DNA Cross-Linking

DOI: 10.1002/anie.201310609

Photoswitchable Formation of a DNA Interstrand Cross-Link by a Coumarin-Modified Nucleotide**

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Abstract: *A coumarin-modified pyrimidine nucleoside (**1**) has been synthesized using a Cu^{I}-catalyzed click reaction and incorporated into oligodeoxynucleotides (ODNs). Interstrand cross-links are produced upon irradiation of ODNs containing **1** at 350 nm. Cross-linking occurs through a [2+2] cycloaddition reaction with the opposing thymidine, 2′-deoxycytidine, or 2′-deoxyadenosine. A much higher reactivity was observed with dT than dC or dA. Irradiation of the dT-**1** and dC-**1** cross-linked products at 254 nm leads to a reversible ring-opening reaction, while such phenomena were not observed with dA-**1** adducts. The reversible reaction is ultrafast and complete within 50–90 s. Consistent photoswitching behavior was observed over 6 cycles of irradiation at 350 nm and 254 nm. To the best of our knowledge, this is the first example of photoswitchable interstrand cross-linking formation induced by a modified pyrimidine nucleoside.*

DNA interstrand cross-links (ICLs) covalently link two DNA strands, which can block DNA replication, transcription, and any other processes requiring strand separation; thus, they have a strong impact on the biological function of nucleic acids. Chemical agents capable of inducing ICLs have been developed for biological applications, such as for DNA damage and repair studies,[1–5] as anticancer agents,[6] for nucleic acid detection,[7,8] for targeting telomeric G-quadruplex structures,[9,10] and for the construction of DNA nanomaterials.[11] Over the past few decades, several research groups have developed novel chemical methods for inducing ICL formation, such as photoirradiation, oxidation, reduction, fluoride induction, and H_2O_2 induction.[12] Among these methods, light induction is particularly important as it is a non-invasive method with high spatio-temporal resolution and control and offers the options of orthogonality. Recently, the research groups of Freccero and Zhou reported the photoinducible formation of quinone methide from biphenyl or binol quaternary ammonium salts[13–15] and formation of vinylidene-quinone methides from 2-alkynylphenols.[16] Quinone methides are highly reactive electrophiles which efficiently cross-link DNA double strands.[11,13–15] Greenberg and co-workers[17–21] described several modified nucleosides that efficiently induced the interstrand cross-linking of DNA upon photoirradiation. Recently, we reported hypoxia-selective ICL formation from nitroimidazole-modified thymidine upon UV irradiation.[22]

Photoactive molecules have also been studied for the construction of DNA-based reversible photoswitches and photo-manipulation of DNA.[23–28] For example, psoralens, a class of naturally occurring photoreactive products, are capable of cross-linking duplex and triplex DNA.[25–28] They have been used as probes of nucleic acid structure and function, for therapeutic gene modulation, and for studying DNA damage and repair.[26–28] Coumarins show many advantages such as high fluorescence quantum yield, large Stokes shift, excellent photostability, and low toxicity. Coumarin derivatives have been widely used in the fields of biology, medicine, cosmetics, and as fluorescent chemosensors for DNA, RNA, and protein detection.[29,30] However, there is no report on the photoactivity of coumarin with DNA. Here, we report the first coumarin-modified nucleoside that induces photoswitchable formation of DNA ICLs upon UV irradiation. A coumarin-modified thymidine efficiently induces ICL formation upon photoirradiation at 350 nm, while the cross-linking can be reversed by irradiation at 254 nm. The cross-linking site was determined by LC-MS/MS as well as by cleavage of gel-purified cross-linked DNA with hydroxyl radicals.

A coumarin moiety has been conjugated to thymidine by a Cu-catalyzed azide-alkyne cycloaddition reaction using azide-modified thymidine **2** and an alkyne-modified coumarin **3**.[18,31] Compound **1** was converted into its phosphoramidite building block **5** under standard conditions (Scheme 1). Oligodeoxynucleotides (ODNs) containing **1** were synthesized by automated solid-phase synthesis using **5** and confirmed by MALDI-TOF-MS analysis (see Table S1 in the Supporting Information).

The DNA duplex was photoirradiated at 350 nm for 50 min using a Rayonet Photochemical Chamber Reactor (Model RPR-100). A wavelength of 350 nm was chosen because near-UV light (> 300 nm) is compatible with living cells and is almost not absorbed by most biological molecules other than the coumarin-modified ODNs. Moreover, the

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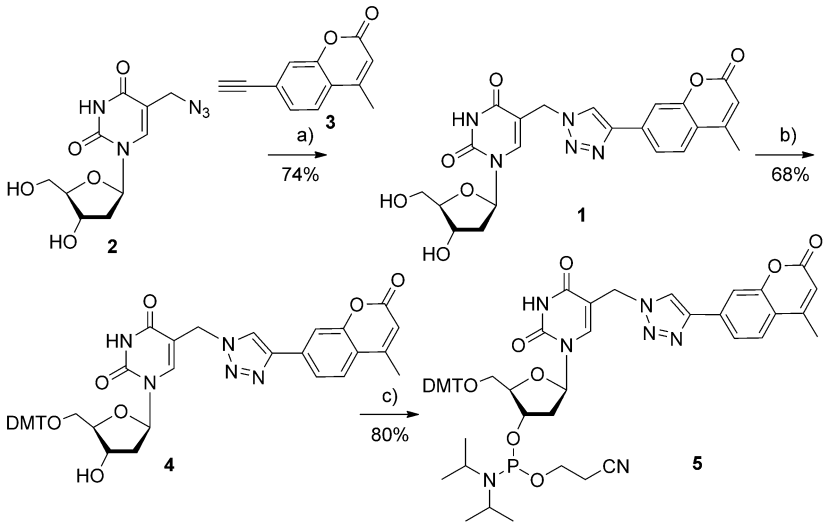
[**] We are grateful for financial support from the National Cancer Institute (1R15CA152914-01), UWM Research Growth Initiative (RGI101X234), WiSys technology foundation (applied research grant award), and the Great Milwaukee Foundation (Shaw Scientist Award; to X.P.) and the National Institute of Environmental Health Sciences (R01 ES019873; to Y.W.). Support from the distinguished graduate student fellowship for H.S. is also acknowledged.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201310609.



Scheme 1. Synthesis of compound **1** and its phosphoramidite building block **5**. Reagents: a) Cu_2SO_4 and sodium ascorbate; b) dimethoxytrityl chloride (DMTCl), pyridine; c) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite, *N*,*N*-diisopropylethylamine and CH_2Cl_2.

coumarin moiety can be excited to singlet and triplet excitation states upon irradiation with light at a wavelength of 350 nm. The UV irradiation of duplex **7** resulted in a new band whose migration is severely retarded relative to unreacted oligonucleotide, indicative of the formation of interstrand cross-linked material, while this was not observed with native DNA duplex **6** (Figure 1). The generation of ICLs induced by **1** followed first-order kinetics with a rate constant (k_{ICL}) of $9.24 \pm 0.25 \times 10^{-4}\ s^{-1}$ (see Figure S1 in the Supporting Information). The cross-linked product was isolated and characterized by mass spectrometry and fluorescence measurements. ODN **7a** showed strong fluorescence with an emission maximum at 392 nm. However, a greatly decreased fluorescence intensity was observed with the cross-linked product **8** (**8a**—**c**, Figure 2). We propose that the cross-link formation may lead to the destruction of the conjugation system of the coumarin moiety.

Figure 1. ICL formation upon UV irradiation at 350 nm for 50 min. Phosphorimage autoradiogram of 20% denaturing PAGE analysis of the ICL products: lane 1, unmodified duplex **6**; lanes 2–5, duplexes **7** and **9**–**11** containing coumarin-modified ODNs hybridized with opposing sequences containing A, C, T, and G; lanes 6–9, duplexes **12**–**15** containing coumarin-modified ODNs hybridized with opposing sequences containing only an A, T, G, and C in opposite sequences.

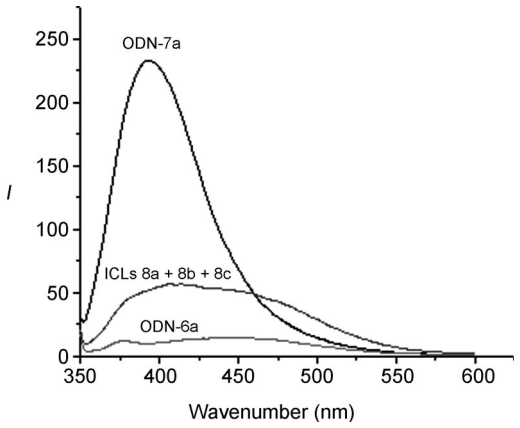


Figure 2. Fluorescence emission spectra of coumarin-modified ODN **7a**, ICLs (**8a**–**c**) induced by coumarin after photoirradiation, and the unmodified ODN **6a**. The reaction mixture contained 5.0 μM DNA, 100 mM NaCl, and 10 mM phosphate buffer (pH 7.0).

Initially, the hydroxyl radical footprinting of gel-purified cross-linked DNA was used to determine which nucleotides were covalently bonded with one another.[17] Each ODN was either 5′- or 3′-^{32}P-labeled. As expected, cross-linking occurred mainly at the position of **1** (see Figure S2 in the Supporting Information). However, the exact cross-linking sites in the complementary strand could not be determined by the cleavage reaction with hydroxyl radicals, possibly because of the presence of multiple reaction sites. To exploit this further, we used LC-MS and MS/MS to determine the identities of the nucleobase(s) in the opposite strand that is (are) cross-linked with the coumarin-modified thymidine moiety. To this end, we digested the isolated ICL products obtained from duplexes **7** and **9** with a cocktail of four enzymes to release the ICL as a dinucleoside remnant, by following previously published procedures,[32] and subjected

the resulting mixture to LC-MS and MS/MS analyses. Our LC-MS/MS results revealed, in the digestion mixture, the presence of dinucleosides, with the coumarin-derived thymidine conjugated with a thymidine (dT), 2′-deoxycytidine (dC, detected as the deaminated product where the dC moiety was converted into a 2′-deoxyuridine, dU), and, to a lower extent, 2′-deoxyadenosine (dA, Figure 3 and see Figures S3–S9 in the Supporting Information).

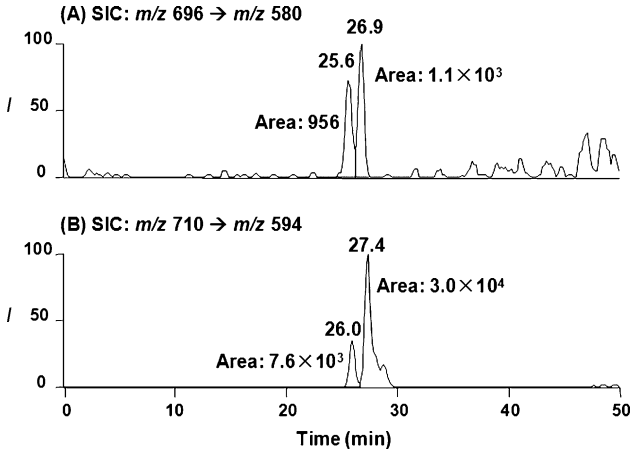
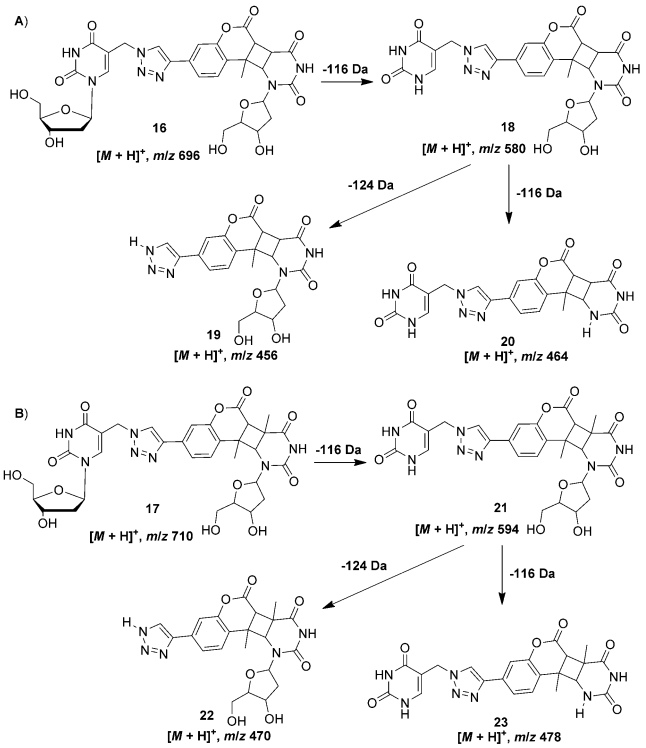


Figure 3. Selected-ion chromatograms (SICs) for monitoring the indicated transitions for the dU-**1** cross-link (A) and dT-**1** cross-link (B) in the digestion mixture of ICL products generated from duplex **7** (**7a**●**6b**).



Scheme 2. The proposed structures of the ICL products and the proposed major fragmentation pathways for the $[M+H]^+$ ion of dU-**1** (A) or dT-**1** cross-link (B) observed in LC-MS/MS.

When **1** is opposite to four different nucleosides, the ICL yields decreased in the order dT (**10**, 69%) > dC (**9**, 55%) > dA (**7**, 43%) > dG (**11**, 24%; Figure 1). This result indicates that **1** has a higher reactivity towards pyrimidines than purines. To fully investigate the reactivity of **1** towards the four canonical nucleosides, we synthesized DNA duplexes **12**–**15** with only dA, dT, dG, or dC surrounding the photo-cross-linking site and examined the cross-linking efficiency and the rate constant for ICL formation. Among these duplexes, the highest ICL yield (87%) was observed with duplex **13** (dT), while no ICLs were found with duplex **14** (dG) (Figure 1). Although duplexes **12** (dA) and **15** (dC) resulted in about 4% and 7% ICLs, respectively, the reaction of dC or dA with **1** is much slower than that of dT with **1**. The rate constants are in the order k_{dT} $(3.5\pm0.5\times10^{-3})$ > k_{dC} $(1.2\pm0.2\times10^{-3})$ > k_{dA} $(0.8\pm0.2\times10^{-3}$; see Figure S10 A–C in the Supporting Information). All these results supported that dT has a much higher reactivity than dC and dA towards the coumarin moiety. The LC-MS/MS analysis of the ICL products obtained from duplex **7** or **9** showed that the coumarin-derived thymidine mainly conjugated with dT in duplex **7** and with dC in duplex **9** (Figure 3 and see Figures S4 and 5 in the Supporting Information). In addition, all cross-linked products are stable to heating in phosphate buffer or 1.0 M piperidine (see Figure S11 in the Supporting Information). Collectively, based on the LC-MS/MS analysis and greatly decreased fluorescence intensity of the ICL adducts and the relative reactivities of **1** towards dT, dC, and dA, we propose that a [2+2] photocycloaddition reaction occurred, thereby forming adduct **16** or **17** (Scheme 2). The MS/MS analysis for monitoring the $[M+H]^+$ ion of dU-**1** revealed two major peaks eluting at 25.6 and 26.9 min in the selected-ion chromatogram (SIC) for the *m*/*z* 696 (**16**) →580 (**18**) transition, which monitors the loss of a neutral 2-deoxyribose (Scheme 2 A). Product ions of *m*/*z* 456 (**19**) and 464 (**20**) were also found in the MS/MS as a result of the losses of neutral 2-deoxyribose and thymine or the second 2-deoxyribose (see Figure S6 A,B,E in the Supporting Information). A similar fragmentation pathway was observed for dT-**1** cross-linking product **17** (Scheme 2 B and see Figure S6 C,D,F in the Supporting Information). It is precedented that a [2+2] cycloaddition reaction occurred between dT and psoralen involving either the 3,4-double bond of the pyrone ring or the 4′,5′-double bond of the furan ring.[25] However, the structure of dA-**1** adduct could not be determined at this point. In this vein, it is worth noting that, upon exposure to 254 nm UV light, thymine was found to undergo [2+2] cycloaddition with its neighboring 3′-adenine to give intrastrand cross-link products.[33,34]

The ICL formation induced by **1** is photoswitchable. Irradiation of duplex **10** at 350 nm efficiently generated ICLs, while the cross-linked product can be split into single-stranded DNAs by irradiation at 254 nm (Figure 4 and see Figure S12 in the Supporting Information). Cleavage of the ICL products to two single-stranded ODNs was ultrafast and complete within 60 s by irradiation at 254 nm ($k_{cleave}=8.0\pm0.5\times10^{-2}\ s^{-1}$, $t_{1/2}=9.0$ s; see Figure S14 A in the Supporting

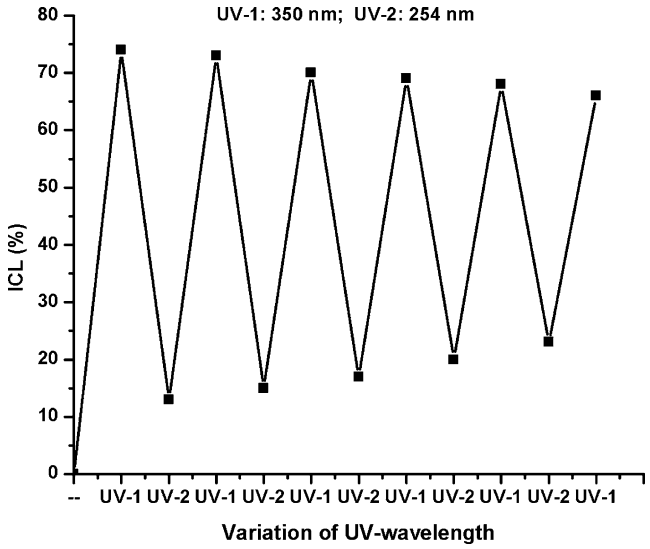


Figure 4. Reversibility of the DNA interstrand photo-cross-linking by a coumarin-modified ODN duplex **10** over six cycles of 50 min UV irradiation at 350 nm and 6 min at 254 nm.

Information). This process was highly reversible, and consistent switching behavior was validated over six cycles of irradiation at 350 nm for 50 min and 254 nm for 6 min (Figure 4 and see Figure S12 in the Supporting Information). A similar phenomenon was observed with duplex **7**. This process was also confirmed by mass spectrometry. The MALDI-TOF-MS analysis showed that the cross-linked DNA products isolated from the duplex **7** irradiated at 350 nm were split into two single-standed ODNs upon irradiation at 254 nm with UV light. The detected masses of two ODNs are 5185 and 4800 Da, which are identical to the coumarin-modified ODN **7a** (calcd: 5186.4 Da) and the complementary strand **6b** (calcd: 4800.2 Da; see Figure S13 in the Supporting Information). So far, neither unsubstituted nor substituted pyrimidine nucleosides have ever been applied as components of DNA photoswitches.[35,36] This feature would be one of the important milestones for photoswitchable interstrand cross-linking of DNA. The photochromic behavior of these compounds was observed by gel electrophoresis analysis followed by UV photoirradiation. Six distinct absorption bands were investigated to verify the ratification of the exceptional photoswitchability.

To determine whether dT-**1**, dC-**1**, and dA-**1** cross-links are reversible, we isolated the ICLs formed from duplex **10** after one cycle of irradiation at 350 nm (ICL-1), one cycle of irradiation at 350 nm/254 nm (ICL-2), and those formed after 10 cycles of irradiation at 350 nm/254 nm (ICL-20). We then digested the isolated ICL products with enzymes to release the ICL as a dinucleoside remnant and subjected the resulting mixture to LC-MS and MS/MS analyses, as described above. We then plotted the ratios of the peak areas found in the SICs to monitor the loss of a 2-deoxyribose from the $[M+H]^+$ ions of dC-**1** (as its deaminated form, that is, **16**, m/z 696→580 transition), dT-**1** (**17**, m/z 710→594 transition), and dA-**1** adducts (m/z 710→594 transition) versus that of the $[M+H]^+$ ion of dA (see Figure S27 in the Supporting Information). The signals for the dT-**1** and dU-**1** cross-links were decreased from ICL-1 to ICL-2 and ICL-20, whereas a significant elevation of the dA-**1** signal was observed from ICL-1 and ICL-2 to ICL-20. These results suggested that the formation of dC-**1** and dT-**1**, but not that of dA-**1**, is reversible. In this context, it is worth noting that, as a result of the differences in the ionization efficiencies for these dinucleosides (dA-**1**, because of the higher proton affinity of adenine than uracil or thymine, is expected to have much better ionization efficiency than dT-**1** and dU-**1** in the positive-ion mode), the ratios of the peak areas displayed in Figure S27 (see the Supporting Information) do not reflect the relative levels of dT-**1**, dU-**1**, and dA-**1** interstrand cross-links. To further substantiate this finding, we examined the reversibility of ICL formation from duplexes **12**, **13**, and **15**. Our data showed that the formation of cross-linked products from duplexes **13** (dT-**1** cross-link) and **15** (dC-**1** cross-link) on irradiation at 350 nm were reversible by irradiation at 254 nm (see Figure S14 B,C in the Supporting Information), while ICL products generated from duplex **12** (dA-**1** cross-link) were not cleaved by irradiation at 254 nm (see Figure S14 D in the Supporting Information). The reversible photo-cross-linking reaction with duplexes **13** and **15** is ultrafast and complete within 90 s and 50 s, respectively (duplex **13**: $k_{\text{dT-1-cleave}} = 5.0 \pm 0.9 \times 10^{-2}\ \text{s}^{-1}$, $t_{1/2} = 13.0$ s; duplex **15**: $k_{\text{dC-1-cleave}} = 8.8 \pm 0.4 \times 10^{-2}\ \text{s}^{-1}$, $t_{1/2} = 8.0$ s; see Figure S14 B,C in the Supporting Information). These data are consistent with our results from LC-MS and MS/MS analysis, and are also in line with the fact that the cyclobutane-type photoproduct formed between thymine and the psoralen moiety can be photoreversed upon irradiation at 254 nm,[37] whereas the UVC-induced dimeric TA photoproduct cannot be photoreversed.[34]

In conclusion, we have developed a novel strategy for photoswitchable interstrand cross-linking of DNA using a coumarin-modified nucleoside (**1**) that can be easily prepared through "click" chemistry. Irradiation at 350 nm of ODNs containing **1** produced a nonfluorescent DNA cross-linking product which can be reverted to the original fluorescent ODNs by irradiation at 254 nm. The mechanism involves the [2+2] photocycloaddition between coumarin moiety and dT, dC, or to a much lower extent dA. The dT-**1** and dC-**1** adducts are photoreversible, while the dA-**1** adduct is not reversible. The reversible interstrand photo-cross-linking reaction is bioorthogonal, does not require additional chemical reagents, and can be controlled in space and time by choosing different irradiation wavelengths. We envision that the method is applicable for in situ DNA manipulation, such as photocontrolled inhibition of DNA transcription or DNA ligation and the regulation of DNA aptamers.[38] It provides a simple and versatile approach for developing reversible DNA fluorescent photoswitches, photoswitchable DNA nanomotors, and smart DNA nanostructures and nanodevices.

Received: December 6, 2013
Revised: March 19, 2014
Published online: ■■ ■■, ■■■■

Keywords: click chemistry · cycloaddition · DNA cross-linking · nucleosides · photoswitch

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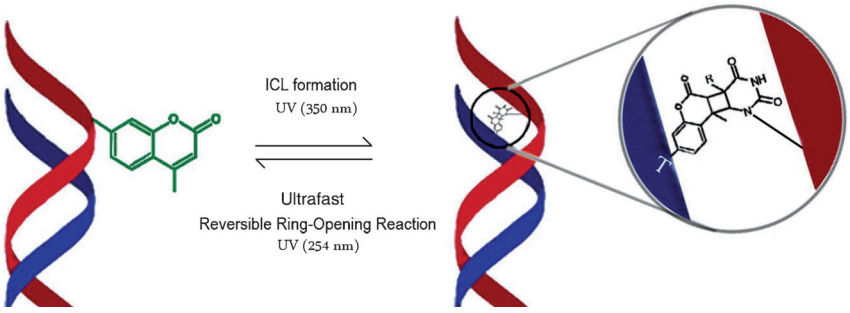
Communications

DNA Cross-Linking

M. M. Haque, H. Sun, S. Liu, Y. Wang, X. Peng* ——— ■■■■–■■■■

Photoswitchable Formation of a DNA Interstrand Cross-Link by a Coumarin-Modified Nucleotide



A [2+2] cycloaddition reaction between a coumarin-modified pyrimidine nucleoside and the opposing thymidine, 2′-deoxycytidine, or 2′-deoxyadenosine on a DNA strand generates photoswitchable interstrand cross-links (ICLs) in the DNA.