Organic & Biomolecular Chemistry

ROYAL SOCIETY OF CHEMISTRY

COMMUNICATION

Cite this: *Org. Biomol. Chem.*, 2021, **19**, 1292

Received 3rd December 2020,
Accepted 20th January 2021

DOI: 10.1039/d0ob02422h

rsc.li/obc

Thermally reversible and irreversible interstrand photocrosslinking of 5-chloro-2′-deoxy-4-thiouridine modified DNA oligonucleotides†

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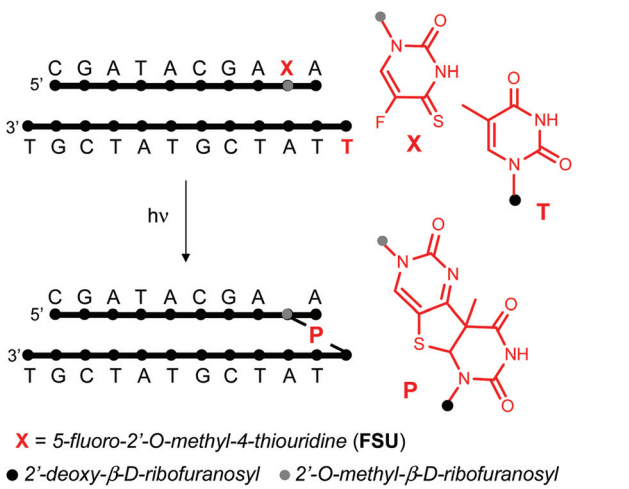
We describe highly efficient interstrand photocrosslinking of a DNA duplex containing 5-chloro-2′-deoxy-4-thiouridine (ClSdU) in one strand, proceeding *via* a two-step photochemical cascade, involving the formation of a thermally reversible crosslink between ClSdU and thymidine in the target strand and its subsequent conversion to a thermally stable fluorescent crosslink. These results show that ClSdU has great potential to be a valuable DNA photocrosslinking reagent for chemical biology applications.

DNA interstrand crosslinking (ICL) agents are widely used in the study of DNA damage and repair mechanisms[1,2] as well as for many other biological and medicinal applications.[3] Particularly valuable are light-activated agents allowing for the sequence-specific generation of interstrand crosslinks in double-stranded DNA oligonucleotide systems.[2,4,5] Despite significant advances in this area of research, there is still a great need for the development of photoactivated DNA cross-linkers. The 5-fluoro and 5-chloro derivatives (**FSU** and **ClSU**, respectively) of highly photoreactive 4-thiouridine (**SU**), studied recently in our lab, appeared very promising in this regard. These compounds, like **SU**, show high photoreactivity toward thymidine (**T**); however, unlike the parent thionucleoside, which forms (5–4) type adducts with **T**,[6] both **FSU** and **ClSU** photoreact with **T** to form a thermally stable tricyclic photoadduct (**P**) characterized by intense fluorescence (φ = 0.49) with maximum emission at 460 nm.[7] DNA interstrand crosslinking utilizing this photoreaction has been already demonstrated for DNA duplexes containing **FSU** placed between two adenosines in one strand. The most efficient and highly specific, both in terms of "direction" and diastereoselectivity, photocrosslinking reaction of **FSU** with **T** was observed for the oligonucleotide system as shown in Scheme 1. The reaction was nearly quantitative and involved the overhanging 5′-**T** in the target oligonucleotide strand only with the formation of a single diastereomer of the crosslink.[9] It should be noted that because of the high chemical instability of 2′-deoxy-5-fluoro-4-thiouridine, the 2′-*O*-methyl analog of **FSU** was incorporated into the investigated probe oligodeoxynucleotides using the solid-phase phosphoramidite method.[8,9] In the present study, we made an attempt to synthesize and investigate the photochemical properties of an analogous oligonucleotide system modified with 5-chloro-2′-deoxy-4-thiouridine (**ClSdU**). The synthesis of **ClSdU** modified oligodeoxynucleotide **5** (ODN **5**) is outlined in Scheme 2. 3′,5′-Di-*O*-acetyl-2′-deoxy-5-chlorouridine, **1**, was obtained by cerium(IV) mediated chlorination of acetylated 2′-deoxyuridine[10] and converted into phosphoramidite **4** following the procedure developed for the synthesis of



Scheme 1 Interstrand photocrosslinking of the DNA duplex modified with 5-fluoro-2′-*O*-methyl-4-thiouridine (**FSU**) and the structure of the fluorescent photoadduct (**P**).[9]

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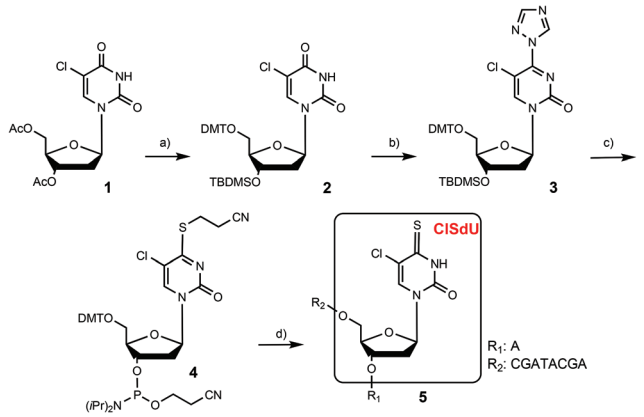
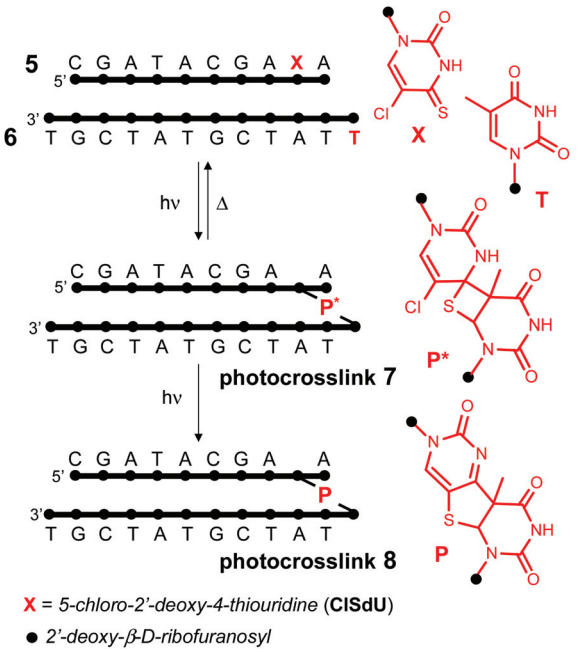
† Electronic supplementary information (ESI) available: Experimental procedures and characterization data. See DOI: 10.1039/d0ob02422h



Scheme 2 Synthesis of ODN **5**, reagents and conditions: (a) (1) NH_3/MeOH, 3 h, (2) DMTCl, pyridine, 24 h, 60%, (3) TBDMSCl, imidazole, DMF, 24 h, 83%, (b) 1,2,4-triazole, TEA, $POCl_3$, CH_3CN, pyridine, 24 h, 93%, (c) (1) $NC(CH_2)_2SH$, DIPEA, CH_3CN, 2 h, 97%, (2) TEA-3HF, 3 h, 62%, (3) $NC(CH_2)_2OP(Cl)N(iPr)_2$, DIPEA, THF, 1 h, 74%, (d) synthesis with an automated DNA synthesizer.



Scheme 3 Thermally reversible (photocrosslink **7**) and irreversible (photocrosslink **8**) interstrand photocrosslinking of the DNA duplex (ODN **5**/ODN **6**) labelled with 5-chloro-2′-deoxy-4-thiouridine with structures of the thietane intermediate (**P***) and the fluorescent photoadduct (**P**).

FSU modified oligonucleotides.[8] The sulfur residue was introduced into position 4 of the pyrimidine ring *via* 1,2,4-triazolyl derivative **3**. The synthesis starting from 3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-2′-deoxyuridine was also attempted, but considerable decomposition was observed during the chlorination step. An alternative order of deacetylation and introducing the DMT group, *i.e.* after the chlorination/sulfuration steps, resulted in a significantly lower yield (~37%). Oligonucleotide **5** was synthesized according to the standard phosphoramidite chemistry on a DNA synthesizer using phosphoramidite **4** and natural DNA phosphoramidites protected with ultramild deprotection groups on the nucleobases. The removal of the thiol-protecting 2-cyanoethyl group and nucleobase-protecting groups followed the procedure used for the synthesis of **FSU** labelled ODNs.[8] After deprotection and HPLC purification, ODN **5** was characterized by MALDI-TOF MS (calcd 3072.5 for $[M + H]^+$, found 3073.5; calcd 3111.5 for $[M + K]^+$, found 3111.5). Like in the case of the analogous oligonucleotide system modified with **FSU**, the photochemical behavior of ODN **5** in the presence of ODN **6** (3′ TGCTATGCTATT) having a complementary sequence with overhanging thymidines at the 3′ and 5′ ends was investigated under the selective excitation of the **ClSdU** residue (Scheme 3). Thus, the reaction mixture containing ODN **5** (10 μM, strand conc.) with a small excess of ODN **6** (12 μM, strand conc.) in 0.1 M phosphate buffer (pH 7.0) was irradiated with a UV laser (80 mW, 355 nm) at 15 °C under aerobic conditions. An excess of **6** relative to **5** was needed to ensure that all the **ClSdU** modified strands were annealed into a duplex. The progress of the reaction was monitored by HPLC (Fig. 1). As shown in Fig. 1b, the analysis of the solution photoirradiated for 4.5 min indicated almost complete disappearance of ODNs **5** and **6** with the concomitant formation of a single photoproduct **7**. The obtained photoreaction mixture containing **7** was heated at 60 °C for 1 hour and analyzed by HPLC. The photoproduct **7** showed thermal instability and was reversed to the starting oligodeoxynucleotides **5** and **6** (Fig. 1c). However, when irradiation was continued for 120 min (80 mW, 355 nm, at 15 °C), intermediate photoproduct **7** was converted to fluorescent photoproduct **8** (Fig. 2b). The thermal instability of photoproduct **7** was determined (Fig. S1–3†) and its half-life at room temperature was estimated (5 h) (Fig. S4†). Photoproduct **8** was isolated and characterized by MALDI-TOF MS (6662.4 calcd for $[M + H]^+$, found 6662.7).

The results indicated that photoproduct **8** had the observed molecular weight of the sum of ODNs **5** and **6** with the loss of a HCl molecule. The melting temperatures of duplex **5**/**6** and photoproduct **8** were measured and a significant difference was noticed (T_m = 40.4 °C and T_m = 76.6 °C, respectively, Fig. S5†). Enzymatic digestion of photoproduct **8** with snake venom phosphodiesterase (SVPD) and alkaline phosphatase (AP) revealed the incompletely digested fragment (Fig. S6†) containing fluorescent adduct **P**. According to the previously proposed enzymatic (SVPD/AP) cleavage pattern and based on the comparison of the UV absorption spectra of incompletely digested fragments (Fig. S7†), the obtained fragment corresponds to the crosslink of **ClSdU** to 3′-overhanging **T** (Scheme 3), as in the case of the analogous duplex modified with **FSU**. The absorption and fluorescence properties of photocrosslink **8** (Fig. 2a and b) were the same as those for the interstrand photocrosslink obtained in our previous studies, concerning photocrosslinking in DNA duplexes labeled with **FSU**.

In the absorption spectrum of photoproduct **7**, the shift of the maximum absorption at 340 nm originating from the 4-thiocarbonyl group of **ClSdU** to a shorter wavelength

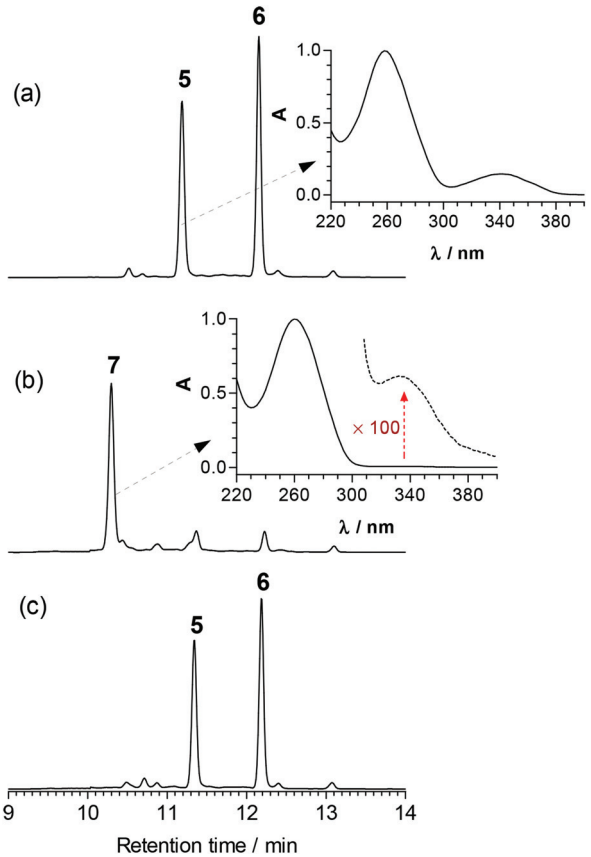


Fig. 1 (a) HPLC analysis of the reaction mixture containing ODN **5** and ODN **6** before irradiation, the inset shows the UV absorption spectrum of **5**; (b) after 4.5 min of irradiation (80 mW, 355 nm, 15 °C), the inset shows the UV absorption spectrum of photocrosslink **7**; and (c) after 4.5 min of irradiation and 1 h of heating of the irradiated solution at 60 °C. The elution profiles were monitored at 260 nm.

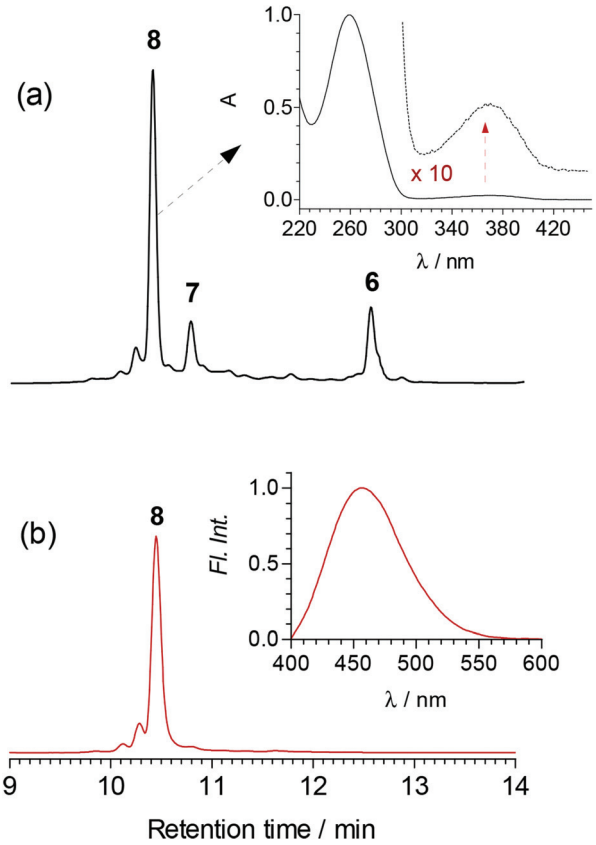


Fig. 2 HPLC analysis of the reaction mixture containing crosslinked oligonucleotide **7** (*cf.* Fig. 1b) after further irradiation for about 120 min (80 mW, 355 nm, 15 °C). (a) The elution profile monitored by UV absorbance at 260 nm, the inset shows the absorption spectrum of photocrosslink **8**. (b) The elution profile monitored by fluorescence detection using an excitation wavelength of 370 nm with monitoring of the emission wavelength at 460 nm. The inset shows the fluorescence emission spectrum of photocrosslink **8**.

(330 nm) was observed (Fig. 1b). However, the thiocarbonyl group is reconstructed when photoproduct **7** is decomposed and is separated into two starting oligonucleotides **5** and **6**. Similar absorption properties of intermediates were reported by Clivio *et al.* during the irradiation of the dinucleotide thymidylyl(3′–5′)-4-thiothymidine leading to the (6–4) photoproducts.[11] According to the accepted mechanism of (2 + 2) cycloaddition of the thiocarbonyl group to the C5–C6 double bond of pyrimidine, the first step of the reaction is the formation of the thietane intermediate.[11,12] In the case of **FSU**, the photocrosslinking reaction is ~4-fold faster compared to **ClSU**[7] and we haven't noticed the formation of any intermediate during the irradiation of oligonucleotides modified with **FSU**.[9] Due to thermal instability, we were not able to fully characterize the structure of photocrosslink **7**. However, based on the observed properties (its thermal reversion to **5** and **6**, absorption properties and its phototransformation to photocrosslink **8**), photocrosslink **7** was characterized as the postulated thietane intermediate (Scheme 3). Thermally unstable intermediates were previously reported in the case of 2-methyl-1,4-napthoquinone[13] and benzophenone[14] DNA crosslinking agents.

Conclusions

We present the incorporation of a photoactive probe – 5-chloro-2′-deoxy-4-thiouridine – into an oligodeoxynucleotide and its photochemical reactivity in the photocrosslinking reaction with thymidine located in the complementary oligodeoxynucleotide, leading to the formation of a fluorescent interstrand photocrosslink. The photocrosslinking of the oligodeoxynucleotide labeled with **ClSdU** allowed for the first time to observe the formation of the postulated thietane intermediate. The results (formation of the thietane intermediate (photocrosslink **7**) during short irradiation and the fluorescent photocrosslink **8** with a permanent covalent bond) may provide a methodology for the reversible or irreversible connection of oligonucleotide strands. Such a versatile methodology would allow to combine two different techniques (reversible and irreversible linking of oligonucleotides) within one analytical tool. Thermally reversible interstrand photocrosslinking of **ClSdU** modified DNA oligonucleotides may find applications in molecular devices used for the rapid capture

and release of intact nucleic acids, whereas the generation of thermally stable, highly fluorescent crosslinks could be used for the detection of specific DNA sequences.

Author contributions

J. N.-K., B. S. and J. M. developed the concept, designed the experiments and analyzed the data. J. N.-K. and K. Z. performed the experiments, J. N.-K. and B. S. prepared the manuscript. All authors have given approval to the final version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This work was partially financially supported by Research Grant No. 2020/04/X/ST4/00418 from the National Science Center (NCN) of Poland.

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