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Photochemical Regulation of Restriction Endonuclease Activity

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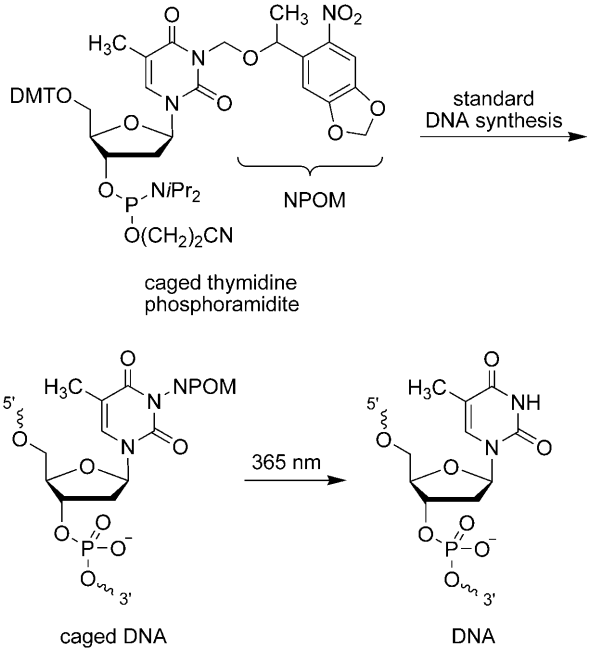
To elucidate biological processes, precise control over these processes is required. Light represents an ideal external control element because it can be easily regulated in a spatial and temporal fashion, and conveys spatiotemporal control of biological activity to the system under study.[1] The photochemical regulation of oligonucleotide function through the installation of light-removable protecting groups (caging groups) on either the phosphate or the nucleotide base has recently received considerable attention.[2–4] Important applications of this technology involve the transient disruption of DNA hybridization to photochemically control DNAzyme activity, the polymerase chain reaction, antisense activity, as well as inhibition of transcription.[4,5] In this context, we demonstrated that a single caging group installed on one base of a typical oligonucleotide 20-mer still enables DNA–DNA and DNA–RNA hybridization, but could disrupt processing of the oligomer by polymerases and inactivate the catalytic ability of DNAzymes.[2] As a result, we became interested in exploring other biologically relevant processes with photocaged DNA that do not involve perturbation of hybridization. Due to the prevalence of DNA–protein interactions both in vivo and in vitro,[6] we hypothesized that it might be feasible to photochemically control such an interaction for restriction endonucleases by the incorporation of our 6-nitropiperonyloxymethyl (NPOM)-caged thymidine nucleotide (Scheme 1) into DNA. Very few studies have been conducted on the effects of unnatural nucleotides on the fidelity and functionality of restriction enzymes. Those that have, primarily involve the effects of endogenous base mutations (for example, methylation events) that do not drastically affect hydrogen bonding and base pair recognition. In many of these cases, the catalytic capabilities of the restriction endonucleases are dramatically decreased, if not abrogated.[7,8]

Restriction endonucleases are enzymes that are capable of the site-specific recognition and cleavage of double-stranded DNA (dsDNA). Based on their unique activity, they have been employed extensively in molecular biology and have facilitated the development of recombinant DNA technology and cloning. To date over 3500 restriction enzymes have been identified, and the number that are commercially available is growing (>600).[9] There are three major classes of restriction enzymes, which differ in their use of cofactors, their target sequence,



Scheme 1. NPOM caged, 5′-dimethoxytrityl (DMT)-protected thymidine phosphoramidite and its incorporation into synthetic DNA. The caged DNA can be effectively decaged through a brief irradiation with UV light at 365 nm.

and the location of the cleavage site relative to their target sequence. The most commonly employed restriction endonucleases are from the type II family, which typically only require Mg^{2+} as a cofactor, recognize a 4–8 base dsDNA sequence, and cleave directly within that sequence. Based on their extensive use in the manipulation of DNA and the site-specific mechanism of action,[8] we investigated the photochemical regulation of these enzymes using our developed nucleobase-caging technology.

Thus, we designed a DNA construct that possesses multiple restriction sites and thymidine residues at various positions relative to the site of cleavage (Table 1). Ideally, this should afford a means to probe the effects of the caging group on restriction endonuclease recognition and function. The EcoRI, BglII, and BamHI sites were selected for this study because they represent commonly employed restriction endonucleases and have thymidine residues at various positions. The noncaged DNA and its complement were synthesized and an initial study was performed to demonstrate the efficient cleavage of the substrate by the three enzymes (see the Supporting Information). Additionally, to ensure that hybridization to the complementary sequence was occurring despite the presence of the caging group, the melting temperature (T_m) of each caged oligonucleotide T^n in presence of the complementary strand was determined on a BioRad MyiQ RT-PCR thermocycler by con-

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Table 1. Synthesized caged restriction enzyme templates.[a]

EcoRI BglII BamHI
1 2 3 4 5 6
5′ GGGTGAATTCAGATCTGGATCCAAAAG 3′

DNA	Sequence	T_m [°C]
T^0	5′-GGGTGAATTCAGATCTGGATCCAAAAG-3′	68.0 ± 0.7
T^1	5′-GGGIGAATTCAGATCTGGATCCAAAAG-3′	62.8 ± 1.1
T^2	5′-GGGTGAAITCAGATCTGGATCCAAAAG-3′	62.5 ± 0.8
T^3	5′-GGGTGAATICAGATCTGGATCCAAAAG-3′	62.5 ± 0.7
T^4	5′-GGGTGAATTCAGAICTGGATCCAAAAG-3′	63.5 ± 0.4
T^5	5′-GGGTGAATTCAGATCIGGATCCAAAAG-3′	63.3 ± 0.3
$T^{2,3,6}$	5′-GGGTGAAIICAGATCTGGAICCAAAAG-3′	50.8 ± 1.2
$T^{4,5}$	5′-GGGTGAATTCAGAICICGGATCCAAAAG-3′	59.5 ± 0.7

[a] Melting temperature of noncaged and caged DNA–DNA hybrids; I denotes the caged thymidine.

ducting a sequence of three heating and cooling cycles (Table 1). It appears that the presence of a single caging group reduces the melting temperature of this DNA sequence by approximately 5 °C, whereas 2–3 caged thymidines reduce the melting temperature by 9–18 °C; however, hybridization was detected for all constructs, and in no case was the disruption sufficient enough to prevent hybridization at 37 °C, the temperature at which the restriction enzyme assays were conducted.

Initially we explored the propensity of the caging group to inhibit EcoRI function based on the position of the caged thymidine residue relative to the site of cleavage using oligomers T^0–T^3, T^5, and $T^{2,3,6}$. The complementary sequence was end labeled with ^{32}P, and hybridized with the corresponding oligomer. The resulting double-stranded DNA, both nonirradiated and irradiated (5 min, 25 W, 365 nm), was digested with the restriction enzyme for 1 h at 37 °C following the manufacturers protocol. The cleavage was then analyzed by polyacrylamide gel electrophoresis (Figure 1).

There was no cleavage of dsDNA in the absence of restriction enzyme (Figure 1, lane 1), whereas the absence of any caged nucleotide resulted in complete cleavage of substrate T^0

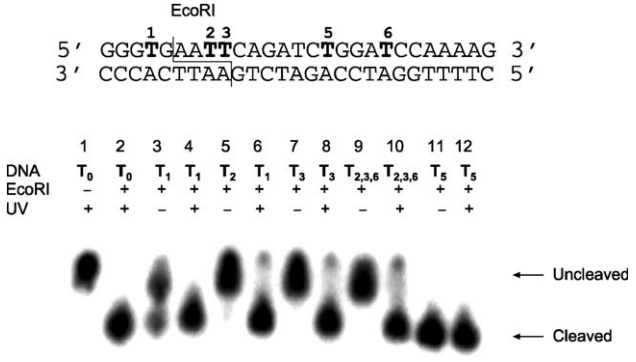

Figure 1. EcoRI digest of caged constructs. Six substrates with caging groups in different positions were digested with EcoRI (1 h, 37 °C) to assess the ability of the caging group to inhibit enzymatic activity. Reactions were irradiated at 365 nm (25 W) for 5 min. Time courses for the digestions are shown in the Supporting Information.

within 1 h (Figure 1, lane 2). The T^1 construct contains a single caged thymidine two bases upstream of the EcoRI cleavage site. Interestingly, digestion of the nonirradiated substrate leads to a mixture of cleaved and uncleaved substrate (Figure 1, lane 3); this suggests that the reaction is inhibited by the presence of the caging group. Upon irradiation of T^1, complete enzymatic cleavage is observed (Figure 1, lane 4). Substrates T^2, T^3, and $T^{2,3,6}$ possess either one or two caging groups within the recognition site of the enzyme, which are either 3 or 4 residues downstream from the cleavage site. The nonirradiated double-stranded oligonucleotides are completely resistant to EcoRI; however, complete cleavage is observed after brief UV irradiation, which removes the caging groups (Figure 1, lanes 5–10; Figure 2). Substrate T^5 possesses a caging

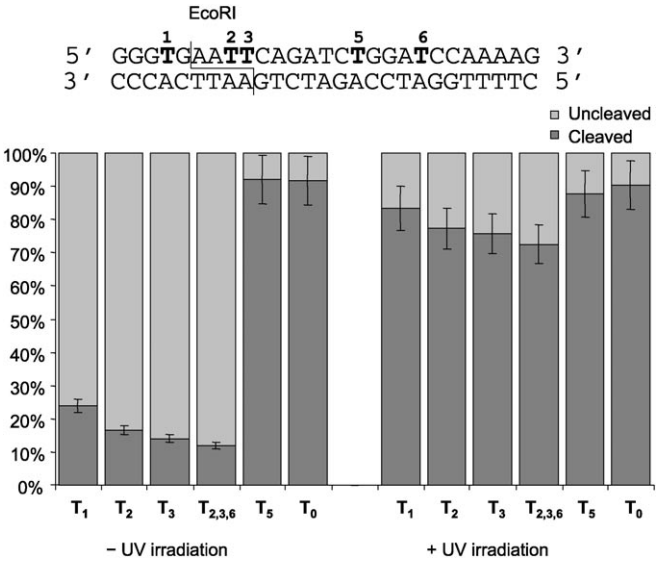

Figure 2. Quantitiative measurement of the EcoRI DNA cleavage with different caged substrates. Reactions were irradiated at 365 nm (25 W) for 5 min. All digests were performed in triplicate and the error bars represent the standard deviation.

group outside the enzymatic recognition and cleavage site; this had no effect on DNA digestion and did not slow the rate of cleavage (Figure 1, lanes 11–12). Conversely, substrate T^4 affords complete cleavage, albeit at a slower rate (see the Supporting Information). This suggests that the caging group does not significantly affect the general binding of the enzyme to the substrate, but instead might inhibit specific recognition of the restriction site. Identical results were observed when labeling the caged DNA strand followed by digestion under identical conditions (data not shown).

To thoroughly assess the binding of the enzyme to the substrate we conducted a gel-shift assay to ascertain the different binding constants for the caged and noncaged dsDNA. Oligomers T^0 and T^2 were hybridized with the radioactively labeled complement, followed by incubation with EcoRI (1 h, 37 °C) at different concentrations in the absence of Mg^{2+} to prevent cleavage activity. This takes advantage of the strong dependence of cleavage activity but not binding on Mg^{2+}.[10,11] The

samples were then analyzed by polyacrylamide gel electrophoresis for the presence of a gel shift, and quantitated by using ImageQuant software. The data were plotted (see the Supporting Information) and analyzed to determine binding constants of 0.54 μM for the noncaged substrate, T^0, and 0.71 μM for the caged substrate, T_2. These values are in agreement with literature binding constants that were obtained under similar conditions (i.e., no Mg^{2+}).[8,11] This suggests that it is feasible for the enzyme to bind the DNA substrate irrespective of the presence of a caging group, albeit at what appears to be a slightly lower affinity. The mechanism of action of many type II restriction endonucleases involves initial non-specific binding to DNA, followed by electrostatic-influenced scanning for the restriction site, and ultimately site-specific cleavage.[8] Thus, our results suggest that the caging group does not significantly inhibit the non-specific binding (based on the gel-shift assay), but does appear to affect the specific recognition of the binding site (based on the cleavage assays). Additionally, because in some cases the caged thymidine is located outside of the recognition site of the enzyme, it does not directly inhibit the recognition of the substrate but leads to a slower cleavage of the substrate. We speculate that this might be a result of steric interference of enzyme recognition as it scans the DNA for the target sequence. This hypothesis is consistent with literature reports of the crystal structures of restriction enzymes as the active site of restriction endonucleases is often responsible for base pair recognition, and the presence of the caged thymidine would represent a significant perturbation of this event.[12]

We next investigated the cleavage reaction of BglII. Here, we employed oligomers T^3–T^5 and $T^{4,5}$ to probe the effect of the caging group. Oligomer T^4 possesses a caging group within the enzyme recognition site that is three nucleotides downstream from the cleavage site. The T^5 oligomer is also caged within the enzyme recognition site five bases downstream from the cleavage site; however, it is located directly in the cleavage site of the opposite strand. Finally, both residues are caged in the $T^{4,5}$ oligomer. As a control oligomer T^3 was also used, which contains a caging group outside the recognition site of this enzyme. As with the EcoRI digest, each double-stranded DNA was digested for 1 h at 37 °C, then analyzed by polyacrylamide gel electrophoresis (Figure 3, and see the Supporting Information). As observed with EcoRI, the digestion of the substrate with BglII can be regulated photochemically by the installation of caged bases. A single caged thymidine completely inhibits enzymatic cleavage if it is located within the enzyme recognition site as observed with the T^4 and T^5 double-stranded DNA. This principle logically extends to the installation of two caging groups, in the case of $T^{4,5}$, in which enzymatic activity is abrogated completely prior to irradiation. In all cases, enzymatic activity is restored upon brief irradiation with UV light at 365 nm (Figure 3). However, complete deactivation of the substrate towards cleavage is not observed in the case of T^3. The degree of DNA cleavage was again analyzed by quantification using ImageQuant software.

Next we examined the application of the nucleobase caging methodology towards the regulation of BamHI on the same

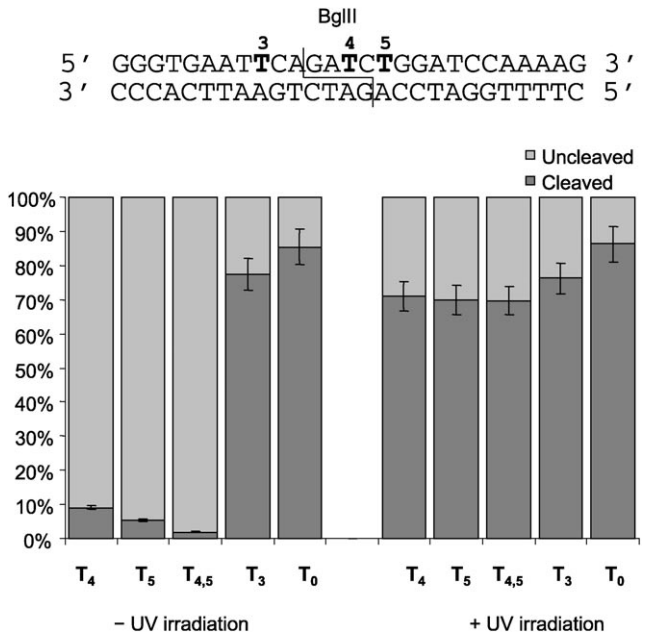

Figure 3. Quantitative assessment of the BglII DNA cleavage with different caged substrates. Reactions were irradiated at 365 nm (25 W) for 5 min. All digests were performed in triplicate and the error bars represent the standard deviation.

substrate. In this investigation we employed the T^4–T^5 and $T^{2,3,6}$ oligomers. Here, only the $T^{2,3,6}$ oligomer possessed a caging group within the recognition site of the enzyme, three bases upstream of the cleavage site. Oligomer T^5 contained a caging group outside of the recognition site, two bases upstream of the cleavage site. The T^4 oligomer was used as a control caged sequence, in which the caging group was located substantially further away from the enzyme recognition and cleavage site. Again, the enzymatic digestions were incubated for 1 h at 37 °C, prior to analysis by gel electrophoresis and quantification using ImageQuant (Figure 4). As observed for the other enzymes, deactivation of DNA cleavage strongly depends on the proximity of the caging group to the cleavage site with complete inhibition in the case of a caged thymidine located directly in the recognition site of BamHI. In all cases, DNA cleavage could be activated by a brief irradiation with UV light of 365 nm. Based on these observations the photochemical regulation of restriction endonuclease activity appears to be generally applicable, as digestion could be regulated by light irradiation for all three enzymes.

Finally, we prepared a substrate for digestion that possessed a restriction site for TaqαI, a hyperthermophilic restriction endonuclease with an optimal activity at 65 °C. We were interested in exploring the scope of the technology by using this enzyme, which is active at high temperatures where DNA hybridization is weaker.[13,14] We examined enzymatic activity at the optimal temperature, and we also probed the enzymatic activity at 37 °C. The caged substrate was again hybridized with its radioactively labeled complement, then subjected to a 2 h digestion (Figure 5).

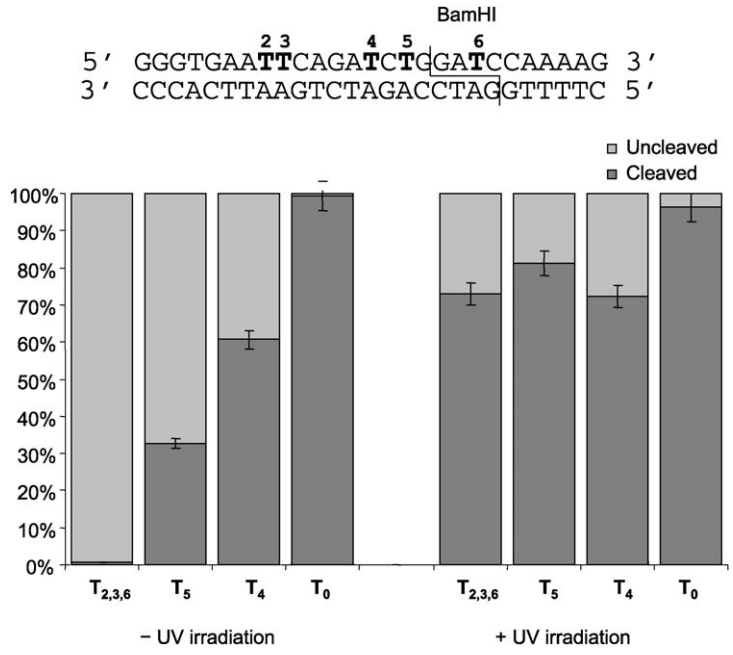

Figure 4. Quantitative assessment of the BamHI DNA cleavage with different caged substrates. Reactions were irradiated at 365 nm (25 W) for 5 min. All digests were performed in triplicate and the error bars represent the standard deviation.

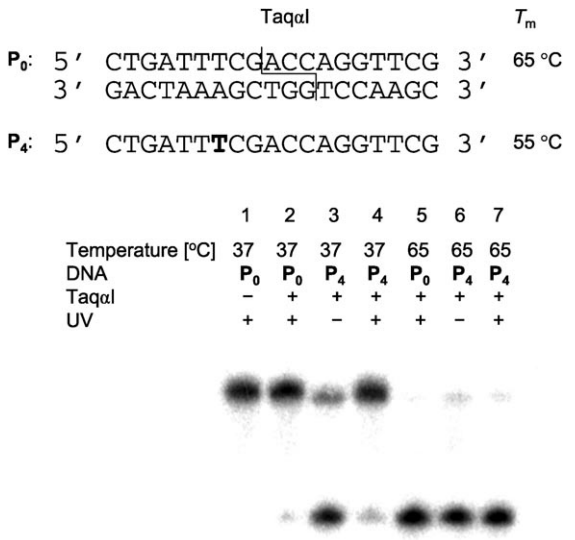

Figure 5. Investigation of the effect of a caging group on the hyperthermophilic restriction enzyme, TaqαI. Reactions were irradiated at 365 nm (25 W) for 5 min.

Interestingly, in the case of TaqαI, the presence of one caging group in P^4 was found to actually activate enzymatic cleavage of the substrate at 37 °C. At this temperature the noncaged substrate P^0 remains uncleaved because the enzyme is not active (Figure 5, lane 1); however, the caged substrate prior to irradiation affords a substantial amount of substrate cleavage (Figure 5, lane 3). This activation is abrogated upon irradiation because very little substrate cleavage is observed in the absence of a caging group (Figure 5, lanes 2 and 4). This unexpected result might occur due to the fact that this enzyme possesses a different mechanism of action than the previously employed endonucleases, and base modifications have little effect on enzyme binding to the DNA target sequence.[13] Whereas only the complementary strand is labeled in Figure 5, both strands are cleaved in the presence of the caging group (see the Supporting Information). At the optimal temperature, the caging group has no effect on enzymatic cleavage because virtually all substrate is cleaved irrespective of the presence of a caging group (Figure 5, lanes 4–6). This is most likely due to the ability of the TaqαI enzyme to ignore base modifications.[13] To ascertain if this effect is specific to the enzyme, or if this substrate is simply prone to non-enzyme-specific degradation as a result of the caging group, we conducted the enzymatic digest with the caged substrate and a variety of restriction endonucleases (see the Supporting Information). However, this effect was only observed in the presence of the TaqαI enzyme, and no other enzyme was capable of cleaving the substrate even after 24 h of incubation.

Based on the enzymatic digests and measured binding constants, we have developed a working hypothesis for the role of the caged thymidine residue on restriction endonuclease activity (Figure 6). It appears that the enzymes are capable of non-specifically binding the caged DNA substrate and subsequently scanning for the restriction site.[8] This is confirmed by gel-shift assays (see the Supporting Information), as EcoRI was indeed able to bind DNA, despite the caging of the restriction site. However, the presence of a caging group inhibits proper identification of this site as dsDNA cleavage is prevented. This is confirmed by the enzymatic digest experiments that demonstrate that the degree of substrate cleavage is dependent upon the position of the caging group relative to the restriction site. Upon irradiation with UV light, the caging group is removed, affording site-specific binding and DNA cleavage by the restriction endonuclease (Figure 6).

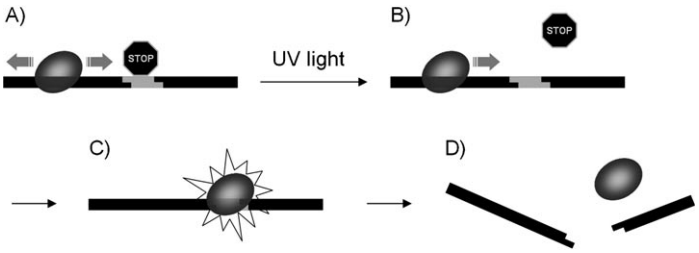

Figure 6. Schematic representation of light-triggered DNA cleavage by restriction endonucleases: A) the restriction enzyme binds to the DNA and scans it for the restriction site, which is blocked by a caging group; B) UV irradiation removes the caging group and this allows access to the restriction enzyme; C) the enzyme finds the restriction site and cleaves it; this leads to D) DNA degradation.

In summary, we have effectively demonstrated both the activation and deactivation of restriction endonucleases via the installation of a photolabile protecting group on the DNA substrate of these enzymes. These results suggest that the interplay of enzyme recognition and cleavage inhibition gives rise to this phenomenon. Based on the developed model, it appears that the caging group can prevent enzyme cleavage when located directly in the enzyme recognition site; however, enzymatic recognition can be affected even when the caging group is located in proximity to the binding site, decreasing the rate of catalysis. Gratifyingly, in all cases, normal endonuclease activity is completely restored upon the photochemical removal of the caging group. These results indicate the possibility of a differential digestion of two cleavage sites with the same restriction enzyme prior and after light irradiation. Moreover, protection of restriction sites by photocaging groups might have implications on the stability of caged DNA in a cellular environment.

Experimental Section

Light-activated restriction enzyme digests: Oligonucleotides were end labeled by using γ^{32}P-ATP (MP Biomedicals, Solon, OH, USA) and T4 kinase (New England Biolabs) at 37 °C for 1 h, and then purified by using TE Midi Select-D, G25 microcentrifuge spin columns (Shelton Scientific, Peosta, IA, USA). The γ^{32}P-end-labeled substrate (10 μL, 1 nmol) was incubated with its complementary strand (10 μL, 1 nmol) at 90 °C for 1 min, and then gradually cooled to 4 °C over 2 h. The dsDNA construct (2 μL, 0.1 nmol) was then subjected to an enzymatic digest (50 μL total volume) according to the manufacturer's protocols with the appropriate buffer (New England Biolabs). Upon completion, the enzyme was deactivated (70 °C, 20 min) and digests were analyzed on a polyacrylamide gel (20%, 400 V, 40 min). Acrylamide gels were visualized by using a Storm phosphorimaging system, and radioactive band intensities were quantified by using Image Quant 5.2.

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Keywords: cage compounds · DNA cleavage · DNA · enzymes · photochemistry

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