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Protected 5-(hydroxymethyl)uracil nucleotides bearing visible-light photocleavable groups as building blocks for polymerase synthesis of photocaged DNA†

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Nucleosides, nucleotides and 2′-deoxyribonucleoside triphosphates (dNTPs) containing 5-(hydroxymethyl)uracil protected with photocleavable groups (2-nitrobenzyl-, 6-nitropiperonyl or 9-anthrylmethyl) were prepared and tested as building blocks for the polymerase synthesis of photocaged oligonucleotides and DNA. Photodeprotection (photorelease) reactions were studied in detail on model nucleoside monophosphates and their photoreaction quantum yields were determined. Photocaged dNTPs were then tested and used as substrates for DNA polymerases in primer extension or PCR. DNA probes containing photocaged or free 5-hydroxymethylU in the recognition sequence of restriction endonucleases were prepared and used for the study of photorelease of caged DNA by UV or visible light at different wavelengths. The nitropiperonyl-protected nucleotide was found to be a superior building block because the corresponding dNTP is a good substrate for DNA polymerases, and the protecting group is efficiently cleavable by irradiation by UV or visible light (up to 425 nm).

Introduction

Photocleavable protecting groups[1] are gaining growing attention due to their applications in organic synthesis, chemical biology and many other areas. In biomolecules, photocaging[1,2] and cleavage of the photoremovable protective groups by light, which is both biocompatible and bioorthogonal, is often used for triggering or switching biological processes. Photocaging of nucleic acids[3] is a particularly active field. The photocaging on the nucleobase (mostly at N3 or exocyclic O^4/N^4 atoms of pyrimidines) is used[4] to prevent the base-pairing of oligonucleotides which only after photodeprotection can hybridize with the complementary strand. On the other hand, photocaging of the phosphate backbone[5] increases the resistance to hydrolysis and influences the interactions with other biomolecules. Photochemical deprotection can therefore either trigger or block biological processes, *i.e.*, primer extension, transcription, antisense effect, RNA interference, translation, *etc*. It is important to mention that all these types of caged oligonucleotides and 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] On the other hand, photocaging in the major-groove site of nucleotides and nucleic acids has been studied less frequently. Photocaged 5-(aminopropargyl)-pyrimidine dNTPs were used for polymerase synthesis of DNA and photochemical release of the reactive amino functions in the major groove.[6] Nitrobenzyl- (NB), and -phenylethyl-caged 5-(hydroxymethyl)pyrimidine or 7-hydroxymethyl-7-deazapurine 2′-deoxyribonucleoside triphosphates (dNTPs) were used as reversible terminators of primer extension in sequencing.[7] On the other hand, 6-*O*-(2-nitrobenzyl)guanosine and 4-*O*-(2-nitrobenzyl)uridine triphosphates[8] were not substrates for RNA polymerases until photochemical decaging.

5-(Hydroxymethyl)uracil (5hmU) is a rare DNA base previously found in bacteriophages,[9] microorganisms,[10] and also eukarytotic genomes.[11] The Schultz group has even evolved[12] a bacterium which replaced thymines with 5hmU in genome showing that this base is biocompatible. Unlike the related 5-(hydroxymethyl)cytosine (5hmC),[13] which is a known epigenetic mark and intermediate in active demethylation of DNA,[14] the biological role of 5hmU is not known so far.[15] In order to

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create an artificial epigenetic system for the regulation or switching of gene expression, we have systematically studied[16] the influence of chemical modifications in the major groove of DNA on bacterial transcription and found that some small modifications are tolerated by the bacterial RNA polymerase and transcription factor and the modified DNA still serves as a template for transcription. Obviously, any bulkier modifications in the major groove inhibited the transcription.[16] Recently we found[17] that the presence of 5hmU or 5hmC in template DNA can even enhance (up to 3.5 times) or inhibit bacterial transcription (depending on the promoter) and thus even hmU could be an epigenetic mark.

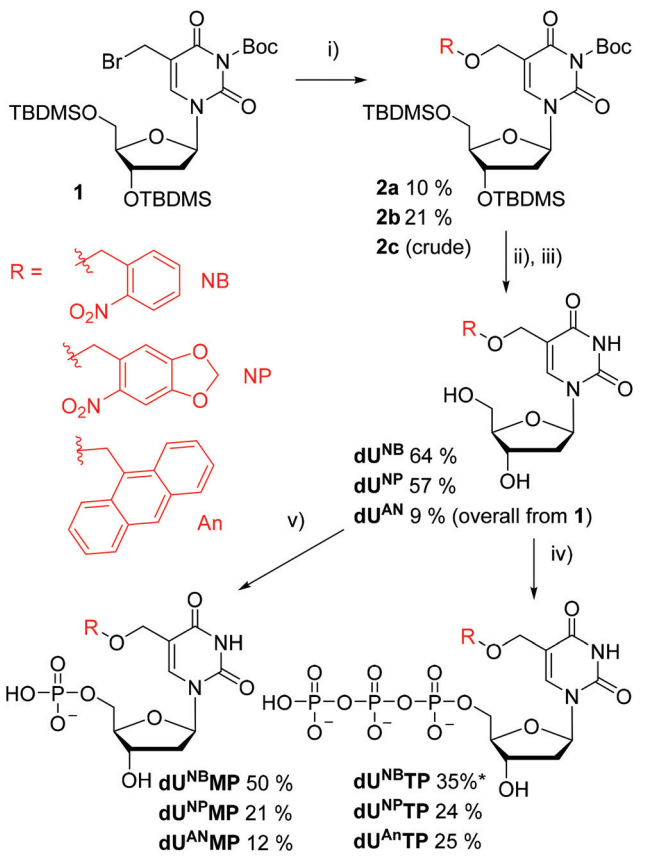
Previously, we have also studied the influence of major-groove modifications on recognition and cleavage of DNA by restriction endonucleases (REs) and found that some small modifications at T/U[18] or A[19] are tolerated (and the REs cleave the modified DNA), whereas all bulky modifications or any modifications at G[20] or C[18] bases inhibit the cleavage. We also developed[21] transient chemical protection of DNA from RE cleavage by polymerase incorporation of bulky 7-(triethylsilylethynyl)-7-deazaadenine which, after desilylation, released 7-ethynyl-7-deazaadenine-modified DNA being fully cleavable by several REs. In order to develop a more biocompatible and bioorthogonal switch, we recently reported[22] the use of 5-[(2-nitrobenzyl)oxymethyl]uracil dNTP as a substrate for DNA polymerases leading to the enzymatic synthesis of DNA photocaged in the major-groove. The presence of the bulky NB group in the major groove blocked specific interactions and cleavage by restriction endonucleases (REs) but, after photodeprotection by irradiation using UV light (365 nm), the 5-hydroxymethyluracil-containing DNA was recognized and cut by REs. It was a good proof of the principle, but for any *in cellulo* or *in vivo* applications, the use of UV light is not desirable[1b] because of its toxicity and limited penetration through tissues. Therefore, it is desirable to develop alternative photocaging groups for 5hmU which would be compatible with the synthesis of modified dNTPs, polymerase incorporation and would be cleavable by visible light. Major challenges in the design and development of new photocaging groups for nucleoside triphosphates are (i) limited chemical stability of some groups (*e.g.* carbonates) during the triphosphorylation steps and (ii) insufficient substrate activities of some very bulky dNTPs for polymerase incorporations to DNA (in particular by PCR). For example, the nitrophenethyl-caged dNTPs were incorporated by polymerases but terminated the primer extension.[7] Here we report on the synthesis of 5hmU dNTPs protected with 6-nitropiperonyl (NP)[23] and anthryl-9-methyl (An)[24] groups and comparison of substrate activities and photocleavage reactions with parent NB-caged nucleotides.

Results and discussion

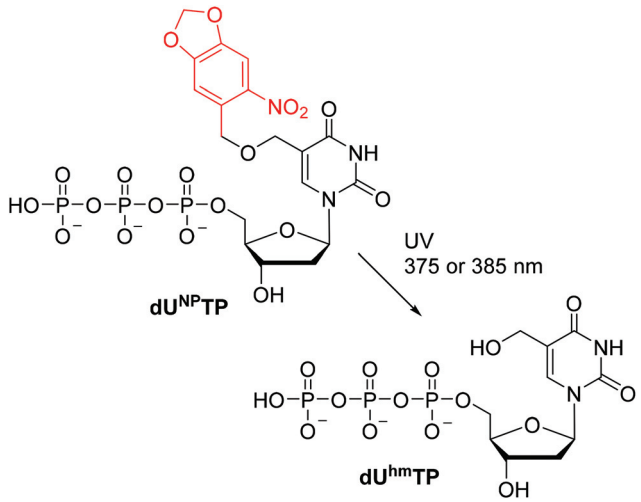
Synthesis

Although there were some reports on selective modification of hydroxymethyl groups in 5-hydroxymethyl-2′-deoxyuridine,[25] for the desired ether formation in the introduction of the photocaging groups, we chose an opposite approach based on nucleophilic substitution of a suitably protected 5-bromomethyl-2′-deoxyuridine derivative (Scheme 1). In analogy to the previous related studies,[7,22,26] we started from 3′,5′-bis-*O*-(TBDMS)-3-Boc-protected 5-bromomethyl-2′-deoxyuridine **1** and performed substitution reactions with the corresponding benzyl-type alcohol in the presence of 2,2,6,6-tetramethylpiperidine in CH_3CN at 50 °C. The addition of AgOTf was important for the precipitation of AgBr and thus shifting the equilibrium towards products. The desired fully protected caged nucleosides **2a** and **2b** were isolated in moderate yields (10 and 21%). The An-caged intermediate **2c** could not be isolated in pure form and was used in the next step in the crude form. Subsequently, the BOC group was removed using sodium bicarbonate[27] in MeOH at 50 °C, whereas the common reagents such as CF_3COOH or TBAF failed. The TBDMS group was then removed using $Et_3N{\cdot}3HF$[28] in THF. The desired photocaged nucleosides **dU^{NB}** and **dU^{NP}** were isolated in good yields of 64 and 57%, whereas the **dU^{An}** was isolated in low 9% overall yield.

Phosphorylation reactions were carried out with $POCl_3$ in trimethyl phosphate at 0 °C. Quenching of the reaction with



Scheme 1 Synthesis of photocaged 2′-deoxyuridine monophosphates and triphosphates (i) ROH, 2,2,6,6-tetramethylpiperidine, silver triflate, CH_3CN (ii) $NaHCO_3$, MeOH (iii) $Et_3N.3HF$, THF (iv) $POCl_3$, $PO(OMe)_3$, $(NHBu_3)_2H_2P_2O_7$ (v) $POCl_3$, $PO(OMe)_3$, 2 M TEAB. *Prepared according to the literature.[22]



Scheme 2 Synthesis of $dU^{hm}TP$.

2 M TEAB gave the desired monophosphates $dU^{NB}MP$, $dU^{NP}MP$ and $dU^{An}MP$ in moderate yields after isolation by HPLC. To form dNTPs, phosphorylation was carried out in the same way followed by the addition of pyrophosphate and tributyl amine in DMF. The targets $dU^{NB}TP$, $dU^{NP}TP$ and $dU^{An}TP$ were obtained in moderate yields after isolation by HPLC. The uncaged 5-hydroxymethyl-2′-deoxyuridine triphosphate $dU^{hm}TP$ was prepared in 27% yield by the photolysis of $dU^{NP}TP$ (with 59% recovery of the starting material) in water using irradiation by UV LEDs at either 375 or 385 nm followed by the separation on HPLC (Scheme 2).

Photophysical properties of modified monophosphates and quantum yields of uncaging reactions

Initially, we recorded the absorption spectra of the model caged monophosphates $dU^{R}MP$ in water and calculated the molar absorption coefficients for selected wavelengths in the range of 355–437 nm (Table 1 and Table S1 and Fig. S1–S7 in the ESI†). The parent $dU^{NB}MP$ exerted an absorption maximum at 264 nm and showed a very low absorption above 300 nm. At 365 nm (under which the NB group is cleaved[22]), the molar absorption coefficient is only 238 M^{-1} cm^{-1} (Table 1, Table S1 and Fig. S3 in the ESI†). On the other hand, the nitropiperonyl-caged nucleotide $dU^{NP}MP$ has a strong absorption maximum at 358 nm and still a relatively significant absorption at 400 nm (1940 M^{-1} cm^{-1}, Table 1 and Fig. S4 in the ESI†). $dU^{An}MP$ showed three absorption maxima at 349, 367 and 386 nm as expected for an anthracene moiety. The spectra of $dU^{NP}MP$ and $dU^{An}MP$ (Table S1, Fig. S4 and S6 in the ESI†) indicate that photochemical deprotection can be possible using light of wavelengths up to 425 nm.

Table 1 Absorption maxima of caged $dU^{R}MP$s in water

Compound	$dU^{NB}MP$	$dU^{NP}MP$	$dU^{An}MP$
λ_{max} (nm) [ε_{max}, (M^{-1} cm^{-1})][a]	264 [n.d.]	252 [n.d.] 358 [5798]	241 [n.d.] 349 [4006] 367 [5527] 386 [5050]

[a] Determined only for relevant maxima >300 nm.

Then we determined the quantum yields (QY) of uncaging. The measurements were carried out at 365 nm in water with a ferrioxalate actinometer[29] according to published procedures[30] giving an absolute value of QY. The QY of photocleavage of $dU^{NP}MP$ was moderate with the value of 4.4 ± 0.8%, whereas the QY value for $dU^{An}MP$ was negligible (in the range of an experimental error). Our attempts to measure QYs of previously published NB-group-protected $dU^{NB}MP$ failed because of two factors. (a) The molar absorption coefficient of $dU^{NB}MP$ at 365 nm was too low and the measurements had to be carried out in too concentrated solutions, and (b) the nitrosobenzene photoproduct formed during the reaction strongly absorbs at 365 nm. The fluorescence quantum yields of modified monophosphates in water were also determined at the excitation wavelength of 365 nm using quinine sulfate dissolved in 0.5 M H_2SO_4 as a standard[31] giving relative values of QYs. $U^{An}MP$ is moderately fluorescent with the quantum yield of 4.48 ± 0.15%; $U^{NB}MP$ and $U^{NP}MP$ are not fluorescent.

Incorporation of caged nucleotides into DNA

Caged triphosphates $dU^{NB}TP$, $dU^{NP}TP$ and $dU^{An}TP$ as well as the uncaged $dU^{hm}TP$ were tested as substrates for DNA polymerases.[32] At first, the primer extension (PEX) reaction was tested using a 19-mer template (for sequences of ONs see Table S4 in the ESI†) encoding for the incorporation of one modified dU^{R} followed by three guanines. Fig. 1a shows the successful incorporation of all modified dU^{R} nucleotides using KOD XL (for successful PEX using Vent (*exo*-) polymerase, see Fig. S12 in the ESI†). The extended modified oligonucleotide (ON) products were confirmed by the MALDI-TOF analysis showing masses corresponding to U^{hm}-containing ONs (Fig. S13 in the ESI†) because the laser irradiation using in MALDI cleaves the photoremovable groups. In the case of An modification, both caged and uncaged products were detected (Fig. S14 in the ESI†). We also performed a simple kinetics of single nucleotide extension (Fig. S15 and S16 in the ESI†) showing that the incorporation of modified $dU^{R}TP$s was only slightly slower compared to that of TTP. Then, the PEX reaction was performed with a more demanding 31-mer template designed for the incorporation of 4 dU^{R} modifications (Fig. 1b). This PEX proceeded successfully with $dU^{hm}TP$, $dU^{NB}TP$ and $dU^{NP}TP$ giving a clean fully extended product. $U^{AN}TP$ appeared to be a slightly worse substrate for DNA polymerase giving the mixture of extended products also containing truncated ONs. The worse outcome was probably due to the bulkiness and hydrophobicity of the anthracene group.

Next, we tested $dU^{hm}TP$, $dU^{NB}TP$, $dU^{NP}TP$ and $dU^{An}TP$ in PCR using either a 98-mer or (a more demanding) 297-mer template. In agreement with our previous communications,[17,22] the PCR reactions with $dU^{hm}TP$ and $dU^{NB}TP$, as well as with the new nitropiperonyl-caged $dU^{NP}TP$, proceeded

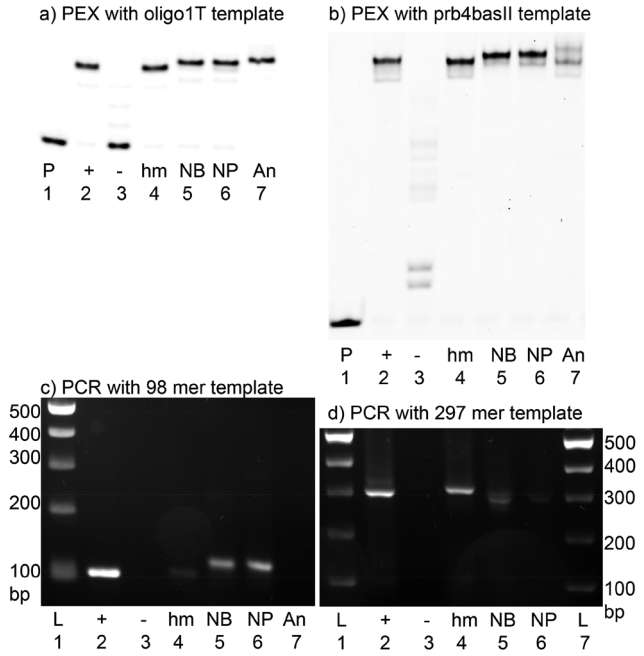
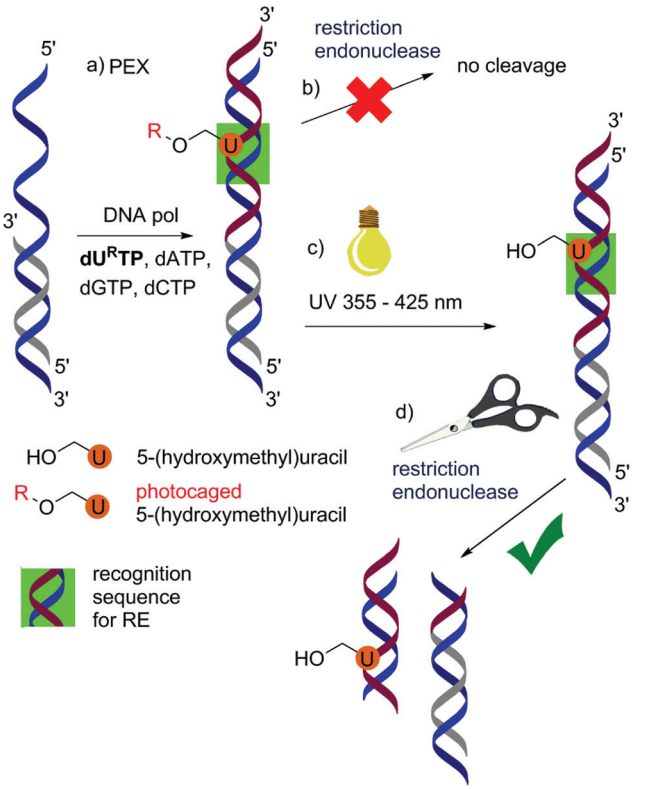


Fig. 1 Denaturing PAGE analysis of KOD XL DNA polymerase catalyzed (a) PEX with oligo1T 19-mer template and (b) PEX with prb4basII 31-mer template: lane 1, P: primer; lane 2 T^{+}: product of PEX with natural dNTPs; lane 3, T^{-}: products of PEX in the absence of dTTP; lane 4, hm: product of PEX with **dU^{hm}TP** and three natural dNTPs; lane 5, NB: product of PEX with **dU^{NB}TP** and three natural dNTPs; lane 6, NP: product of PEX with **dU^{NP}TP** and three natural dNTPs; lane 7, An: product of PEX with **dU^{An}TP** and three natural dNTPs. Agarose gel analysis of PCR with (c) FVL-A 98-mer or (d) 297-mer template: lane 1, L: 100 bp ladder; lane 2, +: product of PCR with natural dNTPs; lane 3, –: product of PCR in the absence of dTTP; lane 4, hm: product of PCR with **dU^{hm}TP** and three natural dNTPs; lane 5, NB: product of PCR with **dU^{NB}TP** and three natural dNTPs; lane 6, NP: product of PCR with **dU^{NP}TP** and three natural dNTPs; lane 7, An: product of PCR with **dU^{An}TP** and three natural dNTPs.



Scheme 3 Caging group prevents the cleavage of DNA by restriction enzymes. When the cage is removed by UV light, DNA cleavage by restriction enzymes is restored.

but the products of amplification were rather weak. The agarose gel of the 98-mer PCR (Fig. 1c) shows the bands of the modified PCR products only after the concentration of the sample, whereas the longer PCR (Fig. 1d) showed only very weak bands with somewhat faster mobility even after the concentration of the sample. However, in all cases, the identity of the modified amplicons was confirmed by sequencing. The bulky and hydrophobic **dU^{An}TP** did not give any amplification products in either of the PCR reaction.

Photochemical deprotection of caged DNA

In order to study the photocleavage of caged DNA, we used the previously described[22] approach utilizing the cleavage of modified DNA by RsaI restriction endonuclease for monitoring the photochemical deprotection of DNA. The caged DNA containing bulky protecting groups is not cleaved by RE, whereas the DNA containing small hydroxymethyluracil modification is fully cleaved (Scheme 3). Therefore, we prepared 30-mer PEX products containing a **dU^{R}** modification in the recognition site for RsaI. We tested the cleavage of the DNA by RE before and after irradiation using different LED diodes. In our previous work, we showed[22] that the NB-caged DNA can be efficiently liberated by irradiation at 355 nm. Now, we tested the photoremoval of all three caging groups with UV or visible light at different wavelengths (355, 375, 385, 400 and 425 nm). Uncaging experiments at 437 nm and higher wavelengths could not be tested because of the photobleaching of the 6-FAM used for labeling and visualization of ONs on gels (Fig. S17†). Considering the negligible absorbances of **dU^{R}MP**s at 437 nm (see Table S1 in the ESI† for molar absorption coefficients at 437 nm), we do not expect that liberation would proceed at 437 nm and higher wavelengths.

Fig. 2 shows the results of photochemical deprotection reactions of modified DNA at 375 and 425 nm (b), whereas Table 1 (and Fig. S22–S27 in the ESI†) gives complete data at all tested wavelengths. ImageJ software was used to quantify the ratios of uncaged DNA (Table 1). Because of different optical power of the different LEDs, the reaction times differed; we tested at least 3 different irradiation times to reach (almost) complete conversion when possible. In all cases, the observed photochemical cleavage of the An group was very inefficient (even at 355 nm) and the conversions were very low (up to 15% even with prolonged time). The cleavage of NB and NP groups proceeded with comparable efficiencies at lower wavelengths (355–385 nm) reaching good conversions (92–95%) in reason-

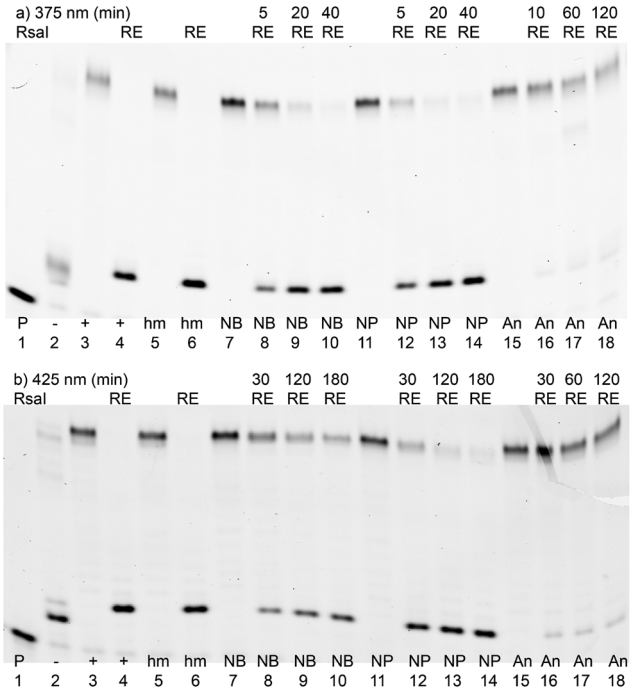


Fig. 2 Denaturing PAGE analysis of PEX with RsT 30-mer template followed by uncaging with the UV LED (a) 375 nm (2.5 mW) (b) 425 nm (10–16 mW): lane 1, P: primer; lane 2, –: product of PEX in the absence of dTTP; lane 3, +: product of PEX with natural dNTPs; lane 4, +: products of PEX with natural NTPs followed by the reaction with RsaI; lane 5, hm: product of PEX with **dU^{hm}TP** and three natural dNTPs; lane 6, hm: product of PEX with **dU^{hm}TP** and three natural dNTPs followed by the reaction with RsaI; lane 7,11,15, products of PEX with **dU^{NB}TP**, **dU^{NP}TP** or **dU^{An}TP** and three natural dNTPs; lanes 8–10, 12–14 and 16–18, product of PEX with **dU^{NB}TP**, **dU^{NP}TP** or **dU^{An}TP** and three natural dNTPs after irradiation with the UV lamp (irradiation time in minutes) followed by reaction with RsaI.

able reaction times (15–120 min). Only at higher wavelengths (400 and 425 nm), the nitropiperonyl group was cleaved significantly faster. At the highest wavelength of 425 nm, the cleavage of the nitrobenzyl group did not reach full conversion, whereas the cleavage of the NP group was still almost quantitative. Taking into account the almost negligible absorbance of the nitrobenzyl group at 400 and 425 nm, it was rather surprising that the NB group was still at least partly cleavable. However, as expected, uncaging of NPs was much faster and more efficient at these wavelengths, and we conclude that this group can be removed with visible light (Table 2).

Conclusions

We prepared three types of photocaged derivatives of 2′-deoxy-5-(hydroxymethyl)uridine nucleoside, mono- and triphosphates protected by 2-nitrobenzyl-, 6-nitropiperonyl- and anthryl-9-methyl groups, and tested their enzymatic incorporations to DNA and photochemical deprotections. The NB- and NP-caged dNTPs (**dU^{NB}TP** and **dU^{NP}TP**) were good substrates for DNA polymerases and worked in both PEX and PCR reactions. The An-linked **dU^{An}TP** was a less efficient substrate but it was still possible to incorporate it to at least some sequences using PEX. The liberation of photocaged DNA was tested by monitoring the cleavage of DNA with REs. We show that both NB- and NP-caged DNA can be efficiently deprotected by UV irradiation, whereas the nitropiperonyl group is suitable for uncaging with visible light at 400 or 425 nm. Therefore, the NP-caging has a better potential for *in cellulo* or *in vivo* photocaging of 5-(hydroxymethyl)uracil in DNA. The An group does not apparently have much potential in DNA photocaging but still the An-substituted dNTP can find some use in polymerase synthesis of DNA bearing a fluorescent extended aromatic system for applications in imaging or selection of hydrophobic aptamers.

Experimental

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

Table 2 DNA uncaging conversions 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	52	60	78	120	95
NP	355	0.8–1.2	10	59	60	79	120	93
An	355	0.8–1.2	60	6	240	10	480	13
NB	375	2.5	5	43	20	82	40	93
NP	375	2.5	5	64	20	90	40	93
An	375	2.5	10	4	60	9	120	10
NB	385	11.0	2	49	5	73	15	92
NP	385	11.0	2	76	5	85	15	95
An	385	11.0	5	9	20	13	60	15
NB	400	21–29	2	32	5	45	20	78
NP	400	21–29	2	59	5	81	20	92
An	400	21–29	10	8	60	11	120	11
NB	425	10–16	30	34	120	56	180	63
NP	425	10–16	30	68	120	86	180	92
An	425	10–16	30	5	60	7	120	7

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

3′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-3-*tert*-butoxycarbonyl-5-(6-nitropiperonyloxy)methyl-2′-deoxyuridine (2b)

6-Nitropiperonyl alcohol (240 mg, 1.22 mmol) and silver triflate (308 mg, 1.20 mmol) were suspended in dry acetonitrile and 2,2,6,6-tetramethylpiperidine (205 μL, 1.21 mmol) was added. The mixture was stirred for 10 min at 50 °C. Then solution of **1** in acetonitrile was added and the reaction was stirred for another 2 h at 50 °C. The solvent was removed under reduced pressure and the mixture was separated by chromatography (hexane : ethyl acetate 5 : 1) to give yellow oil (97 mg, 21%). ^{1}H NMR (500 MHz, DMSO-d$_6$): 0.03, 0.04, 0.078 and 0.081 (4 × s, 4 × 3H, CH_3Si); 0.84 and 0.87 (2 × s, 2 × 9H, $(CH_3)_3C$); 1.51 (s, 9H, $(CH_3)_3COCO$); 2.18 (ddd, 1H, J_{gem} = 13.5 Hz, $J_{2'a,1'}$ = 6.2 Hz, $J_{2'a,3'}$ = 3.2 Hz, H-2′a); 2.31 (ddd, 1H, J_{gem} = 13.5 Hz, $J_{2'b,1'}$ = 7.3 Hz, $J_{2'b,3'}$ = 5.9 Hz, H-2′b); 3.70 (dd, 1H, J_{gem} = 11.4 Hz, $J_{5'a,4'}$ = 4.4 Hz, H-5′a); 3.74 (dd, 1H, J_{gem} = 11.4 Hz, $J_{5'b,4'}$ = 4.6 Hz, H-5′b); 3.84 (td, 1H, $J_{4',5'a}$ = $J_{4',5'b}$ = 4.5 Hz, $J_{4',3'}$ = 3.0 Hz, H-4′); 4.26 (s, 2H, B-CH_2O); 4.35 (dt, 1H, $J_{3',2'b}$ = 5.8 Hz, $J_{3',4'}$ = $J_{3',2'a}$ = 3.1 Hz, H-3′); 4.80 (s, 2H, OCH_2-Ph); 6.10 (dd, 1H, $J_{1',2'b}$ = 7.3 Hz, $J_{1',2'a}$ = 6.2 Hz, H-1′); 6.24 (s, 2H, OCH_2O); 7.26 (s, 1H, H-6″); 7.70 (s, 1H, H-3″); 7.79 (s, 1H, H-6). ^{13}C NMR (125.7 MHz, DMSO-d$_6$): −5.31, −5.28, −4.73 and −4.56 (CH_3Si); 17.94 and 18.23 ($(CH_3)_3\mathbf{C}$); 25.89 and 25.98 ($(\mathbf{C}H_3)_3C$); 27.24 ($(\mathbf{C}H_3)_3COCO$); 39.5 (CH_2-2′); 62.78 (CH_2-5′); 65.02 (B-CH_2O); 68.79 (OCH_2-Ph); 72.11 (CH-3′); 85.56 (CH-1′); 86.71 ($(CH_3)_3\mathbf{C}OCO$); 87.46 (CH-4′); 103.69 (OCH_2O); 105.51 (CH-3″); 107.36 (CH-6″); 110.03 (C-5); 132.28 (CH-1″); 139.86 (CH-6); 141.05 (C-2″); 147.05 (C-4″); 147.82 ($(CH_3)_3C\mathbf{O}CO$); 148.06 (C-2); 152.49 (C-5″); 159.98 (C-4). MS (ESI): *m*/*z* (%):789 (50)[M + Na + H]$^{2+}$, 788 (100) [M + Na]$^+$; HRMS (ESI): *m*/*z* calcd for $C_{35}H_{55}N_3O_{12}NaSi_2$: 788.32195 [M + Na]$^+$; found: 788.32165.

3′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-5-(6-nitropiperonyloxy) methyl-2′-deoxyuridine (3b)

Compound **2b** (131 mg, 0.17 mmol) was dissolved in methanol, $NaHCO_3$ (144 mg, 1.70 mmol) was added and the suspension was stirred at 56 °C overnight. The solvent was removed under vacuum and the residue was extracted with ethyl acetate and purified by column chromatography (hexane : EtOAc 3 : 2) to give **3b** as yellow powder (81 mg, 71%). ^{1}H NMR (500 MHz, DMSO-d$_6$): 0.039, 0.044, 0.080 and 0.082 (4 × s, 4 × 3H, CH_3Si); 0.85 and 0.87 (2 × s, 2 × 9H, $(CH_3)_3C$); 2.12 (ddd, 1H, J_{gem} = 13.4 Hz, $J_{2'a,1'}$ = 6.2 Hz, $J_{2'a,3'}$ = 3.1 Hz, H-2′a); 2.21 (ddd, 1H, J_{gem} = 13.4 Hz, $J_{2'b,1'}$ = 7.8 Hz, $J_{2'b,3'}$ = 6.0 Hz, H-2′b); 3.69 (dd, 1H, J_{gem} = 11.3 Hz, $J_{5'a,4'}$ = 4.3 Hz, H-5′a); 3.74 (dd, 1H, J_{gem} = 11.3 Hz, $J_{5'b,4'}$ = 4.6 Hz, H-5′b); 3.80 (td, 1H, $J_{4',5'a}$ = $J_{4',5'b}$ = 4.5 Hz, $J_{4',3'}$ = 2.9 Hz, H-4′); 4.20 and 4.23 (2 × dd, 2 × 1H, J_{gem} = 11.6 Hz, $J_{CH2,CH2}$ = 0.7 Hz, B-CH_2O); 4.35 (dt, 1H, $J_{3',2'b}$ = 5.9 Hz, $J_{3',4'}$ = $J_{3',2'a}$ = 3.0 Hz, H-3′); 4.78 (d, 2H, $J_{CH2,CH2}$ = 0.7 Hz, OCH_2-Ph); 6.14 (dd, 1H, $J_{1',2'b}$ = 7.8 Hz, $J_{1',2'a}$ = 6.2 Hz, H-1′); 6.23 (s, 2H, OCH_2O); 7.28 (s, 1H, H-6″); 7.65 (s, 1H, H-6); 7.68 (s, 1H, H-3″); 11.50 (bs, 1H, NH). ^{13}C NMR (125.7 MHz, DMSO-d$_6$): −5.35, −5.34, −4.75 and −4.59 (CH_3Si); 17.91 and 18.18 ($(CH_3)_3\mathbf{C}$); 25.86 and 25.94 ($(\mathbf{C}H_3)_3C$); 39.42 (CH_2-2′); 62.90 (CH_2-5′); 65.15 (B-CH_2O); 68.57 (OCH_2-Ph); 72.25 (CH-3′); 84.38 (CH-1′); 87.03 (CH-4′); 103.59 (OCH_2O); 105.39 (CH-3″); 107.41 (CH-6″); 110.55 (C-5); 132.43 (CH-1″); 139.32 (CH-6); 141.02 (C-2″); 146.95 (C-4″); 150.61 (C-2); 152.40 (C-5″); 163.13 (C-4). MS (ESI): *m*/*z* (%): 688 (100) [M + Na]$^+$, 689 (42)[M + Na + 2H]$^{3+}$, 1353 (22) [2M + Na]$^+$; HRMS (ESI): *m*/*z* calcd for $C_{30}H_{48}N_3O_{10}Si_2$: 666.28748 [M + H]$^+$; found: 666.28727.

5-(6-Nitropiperonyloxy)methyl-2′-deoxyuridine (dU^{NP})

Compound **3a** (80 mg, 0.12 mmol) was dissolved in dry THF, triethylamine trihydrofluoride (171 μL, 1.05 mmol) was added and the reaction was stirred overnight at rt. The solvent was removed under reduced pressure and the mixture was separated by chromatography (DCM : MeOH 7 : 1) to give **dU^{NP}** as yellow powder (42 mg, 80%). ^{1}H NMR (500 MHz, DMSO-d$_6$): 2.05–2.15 (m, 2H, H-2′); 3.55 (ddd, 1H, J_{gem} = 11.8 Hz, $J_{5'a,OH}$ = 5.1 Hz, $J_{5'a,4'}$ = 3.9 Hz, H-5′a); 3.59 (ddd, 1H, J_{gem} = 11.9 Hz, $J_{5'b,OH}$ = 5.4 Hz, $J_{5'b,4'}$ = 3.9 Hz, H-5′b); 3.79 (td, 1H, $J_{4',5'a}$ = $J_{4',5'b}$ = 3.9 Hz, $J_{4',3'}$ = 2.9 Hz, H-4′); 4.20 and 4.23 (2 × dd, 2 × 1H, J_{gem} = 11.6 Hz, $J_{CH2,CH2}$ = 0.8 Hz, B-CH_2O); 4.24 (m, 1H, H-3′); 4.79 (d, 2H, $J_{CH2,CH2}$ = 0.8 Hz, OCH_2-Ph); 5.02 (t, 1H, $J_{OH,5'a}$ = $J_{OH,5'b}$ = 5.3 Hz, OH-5′); 5.25 (d, 1H, $J_{OH,3'}$ = 4.3 Hz, OH-3′); 6.16 (t, 1H, $J_{1',2'b}$ = $J_{1',2'a}$ = 6.8 Hz, H-1′); 6.23 (s, 2H, OCH_2O); 7.33 (s, 1H, H-6″); 7.68 (s, 1H, H-3″); 7.99 (s, 1H, H-6); 11.44 (bs, 1H, NH). ^{13}C NMR (125.7 MHz, DMSO-d$_6$): 39.85 (CH_2-2′); 61.49 (CH_2-5′); 65.28 (B-CH_2O); 68.52 (OCH_2-Ph); 70.64 (CH-3′); 84.41 (CH-1′); 87.67 (CH-4′); 103.57 (OCH_2O); 105.32 (CH-3″); 107.41 (CH-6″); 110.34 (C-5); 132.75 (CH-1″); 139.82 (CH-6); 140.92 (C-2″); 146.90 (C-4″); 150.49 (C-2); 152.50 (C-5″); 163.01 (C-4). MS (ESI): *m*/*z* (%): 460 (100) [M + Na]$^+$, 461 (21)[M + Na + H]$^{2+}$, 897 (39)[2M + Na]$^+$, 898 (16) [2M + Na + H]$^{2+}$; HRMS (ESI): *m*/*z* calcd for $C_{18}H_{20}N_3O_{10}$: 438.11432 [M + Na]$^+$; found: 438.11433.

5-(6-Nitropiperonyloxy)methyl-2′-deoxyuridine monophosphate (dU^{NP}MP)

Nucleoside **dU^{NP}** (22 mg, 0.05 mmol) and proton sponge (22 mg, 0.10 mmol) were suspended in trimethyl phosphate (150 μL) in an argon purged flask and the suspension was cooled to 0 °C. Then, redistilled $POCl_3$ (5 μL, 0.06 mmol) was added. The reaction mixture was stirred at 0 °C for 45 min and then aqueous solution of TEAB (2 M, 2 mL) was added and the mixture was evaporated under reduced pressure. The product was purified by HPLC chromatography (0.1 M aq. TEAB 0 → 50% methanol), co-evaporated several times with water and converted to sodium salt on Dowex. Monophosphate **dU^{NP}MP** (6 mg, 21%) was obtained as a yellowish lyophilizate (water). ^{1}H NMR (500 MHz, D_2O): 2.35 (ddd, 1H, J_{gem} = 14.1 Hz, $J_{2'a,1'}$ = 6.5 Hz, $J_{2'a,3'}$ = 4.0 Hz, H-2′a); 2.39 (ddd, 1H, J_{gem} = 14.1 Hz, $J_{2'b,1'}$ = 7.3 Hz, $J_{2'b,3'}$ = 5.9 Hz, H-2′b); 3.91–3.97 (m, 2H, H-5′); 4.13 (m, 1H, H-4′); 4.44 (s, 2H, B-CH_2O); 4.56 (dt, 1H, $J_{3',2'b}$ = 5.8 Hz, $J_{3',2'a}$ = $J_{3',4'}$ = 4.0 Hz, H-3′); 4.87 and 4.91 (2 × d, 2 × 1H, J_{gem} = 14.0 Hz, OCH_2-Ph); 6.15 (m, 2H, OCH_2O); 6.27 (bt, 1H, $J_{1',2'b}$ = $J_{1',2'a}$ = 6.9 Hz, H-1′); 7.15 (s, 1H, H-6″); 7.60 (s, 1H, H-3″); 8.01 (s, 1H, H-6). ^{13}C NMR (125.7 MHz, D_2O): 39.13 (CH_2-2′); 64.21 (d, $J_{C,P}$ = 4.6 Hz, CH_2-5′); 66.25 (B-CH_2O); 69.71

(OCH_2-Ph); 71.75 (CH-3′); 85.91 (CH-1′); 86.74 (d, $J_{C,P}$ = 8.3 Hz, CH-4′); 104.08 (OCH_2O); 106.27 (CH-3″); 109.67 (CH-6″); 111.65 (C-5); 132.13 (CH-1″); 142.10 (C-2″); 142.33 (CH-6); 147.88 (C-4″); 152.74 (C-2); 153.00 (C-5″); 166.72 (C-4). ^{31}P NMR (202.4 MHz, D_2O): 4.55 (s, 1P, P-5′). MS (ESI): *m*/*z* (%): 516 (100) $[M]^-$, 517 (23)[M + H]; HRMS (ESI): *m*/*z* calcd for $C_{18}H_{19}N_3O_{13}P$: 516.06610 $[M]^-$; found: 516.06531.

5-(6-Nitropiperonyloxy)methyl-2′-deoxyuridine triphosphate (dU^{NP}TP)

Nucleoside **dU^{NP}** (40 mg, 0.09 mmol) and proton sponge (40 mg, 0.18 mmol) were suspended in trimethyl phosphate (270 μL) in an argon purged flask and the suspension was cooled to 0 °C. Then, redistilled $POCl_3$ (9 μL, 0.10 mmol) was added. The reaction mixture was stirred at 0 °C for 45 min and then ice-cold solution of $(NHBu_3)_2H_2P_2O_7$ (208 mg, 0.46 mmol) and tributyl amine (90 μL, 0.38 mmol) in anhydrous DMF (4 mL) was added. The reaction mixture was stirred at 0 °C for another 1 h. Then aqueous solution of TEAB (2 M, 2 mL, 4 mmol) was added and the mixture was evaporated under reduced pressure. The residue was co-evaporated several times with water. The product was purified by HPLC chromatography (0.1 M aq. TEAB 0 → 50% methanol), co-evaporated several times with water and converted to sodium salt on Dowex. Triphosphate **dU^{NP}TP** (17 mg, 24%) was obtained as a yellowish lyophilizate (water). ^{1}H NMR (500 MHz, D_2O): 2.34–2.40 (m, 2H, H-2′); 4.18 (qd, 1H, $J_{4',5'a}$ = $J_{4',5'b}$ = $J_{4',3'}$ = 3.5 Hz, $J_{4',P}$ = 2.0 Hz, H-4′); 4.22 (ddd, 1H, J_{gem} = 11.6 Hz, $J_{5'a,P}$ = 5.1 Hz, J_r = 3.2 Hz, H-5′a); 4.26 (ddd, 1H, J_{gem} = 11.6 Hz, $J_{5'b,P}$ = 6.1 Hz, $J_{5'b,4'}$ = 3.6 Hz, H-5′b); 4.40 and 4.47 (2 × d, 2 × 1H, J_{gem} = 12.1 Hz, B-CH_2O); 4.66 (td, 1H, $J_{3',2'a}$ = $J_{3',2'b}$ = 5.4 Hz, $J_{3',4'}$ = 3.7 Hz, H-3′); 4.84 and 4.92 (2 × d, 2 × 1H, J_{gem} = 14.0 Hz, OCH_2-Ph); 6.15 (t, 2H, $J_{CH2,LR}$ = 1.3 Hz, OCH_2O); 6.26 (t, 1H, $J_{1',2'b}$ = $J_{1',2'a}$ = 6.7 Hz, H-1′); 7.13 (s, 1H, H-6″); 7.58 (s, 1H, H-3″); 7.96 (s, 1H, H-6). ^{13}C NMR (125.7 MHz, D_2O): 39.31 (CH_2-2′); 65.65 (d, $J_{C,P}$ = 5.5 Hz, CH_2-5′); 66.29 (B-CH_2O); 69.77 (OCH_2-Ph); 70.73 (CH-3′); 85.91 (CH-1′); 86.20 (d, $J_{C,P}$ = 9.1 Hz, CH-4′); 104.11 (OCH_2O); 106.27 (CH-3″); 109.84 (CH-6″); 111.55 (C-5); 132.11 (CH-1″); 142.08 (CH-6); 142.15 (C-2″); 147.90 (C-4″); 152.47 (C-2); 152.94 (C-5″); 166.06 (C-4). ^{31}P NMR (202.4 MHz, D_2O): −20.95 (t, 1P, $J_{\beta,\alpha}$ = $J_{\beta,\gamma}$ = 19.8 Hz, P_β); −10.57 (d, 1P, $J_{\alpha,\beta}$ = 19.5 Hz, P_α); −5.11 (bd, 1P, $J_{\gamma,\beta}$ = 20.0 Hz, P_γ). MS (ESI): *m*/*z* (%): 516 (41) $[M + H\text{-}HP_2O_6]^-$, 596 (100) $[M + 2H\text{-}HPO_3]^-$, 618 (60) $[M + H + Na\text{-}HPO_3]^-$; HRMS (ESI): *m*/*z* calcd for $C_{18}H_{20}N_3O_{19}P_3Na$: 697.98070 $[M + H + Na]^-$; found: 697.97937.

PEX – multiple modifications

The reaction mixture (20 μL) contained KOD XL DNA polymerase (2.5 U μL^{-1}, 0.02 μL), mixture of dATP, dCTP and dGTP (1 mM, 1 μL), dTTP (1 mM, 1 μL) or modified **dU^{R}TP** (1 mM, 2 μL), 6-FAM labelled primer 248sh (3 μM, 1 μL), 31-mer template TINA-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 the 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) heating was followed for 5 min at 95 °C. The reaction mixtures were separated using 12.5% denaturing PAGE.

Uncaging of DNA

The reaction mixture (20 μL) contained KOD XL DNA polymerase (2.5 U μL^{-1}, 0.2 μL), mixture of dATP, dCTP and dGTP (1 mM, 0.2 μL), dTTP or modified dUTP (1 mM, 0.3 μL) 6-FAM labelled primer 248sh (3 μM, 1 μL) and 30-mer template RsT (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 that, the reaction samples were either:

(a) denatured by the addition of the 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 heated at 95 °C for 5 min,

(b) incubated with RSaI (0.8 μL) in CutSmart buffer (2.2 μL) and denatured by addition of the stop solution (23 μL) and heated at 95 °C for 5 min, or

(c) irradiated with a UV LED followed by incubation with RSaI (0.8 μL) in CutSmart buffer (2.2 μL) and denatured by the addition of the 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.

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