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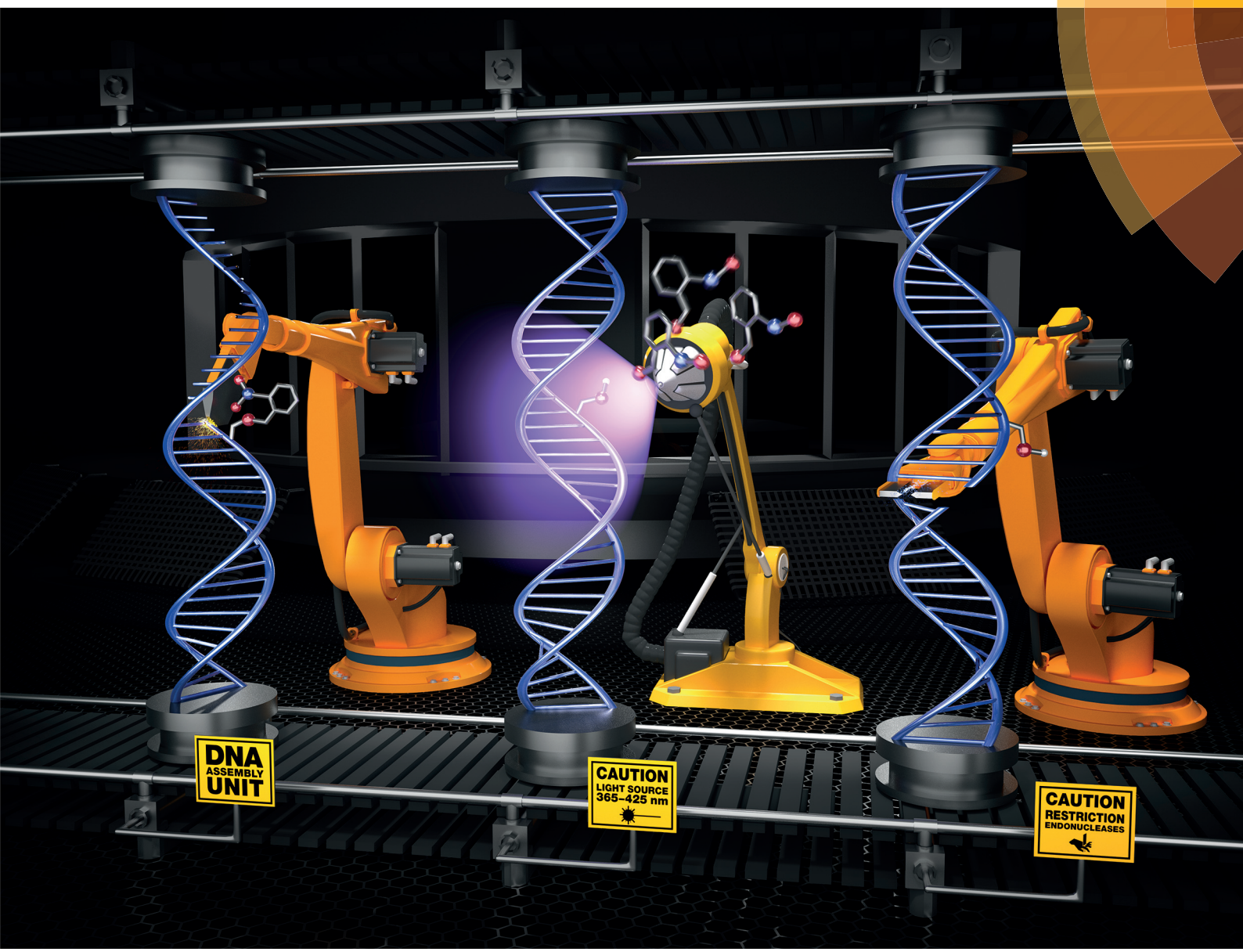

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Protected 2′-deoxyribonucleoside triphosphate building blocks for the photocaging of epigenetic 5-(hydroxymethyl)cytosine in DNA†

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2′-Deoxyribonucleoside triphosphates (dNTPs) containing 5-(hydroxymethyl)cytosine (5hmC) protected with photocleavable groups (2-nitrobenzyl or 6-nitropiperonyl) were prepared and studied as substrates for the enzymatic synthesis of oligonucleotides and DNA containing a photocaged epigenetic 5hmC base. DNA probes containing photocaged or free 5hmC in the recognition sequence of restriction endonucleases were prepared and used for the study of the photorelease of caged DNA by UV or visible light at different wavelengths. The nitrobenzyl-protected dNTP was a slightly better substrate for DNA polymerases in primer extension or PCR, whereas the nitropiperonyl-protected nucleotide underwent slightly faster photorelease at 400 nm. However, both photocaged building blocks can be used in polymerase synthesis and the photorelease of 5hmC in DNA.

Introduction

5-(Hydroxymethyl)cytosine (5hmC) is an epigenetic DNA base[1] which is not only an intermediate in active DNA demethylation[2] but also an epigenetic signal regulating gene expression.[3,4] Several protected 5hmC 2′-deoxyribonucleoside building blocks for the phosphoramidite synthesis of modified oligonucleotides (ONs) have been reported,[5] as well as the synthesis and use of the corresponding unprotected 5hmC 2′-deoxyribonucleoside triphosphate (**dC^{hm}TP**) in the polymerase synthesis of modified DNA.[6] For a deeper study of the role of 5hmC in the regulation of transcription,[3,4] genomic stability and active demethylation,[2] it might be advantageous to have a masked/caged 5hmC, which can be released in DNA on demand.

Photocleavable protecting groups[7] play an important role in chemical biology. The photocaging of biomolecules[7,8] and cleavage of the photolabile protective groups by light is often used for triggering or switching biological processes. In nucleic acids,[9] photocaging is mostly introduced at nucleobases[10] to prevent base-pairing and hybridization or at the sugar[11] or phosphodiester backbone[12] to prevent hydrolysis or interactions with other biomolecules. Photocaging in the major-groove of DNA has been studied less frequently.[13,14] Nitrobenzyl- (NB), and phenylethyl-protected 5-(hydroxymethyl)pyrimidine or 7-hydroxymethyl-7-deazapurine-2′-deoxyribonucleoside triphosphates (dNTPs) were used as reversible terminators of primer extension in sequencing.[14] We have recently reported the use of NB[15] or 6-nitropiperonyl (NP)[16] caged 5-hydroxymethyluracil dNTPs for the polymerase synthesis of photocaged DNA that can release 5hmU upon irradiation by UV or visible light (up to 425 nm for NP-protection). Here, we report on the photocaging of dNTPs derived from epigenetic 5hmC, their polymerase incorporation to DNA and photorelease.

Results and discussion

Synthesis

Photocaged 2′-deoxycytidines were prepared from previously reported modified 2′-deoxyuridines **1**[15,16] (Scheme 1). The silyl-protected deoxyuridines **1a** and **1b** were converted to the corresponding deoxycytidines **2a** and **2b** in two steps consisting of an activation of the oxo group through reaction with 2,4,6-triisopropyl-benzenesulfonyl chloride and DMAP, followed by nucleophilic substitution using ammonia. Protective groups were removed using $Et_3N{\cdot}3HF$ in THF to yield the desired caged nucleosides **dC^{NB}** and **dC^{NP}** in moderate yields of 41 and 39%, respectively. Triphosphorylation reactions were carried out under standard conditions.[17] The nucleosides were

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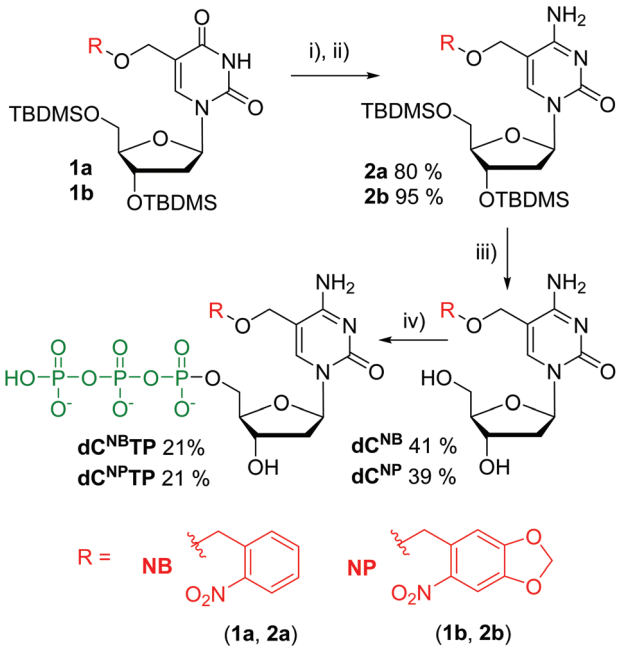


Scheme 1 Synthesis of photocaged 2′-deoxycytosine triphosphates (i) 2,4,6,-triisopropylbenzenesulfonyl chloride, DMAP, CH_3CN or DCM, (ii) NH_3 (g) or NH_4OH, (iii) $Et_3N{\cdot}3HF$, THF, (iv) 1. $POCl_3$, $PO(OMe)_3$, 2. $(NHBu_3)_2H_2P_2O_7$, 3. TEAB.

treated with $POCl_3$ in trimethyl phosphate at 0 °C followed by an addition of pyrophosphate and tributyl amine in DMF and, finally, treatment with triethylammonium bicarbonate (TEAB). The targets **dC^{NB}TP** and **dC^{NP}TP** were obtained in moderate yields after isolation with HPLC.

Fig. 1 shows the absorption spectra of the **dC^{NB}TP** and **dC^{NP}TP** in water and calculated molar absorption coefficients at the absorption maxima (for more detailed analysis of absorption spectra, see Fig. S1–S4 in the ESI†). While the NB-caged nucleotide **dC^{NB}TP** exerted an absorption maximum at 272 nm and showed a low absorption above 300 nm, the nitropiperonyl nucleotide **dC^{NP}TP** exerted another maximum at 359 nm and still some non-negligible absorbance at 400 nm indicating its potential for photorelease with visible light.

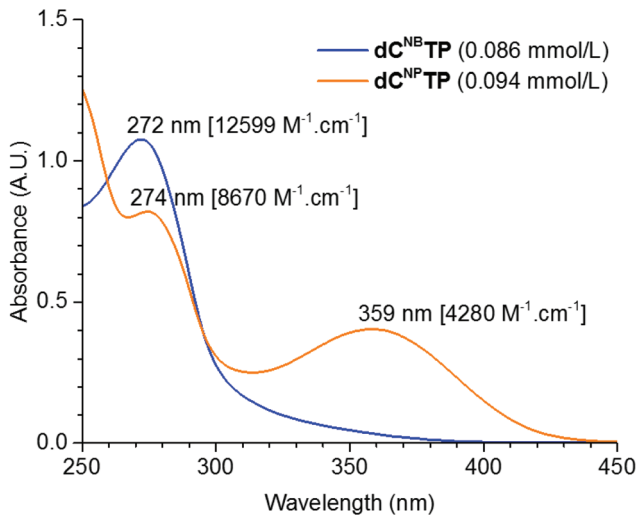


Fig. 1 Absorption spectra of **dC^{NB}TP** and **dC^{NP}TP**.

Incorporation of caged nucleotides into DNA

Caged triphosphates **dC^{NB}TP** and **dC^{NP}TP** as well as the uncaged **dC^{hm}TP** were tested as substrates for DNA polymerases.[18] The primer extension (PEX) reaction was studied using a 19-mer template (for the sequences of ONs, see Table S2 in the ESI†) encoding for the incorporation of one modified **dC^{R}** followed by three guanines. Fig. 2a shows the successful incorporation of all modified **dC^{R}** nucleotides using KOD XL (for successful PEX using Vent (*exo*-) polymerase, see Fig. S5 in the ESI†). The identity of the extended modified oligonucleotide (ON) products was confirmed by MALDI-TOF analysis showing masses corresponding to **C^{hm}**-containing ONs (Fig. S8 and S9 in the ESI†) since the laser irradiation used in MALDI cleaves the photocaging groups. Then we conducted a simple kinetic study of single nucleotide extension (Fig. S10 and S11 in the ESI†), which indicated that the incorporation of modified **dC^{R}TP**s was only slightly slower compared to that of natural dCTP. The PEX reaction using a longer 31-mer template encoding for the incorporation of 4 **dC^{R}** modifications (Fig. 2b) also proceeded successfully with

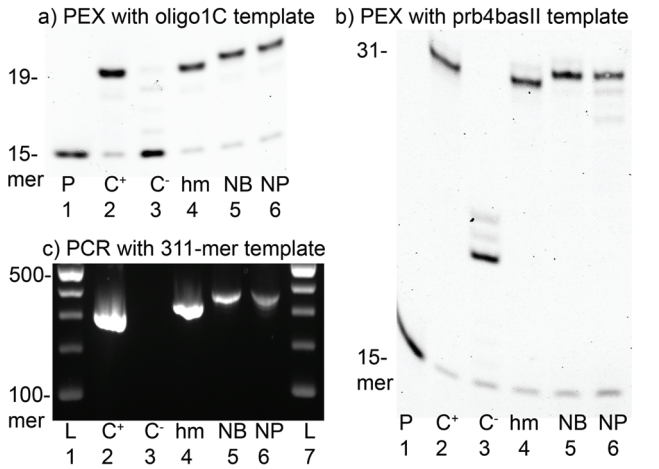


Fig. 2 Denaturing PAGE analysis of the KOD XL DNA polymerase synthesis of modified DNA. (a) PEX with oligo1C 19-mer template and (b) PEX with prb4basII 31-mer template: lane 1, P: primer; lane 2, C^+: product of PEX with natural dNTPs; lane 3, C^-: products of PEX in the absence of dCTP; lane 4, hm: product of PEX with **dC^{hm}TP** and three natural dNTPs; lane 5, NB: product of PEX with **dC^{NB}TP** and three natural dNTPs; lane 6, NP: product of PEX with **dC^{NP}TP** and three natural dNTPs. (C) Agarose gel analysis of PCR with the 311-mer template: lanes 1 and 7, L: 100 bp ladder; lane 2, C^+: product of PCR with natural dNTPs; lane 3, C^-: product of PCR in the absence of dCTP; lane 4, hm: product of PCR with **dC^{hm}TP** and three natural dNTPs; lane 5, NB: product of PCR with **dC^{NB}TP** and three natural dNTPs; lane 6, NP: product of PCR with **dC^{NP}TP** and three natural dNTPs.

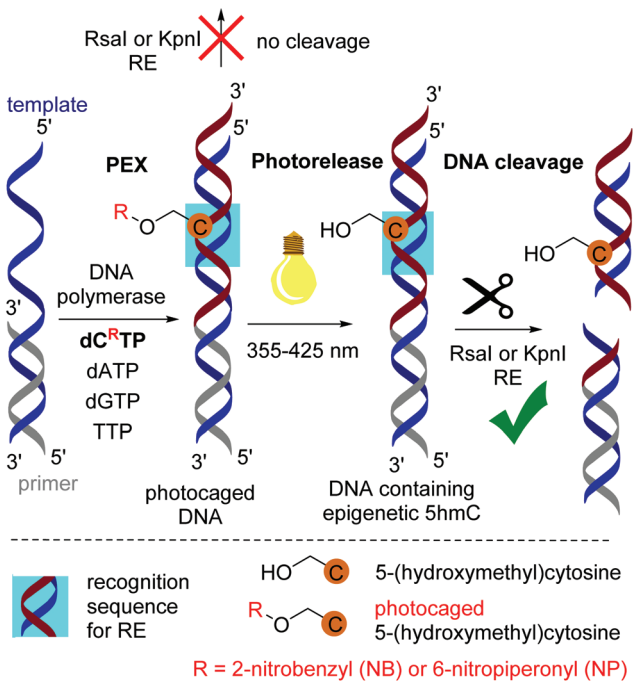
dC^{hm}TP, **dC^{NB}TP** or **dC^{NP}TP** giving clean, fully extended products.

Finally, we tested **dC^{hm}TP**, **dC^{NB}TP** and **dC^{NP}TP** as substrates in PCR amplifications using 98-mer (Fig. S21†) and 311-mer templates. The PCR reactions with **dC^{hm}TP** or **dC^{NB}TP** proceeded very well giving significant amounts of the modified amplicons (Fig. 2c). The use of bulkier **dC^{NP}TP** gave a somewhat weaker band of the full-length product, but the amplification still worked out. These results indicate that the photocaged 5hmC triphosphates (**dC^{NB}TP** and **dC^{NP}TP**) are comparable or better substrates for the PCR reaction, than analogous photocaged 5-(hydroxymethyl)uracil dNTPs reported previously (although the templates were different).[15,16]

Photochemical release of the caged DNA

In order to study the photorelease of the caged DNA, we used the previously described approach utilizing the cleavage of modified DNA by restriction endonucleases (REs) for monitoring the photochemical deprotection of DNA.[15,16] We have repeatedly shown[19,20] that modified DNA containing bulky groups in the major groove is not cleaved by RE, whereas non-modified DNA or DNA containing small modifications at T or A is fully cleaved by some REs. However, DNA duplexes containing modified C[20] or G[21] bases are typically not recognized and cleaved by most REs. Therefore, the first goal was to identify some REs that would recognize and cleave DNA containing 5hmC and not cleave the photocaged DNA. We prepared the corresponding modified DNA duplexes containing 5hmC or the photocaged **dC^{NB}** by PEX. The sequences always contained restriction sites for one of the tested 6 different REs (AflII, EcoRI, KpnI, PvuII, RsaI and HF-ScaI). Then we studied the cleavage of the modified DNA by REs (see Fig. S16 in the ESI†). Luckily, we found two enzymes (KpnI and RsaI) that fully cleaved hmC-modified DNA and did not cleave the photocaged DNA (Fig. S16†). Subsequently, we prepared 30-mer PEX products containing a **dC^{R}** modification in the recognition site for KpnI or RsaI. We tested the cleavage of the DNA by RE before and after irradiation using different LED diodes (Scheme 2). Analogous to our previous work on photocaged 5hmU DNA,[16] we tested the photorelease of each caging group from 5hmC with UV or visible light at three different wavelengths (355, 400 and 425 nm).

Fig. 3 shows the results of the photochemical uncaging reactions of caged DNA at 355 (a) and 400 nm (b), whereas Table 1 (and Fig. S18–S20 in the ESI†) gives the complete data at all tested wavelengths. ImageJ software was used to quantify the ratios of caged to uncaged DNA, which correspond to the reaction conversions (Tables 1 and 2). Because of a different optical power of the different LEDs, the reaction times differed. In all cases, we tested at least 3 different irradiation



Scheme 2 PEX synthesis of photocaged DNA, photorelease and cleavage of DNA by the restriction enzyme.

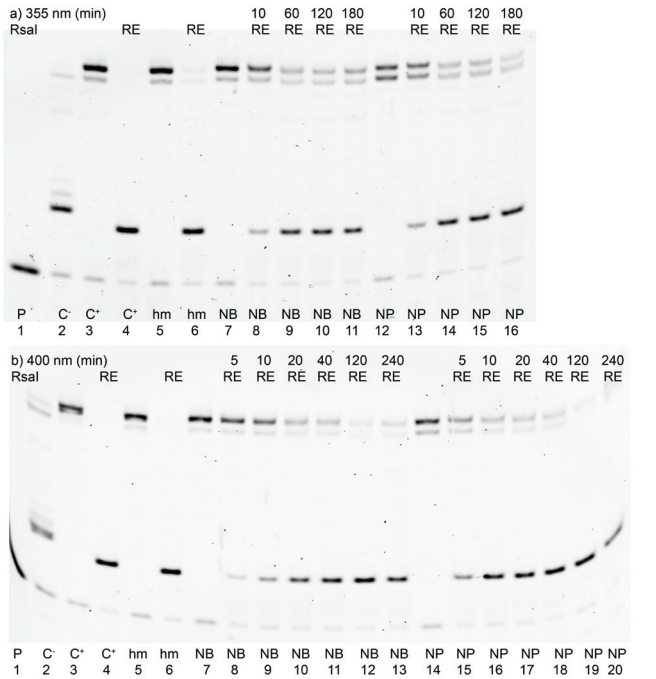


Fig. 3 Denaturing PAGE analysis of PEX with RsC 30-mer template followed by uncaging with a UV LED (a) 355 nm (0.8–1.2 mW), (b) 400 nm (21–29 mW): lane 1, P: primer; lane 2, C^{-}: product of PEX in the absence of dCTP; lane 3, C^{+}: product of PEX with natural dNTPs; lane 4, C^{+}: products of PEX with natural dNTPs followed by reaction with RE; lane 5, hm: product of PEX with **dC^{hm}TP** and three natural dNTPs; lane 6, hm: product of PEX with **dC^{hm}TP** and three natural dNTPs followed by reaction with RE; lane 7: products of PEX with **dC^{NB}TP**; lanes 8–11, or 8–13: product of PEX with **dC^{NB}TP** and three natural dNTPs after irradiation with a UV lamp (irradiation time in minutes) followed by reaction with RE; lane 12 or 14: products of PEX with or **dC^{NP}TP** and three natural dNTPs; lanes 13–16 or 15–20: products of PEX with **dC^{NP}TP** and three natural dNTPs after irradiation with a UV lamp (irradiation time in minutes) followed by reaction with RE.

Table 1 KpC template – DNA uncaging conversion quantification evaluated from gels using ImageJ software

Caging group	Λ (nm)	Optical power[a] (mW)	Time 1 (min)	Conv. (%)	Time 2 (min)	Conv. (%)	Time 3 (min)	Conv. (%)
NB	355	0.8–1.2	10	16	60	67	120	66
NP	355	0.8–1.2	10	24	60	62	120	71
NB	400	21–29	5	9	20	43	120	81
NP	400	21–29	5	27	20	65	120	78
NB	425	10–16	30	2	120	11	180	28
NP	425	10–16	30	11	120	44	180	49

[a] Optical power of the LED diodes supplied by the manufacturer.

Table 2 RsC template – DNA uncaging conversion quantification evaluated from gels using ImageJ software

Caging group	Λ (nm)	Optical power[a] (mW)	Time 1 (min)	Conv. (%)	Time 2 (min)	Conv. (%)	Time 3 (min)	Conv. (%)
NB	355	0.8–1.2	10	19	60	57	120	61
NP	355	0.8–1.2	10	19	60	60	120	61
NB	400	21–29	5	10	20	55	120	85
NP	400	21–29	5	36	20	71	120	95
NB	425	10–16	60	15	180	27	300	42
NP	425	10–16	60	31	180	65	300	65

[a] Optical power of the LED diodes supplied by the manufacturer.

times to reach complete (or almost complete) conversion whenever possible. The cleavage of NB and NP groups proceeded with comparable efficiencies at lower wavelengths (355 nm) reaching good conversions (60–70%) in reasonably short reaction times (10–120 min). Only at higher wavelengths (400 and 425 nm) was the nitropiperonyl group removed significantly faster. When using visible light (at 425 nm), the removal of the photocaging groups never reached full conversion even after 300 min of irradiation (maximum conversion for the NP group was *ca.* 65%). On the other hand, at 400 nm, both groups were uncaged efficiently reaching high or almost quantitative conversions (80–95%). Apparently, both NB and NP photocaging groups at 5hmC can be cleaved with UV or visible light but the cleavage at 425 nm is less efficient compared to that of NP-protected 5hmU.[16]

Conclusions

We prepared two types of photocaged derivatives of 2′-deoxy-5-(hydroxymethyl)cytosine dNTPs protected by 2-nitrobenzyl or 6-nitropiperonyl groups, and tested their enzymatic incorporations into DNA and photochemical deprotections. The NB- and NP-caged dNTPs (**dC^{NB}TP** and **dC^{NP}TP**) were good substrates for DNA polymerases and worked in PEX reactions, as well as in PCR amplifications. The liberation of the photocaged DNA was tested using monitoring by cleavage of DNA with REs, which tolerate hmC. We showed that both NB- and NP-caged DNA can be efficiently deprotected by UV irradiation, whereas the nitropiperonyl group is more suitable for uncaging with visible light at 400 or 425 nm. Although the visible-light photorelease of 5hmC from NB-caged DNA is slightly less efficient than the uncaging of the corresponding NP-caged 5hmU-containing DNA,[16] the NB-protection of 5hmC still has a good potential for photocaging of DNA containing this important epigenetic base and for some biological applications including studies of active demethylation and regulation of transcription *in cellulo* or *in vivo*.

Experimental

The full experimental part is given in the ESI.† Selected typical experiments are given below.

PEX – multiple modifications

The reaction mixture (20 μL) contained KOD XL DNA polymerase (2.5 U μL^{-1}, 0.02 μL), a mixture of dATP, dGTP and dTTP (1 mM, 1.5 μL), dTTP (1 mM, 3 μL) or modified **dC^{R}TP** (1 mM, 1.5 μL or 3 μL for **dC^{NB}TP** and **dC^{NP}TP**), 6-FAM labelled primer 248sh (3 μM, 1 μL), 31-mer template prb4basII (3 μM, 1.5 μL) and 10× buffer for KOD XL DNA polymerase (2 μL) supplied by the manufacturer. Reaction mixtures were incubated for 30 min at 60 °C in a thermal cycler. After reaction samples were denatured by the addition of stop solution (20 μL, 80% [v/v] formamide, 20 mM EDTA, 0.025%, [w/v] bromophenol blue, 0.025% [w/v] xylene cyanol, PCR water) followed by heating for 5 min at 95 °C. Reaction mixtures were separated using 12.5% denaturing PAGE.

Uncaging of DNA

KpC template. The reaction mixture (20 μL) contained KOD XL DNA polymerase (2.5 U μL^{-1}, 0.02 μL), mixture of dATP, dGTP and dTTP (1 mM, 0.5 μL), dCTP or modified **dC^{R}TP** (1 mM, 0.5 μL or 1 μL for **dC^{NB}TP** and **dC^{NP}TP**), 6-FAM labelled primer 248sh (3 μM, 1 μL), 30-mer template KpC (3 μM, 1.5 μL) and 10× buffer for KOD XL DNA polymerase (2 μL) supplied by the manufacturer.

RsC template. The reaction mixture (20 μL) contained KOD XL DNA polymerase (2.5 U μL^{-1}, 0.02 μL), mixture of dATP, dGTP and dTTP (1 mM, 0.2 μL), dCTP or modified **$dC^{R}TP$** (1 mM, 0.2 μL or 0.25 μL for **$dC^{NB}TP$** and **$dC^{NP}TP$**), 6-FAM labelled primer 248sh (3 μM, 1 μL), 30-mer template RsC (3 μM, 1.5 μL) and 10× buffer for KOD XL DNA polymerase (2 μL) supplied by the manufacturer.

Reaction mixtures were incubated for 30 min at 60 °C in a thermal cycler.

After reaction the samples were either:

(a) Denatured by the addition of stop solution (23 μL, 80% [v/v] formamide, 20 mM EDTA, 0.025%, [w/v] bromophenol blue, 0.025% [w/v] xylene cyanol, PCR water) and water (3 μL) and heating at 95 °C for 5 min.

(b) Incubated with RE (1.6 μL) in CutSmart or 1.1 NEBuffer (2.2 μL) and denatured by the addition of stop solution (23 μL) and heating at 95 °C for 5 min.

(c) Irradiated with a UV LED followed by incubation with RE (1.6 μL) in CutSmart or 1.1 NEBuffer (2.2 μL) and denaturation by the addition of stop solution (23 μL) and heating at 95 °C for 5 min.

Reaction mixtures were separated using 12.5% denaturing PAGE.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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