Photocaged DNA

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# Polymerase Synthesis of Photocaged DNA Resistant against Cleavage by Restriction Endonucleases**

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**Abstract:** *5-[(2-Nitrobenzyl)oxymethyl]-2′-deoxyuridine 5′-O-triphosphate was used for polymerase (primer extension or PCR) synthesis of photocaged DNA that is resistant to the cleavage by restriction endonucleases. Photodeprotection of the caged DNA released 5-hydroxymethyluracil-modified nucleic acids, which were fully recognized and cleaved by restriction enzymes.*

Photocaging of biomolecules,[1] and nucleic acid components in particular,[2] is increasingly popular and useful as the photocleavage of protecting groups is inherently biocompatible and bioorthogonal. In nucleic acids (both DNA and RNA) the photolabile protecting groups are either linked to the Watson–Crick edge of nucleobases[3] (typically at N3 or exocyclic $O^4$/$N^4$ atoms of pyrimidines) or at the phosphodiester backbone.[4] The former type is unable to form duplexes,[3] whereas the phosphate-caged oligonucleotides hybridize well, but are resistant to enzymatic cleavage.[4] Recently, non-aromatic 5-arylsulfanyl-5,6-dihydro-pyrimidines were used[5] as caged nucleobases, both for DNA and RNA, to modulate secondary structures in which the natural nucleic acids were released by radical photodisproportionation. All of these types of caged nucleic acids can only be synthesized chemically because the corresponding caged (deoxy)nucleoside triphosphates (NTPs or dNTPs) are not suitable substrates for polymerases.[3–5] Major-groove photocaged 5-(aminopropargyl)-pyrimidine dNTPs were also reported and used for the polymerase synthesis of DNA and the photochemical release of the reactive amino functions.[6] A series of α-branched 2-nitrobenzyl-protected 5-(hydroxymethyl)pyrimidine and 7-hydroxymethyl-7-deazapurine dNTPs have been studied as photocleavable reversible terminators for sequencing.[7]

In our systematic study of the influence of the major-groove modifications of DNA on the sequence-specific recognition and cleavage by restriction endonucleases (REs) we found[8] that certain small modifications are tolerated by some REs at position 5 of uracil and at position 7 of 7-deazaadenine, whereas all bulky modifications or any modifications on cytosine completely prevent the cleavage. This fact was later applied[9] in the first transient chemical protection of DNA from RE cleavage by polymerase incorporation of 7-(triethylsilylethynyl)-7-deazaadenine nucleotides to form RE-resistant protected DNA which, after desilylation by treatment with ammonia, released ethynyl-modified DNA fully cleavable by several REs. Recently, this modification was used[10] for a new protecting-group strategy for gene cloning and expression. However, the need of treatment with non-biocompatible ammonia to deprotect the modified DNA prevents any in vivo applications of the silylethynyl protection.

Therefore, we turned our attention to photocaging of the major groove by bulky photolabile groups. Metzker et al. found[7] that α-nonsubstituted 2-nitrobenzyl-protected 5-(hydroxymethyl)uracil dNTP (**dU$^{NB}$TP**) was a good substrate for DNA polymerases and was a poor terminator (the extension of the primer next to this modification was efficient). They also reported[7] that both **dU$^{NB}$TP** and the **U$^{NB}$**-containing DNA sequence can be easily deprotected to 5-(hydroxymethyl)uracil-containing nucleotides (**dU$^{HM}$TP**) or DNA by UV irradiation. This makes the **dU$^{NB}$TP** a very good candidate for construction of major-groove photocaged DNA. Moreover, the 5-(hydroxymethyl)uracil-modified DNA is interesting in itself, as this noncanonical nucleobase was found to be present in some genomes.[11] Recently, U$^{HM}$ was found to influence binding of transcription factors and chromatine remodelling in mouse embryonic stem cells.[12] The related 5-(hydroxymethyl)cytosine and its oxidized derivatives 5-formyl- and 5-(carboxy)cytosine are of paramount importance as newly discovered epigenetic markers,[13] which are not only intermediates in the demethylation of cytosine but also act as specific signals for gene expression.[14] Here we report the use of nitrobenzyl-protected triphosphate **dU$^{NB}$TP** for the polymerase construction of major-groove photocaged DNA that is resistant to RE cleavage, and the photodeprotection leading to U$^{HM}$-modified DNA.

The synthesis of **dU$^{NB}$TP** was performed according to reported procedures.[7,15] Analogously, the hydrolytically stable model 5′-*O*-monophosphate **dU$^{NB}$MP** was prepared in order to study the kinetics of the photocleavage. The photolysis of **dU$^{NB}$MP** in water using a weak light-emitting diode (365 nm, 1 mW) showed that the reaction is completed within 6 h, giving a clean conversion to the hydroxymethyl

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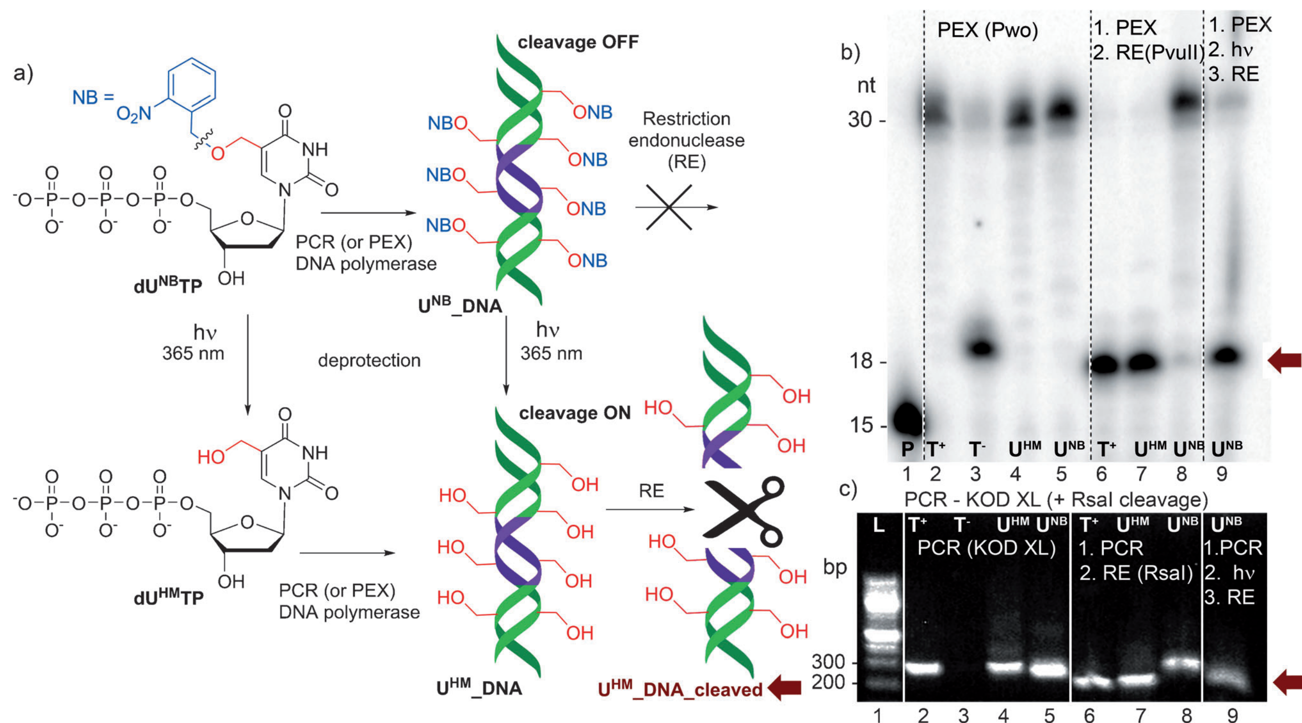


***Figure 1.*** a) Polymerase synthesis of $U^{NB}$- or $U^{HM}$-modified DNA, photochemical deprotection and/or cleavage by restriction endonucleases. b) PAGE analyses of PEX (Pwo polymerase, 30-mer template $temp^{PvuII}$), deprotection (UV light), and RE cleavage (PvuII) experiments. Lane 1, P: primer; lane 2, $T^{+}$: product of PEX with natural dNTPs; lane 3, $T^{-}$: product of PEX with dATP, dGTP and dCTP, lane 4, $U^{HM}$: product of PEX with dATP, dCTP, dGTP, **dU^HM^TP**; lane 5, $U^{NB}$: product of PEX with dATP, dCTP, dGTP, **dU^NB^TP**; lane 6, $T^{+}$: product of PEX with natural dNTPs followed by cleavage with a RE (PvuII); lane 7, $U^{HM}$: product of PEX with dATP, dCTP, dGTP, **dU^HM^TP** followed by cleavage with RE (full cleavage); lane 8, $U^{NB}$: product of PEX with dATP, dCTP, dGTP, **dU^NB^TP** followed by cleavage with RE (no cleavage); lane 9, $U^{NB}$: product of PEX with dATP, dCTP, dGTP, **dU^NB^TP** followed by photodeprotection (365 nm) and cleavage with RE (full cleavage). c) Agarose gel analysis of PCR, photodeprotection, and RE (RsaI) cleavage experiments with 287-mer template. Lane 1, L: DNA ladder; lane 2, $T^{+}$: product of PCR with natural dNTPs; lane 3, $T^{-}$: product of PCR with dATP, dCTP, dGTP; lane 4, $U^{HM}$: product of PCR with dATP, dCTP, dGTP, **dU^HM^TP**; lane 5, $U^{NB}$: product of PCR with dATP, dCTP, dGTP, **dU^NB^TP**; lane 6, $T^{+}$: cleavage of the unmodified PCR product with RsaI; lane 7, $U^{HM}$: treatment of the $U^{HM}$-modified PCR product with RsaI (full cleavage); lane 8, $U^{NB}$: treatment of the $U^{NB}$-modified PCR product with RsaI (no cleavage); lane 9, $U^{NB}$: photodeprotection of the $U^{NB}$-modified PCR product followed by treatment with RsaI (full cleavage); cleavage products marked with magenta-colored arrow.

derivative **dU^HM^MP** (preparative experiment using a stronger LED (0.8 W) gave the isolated product in 76% yield in 40 min, see Figure S1 in the Supporting Information). Then, an analogous preparative photolysis of **dU^NB^TP** was performed for 50 min. to give the desired **dU^HM^TP** (Figure 1a) in 62% yield with only minor amounts of hydrolyzed by-products, which were separated by semipreparative HPLC.

Both dNTPs (**dU^NB^TP** and **dU^HM^TP**) were then tested as substrates for DNA polymerases in primer extension (PEX) or PCR. All tested polymerases (KOD XL, Pwo, and Vent(*exo-*)) successfully incorporated the dNTPs into a mixed sequence in primer extension using a 15-mer primer and 31-mer template (Figure 1b and Figure S3 in the Supporting Information). The PCR synthesis of a 98 bp and 287 bp DNA was also successful with both these **dU^X^TPs** (Figure 1c and Figures S8 and S9 in the Supporting Information) using KOD XL (but not Pwo and Vent(*exo-*)) polymerase.

Next, PEX was used for the synthesis of $U^{X}$-modified DNA containing recognition sequences specific for several REs (PvuII, RsaI, KpnI, AflII) and the products were treated with the corresponding RE. As expected, the presence of a bulky NB modification blocked the cleavage by all REs in all cases, whereas the sequences that contained small HM modifications were fully cleaved by the same enzymes (Figure 1b and Figures S4 and S5 in the Supporting Information). The 287-mer PCR products modified by $U^{NB}$ or $U^{HM}$ bases (159 modifications in the dsDNA) contained one recognition sequence (5′-GT/AC-3′) for RsaI RE. The treatment of these DNA constructs with RsaI also confirmed the previous observation that the $U^{HM}$ modifications are fully tolerated and recognized by the RE, whereas the presence of the bulky $U^{NB}$ bases protects the DNA against the RE cleavage (Figure 1c and Figure S9 in the Supporting Information). All modified PCR products and products of photolysis and RE cleavage were then successfully used as templates for another PCR with natural dNTPs, and sequenced to confirm the fidelity and specificity of the replication with the modified **dU^X^TPs** and the site of restriction. We also prepared a 99 bp PCR product that contained $U^{NB}$ modifications and cloned it to a plasmid both without and after photodeprotection. The plasmids were then transfected to *E. coli*, and the replicated plasmids from transformed colonies were isolated and sequenced (see


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Figure S13 in the Supporting Information). These experiments fully confirmed the fidelity of incorporation, the biocompatibility of the modified nucleobase, and the absence of DNA damage as a result of UV irradiation.

Our last goal was to develop the photodeprotection to convert the RE-resistant $U^{NB}$-modified DNA to RE-cleavable $U^{HM}$-modified DNA. A model PEX experiment was performed using a 19-mer biotinylated template (*Temp*$^{oligo1T\text{-}bio}$) with **dU$^{NB}$TP** followed by magnetoseparation to get a single-stranded oligonucleotide (ON) that contained one $U^{NB}$ modification. This ON was then photolyzed by irradiation with UV light (366 nm) for 24 h. Both the PEX product and the product of photolysis were characterized by MALDI-TOF to confirm the conversion of the $U^{NB}$ modification (*m*/*z* 6118) to $U^{HM}$ (*m*/*z* 5983; see Figure S7 in the Supporting Information). Next, the preparative photolysis was performed with the $U^{NB}$-modified PEX products (dsDNA) containing sequences specific for REs. The reaction time that was needed to reach full conversion depended on the sequence, but all photolysis reactions were completed within a maximum of 6 h. Finally, the photolysis products were treated with the corresponding RE. In all cases, the DNA was fully (or almost fully) cleaved (Figure 1 b and Figures S4 and S5 in the Supporting Information). The same photolysis of the $U^{NB}$-modified 287-mer PCR product followed by treatment with RsaI gave clean product of DNA cleavage (214-mer, Figure 1 c and Figure S9 in the Supporting Information). In order to confirm the full conversion of the photodeprotection, we measured UV spectra of the $U^{NB}$-containing PCR products before and after irradiation to show the disappearance of the peak at 264 nm (absorbtion of the NB group) and performed the digestion of the PCR products by Benzonase Digest Mix[16] followed by HPLC analysis, which also confirmed the conversion of $U^{NB}$ to $U^{HM}$.

In conclusion, we have shown that **dU$^{NB}$TP** can be efficiently used for polymerase synthesis of major-groove-photocaged DNA both by PEX (for short sequences containing several modifications) and by PCR (for long DNA sequences with a high frequency of modifications). These photocaged DNA sequences are replicable by DNA polymerases (including plasmid replication in *E. coli*), but are fully protected against specific recognition and cleavage by REs. Simple photolysis of the bulky $U^{NB}$-containing DNA molecules by UV irradiation converts them to nucleic acids that bear just small $U^{HM}$ modifications, which are recognized and cleaved by REs.

The good substrate activity of **dU$^{NB}$TP** in polymerase incorporations makes it suitable for in vitro selection applications for the development of functional DNA molecules (DNAzymes, aptamers) triggerable by light. DNA sequences that contain naturally occuring $U^{HM}$ can be synthetized either directly (by polymerase incorporation of **dU$^{HM}$TP**) or indirectly through photolysis of $U^{NB}$. The photodeprotection is bioorthogonal and any future biological applications of this system should thus be extendable to in vivo use.[1,2] The masking and modulation of sequence-specific recognition and cleavage of the DNA sequences by REs is applicable by itself in gene manipulation and cloning,[9,10] but hopefully also can be further extended to the modulation of binding of transcription factors in the regulation of gene expression. Follow-up studies along these lines are under way in our group.

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