcription reached the level of unmodified template. The most significant effect was observed for T/hmU at position −35 and for the combination of three pyrimidines in the −35 region. Interestingly, we have not observed an increase in transcription, compared to the natural template, in any case. Since in our previous works, we have shown that the templates fully modified with hmU or hmC exert increased transcription (up to 350% or 250%, respectively),[10,11] it seems that a combination of several hydroxymethylpyrimidines at several positions in the promoter is needed for the enhancement. The new photocaged phosphoramidites **5a** and **5b** will enable further, more-detailed studies of the influence of pyrimidines with epigenetic modifications on transcription with both bacterial and eukaryotic RNA polymerases, to decipher the biological roles of those epigenetic modifications and to develop optical switches for transcription.[11,18]

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.0c03462.

Full experimental part with synthetic procedures, characterization of compounds, detailed gels of PCR and transcription experiments (PDF)

FAIR data, including the primary NMR FID files, for compounds **3a**, **3b**, **4a**, **4b**, **5a**, and **5b** (ZIP)

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Notes

The authors declare no competing financial interest.

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