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Hypoxia-Selective DNA Interstrand Cross-Link Formation by Two Modified Nucleosides

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Abstract

Two nitroimidazole modified thymidines **1a** and **1b** have been synthesized and incorporated into DNA oligomers. The 350 nm photolysis of **1a** and **1b** generated a 5-(2'-deoxyuridiny)methyl radical that induced DNA interstrand cross-links (ICL). Higher ICL yield was observed under hypoxic conditions than aerobic conditions. The photoproducts of monomers or DNA oligomers were isolated and characterized by NMR and/or mass spectroscopy.

Keywords

nucleosides; DNA damage; hypoxia-selective; DNA cross-linking; UV-photolysis

DNA interstrand cross-links (ICLs) are deleterious to cells because of their unyielding obstruction to replication and transcription.^[1, 2] For example, a single unrepaired ICL can be sufficient to kill an eukaryotic cell. Many anticancer agents, such as mitomycin C, nitrogen mustard, and chlorambucil, can kill cancer cells by alkylating DNA opposing strands. However, these agents are also toxic to healthy cells. One approach to reduce the toxicity of the cross-linking agents for normal cells is to trigger site-selective crosslink formation in tumor cells. Hypoxia appears to be a common and unique property of cells in solid tumors, and is an important potential mechanism for the tumor-specific activation of prodrugs.^[3–5] Therefore, it is of great interest to develop agents that can form DNA ICLs selectively under hypoxic conditions.

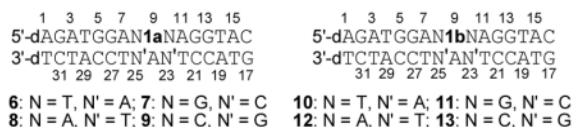
Exogenous molecules have been developed that sensitize DNA to γ -radiolysis under anoxic conditions, such as nitroimidazole and nitrophenyl derivatives.^[6, 7] Coupling of a nitroaryl group with active species resulted in hypoxia-targeting anticancer drugs.^[8] The non-native nucleosides 5-bromo- and 5-iodo-2'-deoxyuridine are also used as radiosensitizers.^[9] Recently, Greenberg's group has reported that a phenyl selenide-modified thymidine (dT-SePh) produced DNA interstrand cross-links (ICLs) upon UV-photolysis or γ -radiolysis via a nucleotide radical.^[10–13] The ICL formation is independent from oxygen.^[10, 12a] We expect that the introduction of a hypoxic radiosensitizer to the modified nucleosides will

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make them more sensitive to photolysis under hypoxic condition. All these encourage us to synthesize two nitroimidazole-modified pyrimidine nucleosides (**1a**, **1b**), and determine their radiosensitivity and hypoxia selectivity in respect to DNA ICL formation. Our investigations showed that **1a** and **1b** can form ICL selectively under hypoxic condition.

Compounds **1a** and **1b** were synthesized starting from thymidine (dT), which was protected in the sugar moiety with an acetyl group (**2**) (Scheme 1). The bromination of **2** followed by the introduction of nitroimidazole group resulted in **3a** and **3b**. Deprotection of **3a** and **3b** yielded **1a** and **1b**, which were converted to their phosphoramidite building blocks **5a** and **5b**. Oligonucleotides (**6–13**) containing **1a** or **1b** were synthesized via automated solid-phase synthesis using **5a** or **5b**. The coupling yields were more than 98%.



The nitroimidazole slightly decreased the duplex stability (**6** and **10**) with a ΔT_m of 3–4 °C relative to when dT was present (duplex **14**) (Table 1). Similar to dT, **1a** and **1b** show mismatch discrimination towards dC ($\Delta T_m = -10.8$ and -8 °C), dG (-7.7 and -5.7 °C), and dT (-10.4 and -8.6 °C). These data suggest that **1a** and **1b** form a Watson-Crick base pair with dA.

All DNA duplexes were photo-irradiated at 350 nm using a Rayonet Photochemical Chamber Reactor (Model RPR-100). A wavelength of 350 nm was chosen because near-UV light (> 300 nm) is not/slightly absorbed by most biological molecules and is compatible with living cells.^[14] Both compounds have absorbance at 350 nm ($A = 0.08$ for 0.023 mM **1a** and $A = 0.19$ for 0.023 mM **1b**) (Supporting Information, SI, Figure S46). Furthermore, the nitroimidazole derivatives can be excited by 350 nm to form a radical anion transition state.^[15] The UV-photolysis of **6** resulted in a new band whose migration is severely retarded relative to unreacted oligonucleotide, indicative of interstrand cross-linked material (Figure 1). The cross-linking yield under hypoxic condition (3.6%) is 1.6 times higher than that under aerobic condition (2.2%) (Figure 1, lanes 5 vs. 6). We also examined cross-link formation with duplexes **7–9** in which **1a** is flanked by different sequences (Figure 1, lanes 8 vs. 9, 11 vs. 12, 14 vs. 15). In all cases, hypoxic conditions resulted in higher cross-linking yield than aerobic conditions (Figure 2A). This further proved the hypoxia-selective cross-linking formation induced by **1a**. In a control experiment, an otherwise identical duplex (**14**) containing dT in place of **1a** was irradiated by UV, while less than 0.2% ICL was observed under both aerobic and anaerobic conditions. This indicated that the nitroimidazole group in **1a** plays an integral role in ICL formation and hypoxia-selectivity in **6–9**. In order to determine the generality of this property, we synthesized 2-nitroimidazole modified thymidine (**1b**), and examined its ability for ICL formation. Similarly, higher cross-linking yield was observed under hypoxic conditions than that under aerobic conditions when the duplexes containing **1b** (**10–13**) were irradiated at 350 nm (Figure 2B).

There are two possible mechanisms for the ICL formation, either through a 5-(2'-deoxyuridinyl)methyl radical **16**^[12] or via formation of a carbocation derived from the protonated **1a** or **1b**.^[16] However, the carbocation mechanism is considered particularly unlikely as the incubation of duplexes **6** and **10** at 37 °C in a buffer of pH 5–9 produced no ICL, whereas the incubated reaction mixture afforded the cross-linking adduct after 350 nm photolysis (SI, Figure S2). The support for the radical mechanism was obtained by determining the rate constant for ICL formation in **6** by measuring the dependence of its formation on the concentration of thiol (RSH, β -mercaptoethanol).^[12, 13] The estimated rate

constant (k_{ICL}) for ICL formation induced by **1a** was $3.5 \pm 0.3 \times 10^4 \text{ s}^{-1}$ when the upper limit for alkyl radical trapping by a thiol ($K_{\text{RSH}} = 1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$) is used (eq 1) (Figure 3).^[12] This is consistent with the rate constant for ICL formation from 5-(2'-deoxyuridinyl)methyl radical **16** ($k_{\text{ICL}} = 3.8 \times 10^4 \text{ s}^{-1}$) that was observed by Greenberg *et al.*^[12]

$$\frac{[\text{ssDNA}]}{[\text{ICL}]} = \frac{K_{\text{RSH}}}{K_{\text{ICL}}} [\text{RSH}] + \frac{K_x}{K_{\text{ICL}}} \quad (1)$$

The formation of nucleotide radical **16** was further proved by the analysis of the product formed by 350 nm photolysis of the monomer. When **1a** was irradiated for 3h under aerobic conditions, 5-hydroxymethyl-2'-deoxyuridine (**23**) was observed. Compound **23** was confirmed using an authentic sample (SI, Figure S45). The formation of a hydroxymethyl derivative from **16** was also reported by Greenberg and Cadet.^[12, 17] All these indicated that 350 nm photolysis of **1a** released nitroimidazole, and generated 5-(2'-deoxyuridinyl)methyl radical **16** which finally produced ICL formation. The details and mechanism for the ICL formation by **16** has been reported by Greenberg *et al.*^[11, 12] The final ICL product induced by **1a** and **1b** is stable to heating in phosphate buffer, which is consistent with the previous report (SI, Figure S1, lanes 3 and 6).^[11, 12] However, the cleavage bands were observed upon piperidine treatment (SI, Figure S1, lanes 2 and 5). This indicated that apart from the cross-linking reaction, other side reactions took place.

While the reason for the hypoxia selective ICL formation is unclear, an attractive mechanism appears to the formation of a radical anion **15** via a photo-induced single electron transfer (PSET) from dT8 or other nucleotides in the duplex DNA to **1a** or **1b**.^[18] The resulting anion would spontaneously release nitroimidazole anion to generate 5-(2'-deoxyuridinyl) methyl radical **16** which directly produces ICLs (**17** and **18**). Oxygen molecules can reoxidize the one-electron reduced radical anion **15** to the original agent **1a** or **1b**, thereby decreasing the ICL yield. This was further proved by the slower rate of disappearance of monomers upon 350 nm photolysis in the presence of air ($K_{\text{1a-air}} = 4.9 \times 10^{-6} \text{ s}^{-1}$; $K_{\text{1b-air}} = 1.7 \times 10^{-4} \text{ s}^{-1}$) than that in the presence argon ($K_{\text{1a-Ar}} = 6.1 \times 10^{-6} \text{ s}^{-1}$; $K_{\text{1b-Ar}} = 2.4 \times 10^{-4} \text{ s}^{-1}$). In addition to the ICL formation, direct strand breaks (DSB) (2–7%, SI, Figure S1, lanes 1 and 4) and alkaline labile lesions (10–15%, SI, Figure S1, lanes 2 and 5) were observed specially at the positions of the flanking dT8 and the modified nucleosides **1a,b**. We believe that 5-(2'-deoxyuridinyl)methyl radical in **16** would abstract hydrogen of the sugar moiety of the thymidine radical cation producing a direct strand break at dT8. Greenberg *et al.* has shown that nucleotide radical can abstract C-2' hydrogen from the 5'-adjacent nucleotide.^[18] In addition, the thymidine radical cation in **16** can react with water to form alkaline labile lesions such as thymine glycol and/or 5-hydroxyl-5,6-dihydropyrimidine derivatives (**17** and **18**) which produce cleavage bands upon piperidine treatment (SI, Figure S3A, C, lane 3 and SI, Figure S4A, C, lane 3).^[15, 19, 20]

In an effort to determine the structure of the cross-linked product and possible side reactions, we used LC-mass to characterize the materials isolated from denaturing gel. A series of discrete peaks were observed, while the exact mass of ICL products was not obtained. Many peaks are smaller than the calculated mass of ICL product having nitroimidazole (9872.15), but larger than the calculated mass of ICL product losing nitroimidazole (9759.49 for **6** and **10**) (SI, Figure S43 and S44). The differences between these peaks are usually 17 or 18, which indicates a hydroxyl group or a water molecule was added. For example, a peak of 9810 corresponds to the addition of three hydroxyl groups and 9830 corresponds to the addition of two water molecules and two hydroxyl groups (SI, Figure S43). This further prove the formation of thymine glycol and/or 5-hydroxyl-5,6-dihydropyrimidine derivatives

and other DNA lesions with the addition of the hydroxyl group or water molecules. The alkaline labile lesions were also observed at the positions 20–23 of the complementary strand which is evidenced by the formation of the cleavage bands at these positions caused by the piperidine treatment of the ICL product (SI, lane 3 in Figure 3B, D and Figure 4B, D). During LC-mass analysis, the exact mass of **19** with an aldehyde on the 5-methyl group was observed (observed $m/z = 4974.6056$, calculated $m/z = 4974.2792$) (Scheme 2 and SI, Figure S42). We believe that **19** is derived from the peroxy radical **20**, as proposed by Greenberg and Cadet.^[12, 17]

Hydroxyl radical cleavage of gel-purified cross-linked DNA was used to determine which nucleotides were covalently bonded to one another.^[11] Each oligonucleotide was either 5'-³²P-labelled or 3'-³²P-labelled. As expected, cross-linking occurred mainly with the position of **1a** or **1b** where **16** was generated (Figure 4A, C). The majority of the cross-links in the complementary strand involve reaction with dA₂₃. Partial ICL took place with dA₂₄ (Figure 4B, D and SI, Figure S47).

In summary, this work demonstrated that coupling of a hypoxia-radiosensitizer (e.g. nitroimidazoles) with a pyrimidine nucleoside allows for more efficient DNA interstrand cross-link formation under hypoxic conditions than that under aerobic conditions. The mechanism involves the release of a 5-(2'-deoxyuridinyl) methyl radical from a nucleoside radical anion which is generated via a photo-induced single electron transfer (PSET) within duplex DNA. The presence of oxygen quenches the radical anion therefore decreasing the ICL yield. Work is in progress to determine their hypoxia-selectivity in hypoxic cells, and their potential as hypoxia-targeting anticancer drugs.

Experimental Section

General Methods

Unless otherwise specified, chemicals were purchased from Aldrich or Fisher Scientific and were used as received without further purification. T₄ polynucleotide kinase was obtained from New England Biolabs. Oligonucleotides were synthesized via standard automated DNA synthesis techniques using an Applied Biosystems model 394 instrument in a 1.0 μM scale using commercial 1000Å CPG-succinyl-nucleoside supports. Deprotection of the nucleobases and phosphate moieties as well as cleavage of the linker were carried out under mild deprotection conditions using a mixture of 40% aq. MeNH₂ and 28% aq. NH₃ (1:1) at room temperature for 2 h. Thermal denaturation temperatures (T_m values) were measured on a Cary 100 UV/Vis spectrometer equipped with a thermoelectrical temperature controller. The temperature was changed at a rate of 1°C/min. Radiolabeling was carried out according to the standard protocols. [²¹γ-³²P]ATP and [α-³²P]ATP was purchased from Perkin-Elmer Life Sciences. Quantification of radiolabeled oligonucleotides was carried out using a Molecular Dynamics Phosphorimager equipped with ImageQuant Version 5.2 software. ¹H NMR and ¹³C NMR spectra were taken on either a Bruker DRX 300 or DRX 500 MHz spectrophotometer. High resolution mass spectrometry was performed at the University of Kansas Mass Spectrometry Lab. ESI-MS for oligonucleotides and cross-linking DNA was determined by Center for Computational and Integrative Biology of Harvard Medical School.

Sample preparation under hypoxic conditions

For preparing samples under reduced O₂ tension conditions, samples were degassed to remove the dissolved O₂ using high vacuum pump (1 min), then purged with argon (1.0 min) (three cycles).

Interstrand cross-link formation and kinetics study with duplex DNA

The ^{32}P -labelled oligonucleotide (1.0 μM) was annealed with 1.5 equiv of the complementary strand by heating to 65 $^{\circ}\text{C}$ for 3 min in buffer (10 mM potassium phosphate, pH 7.5, and 100 mM NaCl), followed by slow-cooling to room temperature overnight. The ^{32}P -labeled oligonucleotide duplex (2 μL , 1.0 μM) was mixed with 1 M NaCl (2 μL), 100 mM potassium phosphate (2 μL , pH 7.5) and appropriate amount of autoclaved distilled water to give a final volume of 20 μL . The mixture was degassed to remove the air and purged with argon, then UV-irradiated in a Rayonet Photochemical Chamber Reactor (Model RPR-100, sixteen bulbs, 350 nm light wavelength) for 2h. The reaction was quenched by an equal volume of 90% formamide stop/loading buffer, then electrophoresed on a 20% denaturing polyacrylamide gel at 1200 V for approximately 4 h. For kinetics study, aliquots (final concentration: 50 nM ^{32}P -labeled oligonucleotide duplex, 100 mM NaCl, 10 mM potassium phosphate) mixed with appropriate concentration of 2-mercaptoethanol were irradiated under argon for 2h and immediately quenched by 90% formamide loading buffer, and stored at -20°C until subjecting to 20% denaturing PAGE analysis.

Hydroxyl radical reaction (Fe-EDTA reaction)

The cross-linked DNAs formed in duplexes **6** and **10** were purified by 20% denaturing PAGE. The band containing cross-linked product was cut, crushed, and eluted with 200 mM NaCl, 20 mM EDTA (2.0 mL). The crude product was further purified by C18 column eluting with H_2O (3×1.0 mL) followed by $\text{MeOH}:\text{H}_2\text{O}$ (3:2, 1.0 mL). Fe(II)-EDTA cleavage reactions of ^{32}P -labelled oligonucleotide (0.1 μM) were performed in a buffer containing 50 μM $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$, 100 μM EDTA, 5 mM sodium ascorbate, 0.5 M NaCl, 50 mM sodium phosphate (pH 7.5) and 1 mM H_2O_2 for 3 min at room temperature (total substrate volume 20 μL), then quenched with 100 mM thiourea (10 μL). Samples were lyophilized, dissolved in 20 μL H_2O : 90% formamide loading buffer (1:1) and subjected to 20% denaturing PAGE analysis.

Kinetic study of monomer reaction upon UV photolysis (350 nm)

Compound **1a** or **1b** (8 mg) was dissolved in a mixture of 0.93 mL $\text{DMSO}-d_6$ and 0.07 mL D_2O . The solution was equally divided into two portions which were placed in two NMR tubes. One sample was degassed to remove air under high vacuum, then purged with Ar_2 for 5 min. Both samples were exposed to UV irradiation at 350 nm. ^1H NMR spectra were recorded at different time (2, 6, 12, 18, 24, 36 h for **1a**; 5, 10, 30, 60, 90, 120, 150, 180, 240 min for **1b**). The reaction yield was determined by calculating the decrease of the signal at 8.30 ppm which corresponds to imidazole. The peak at 2.50 ppm (DMSO) was used as a reference to quantify other peaks.

Stability study of ICL product formed with **6** or **10**

After the cross-link reaction, the reaction mixtures (0.35 μM DNA duplex, 20 μL) were co-precipitated with calf thymus DNA (2.5 mg/mL, 5 μL) and NaOAc (3 M, 5 μL) in the presence of EtOH (90 μL) at -80°C for 30 min, followed by centrifuging for 5 min at 15000 rpm. The supernatant was removed, and the pellet was washed with cold 75% EtOH and lyophilized for 30 min in a Centrivap Concentrator of LABCONCO at 37 $^{\circ}\text{C}$. The dried DNA fragments were dissolved in H_2O (30 μL) and divided into three portions. One portion (10 μL) was incubated with piperidine (2 M, 10 μL) at 90 $^{\circ}\text{C}$ for 30 min, and the second portion (10 μL) was incubated with 0.1 M NaCl and 10 mM potassium phosphate buffer (pH 7, 10 μL) under the same condition, and the third portion was used as a control sample. The samples were subjected to electrophoresis on a 20% denaturing polyacrylamide gel.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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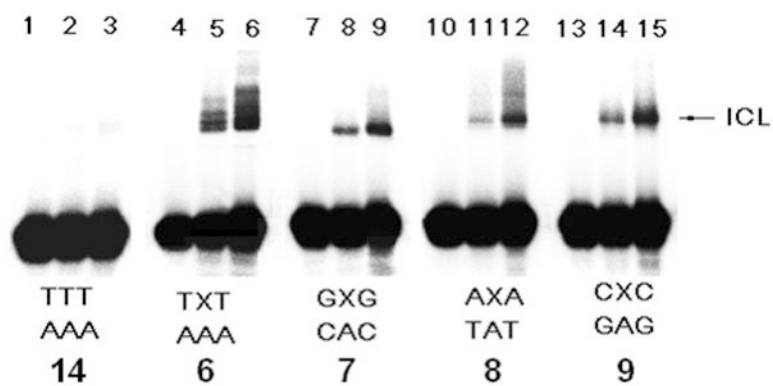


Figure 1.

Hypoxia-selective interstrand cross-link formation by **1a** upon UV-photolysis at 350 nm for 2 h under aerobic and anaerobic conditions. Phosphorimage autoradiogram of 20% denaturing PAGE analysis of the ICL product from duplexes containing **1a** (**6-9**): Lanes 1, 4, 7, 10 and 13, DNA duplexes without UV irradiation; Lanes 2, 5, 8, 11 and 14, DNA duplexes irradiated under aerobic condition; Lanes 3, 6, 9, 12 and 15, DNA duplexes irradiated under anaerobic condition.

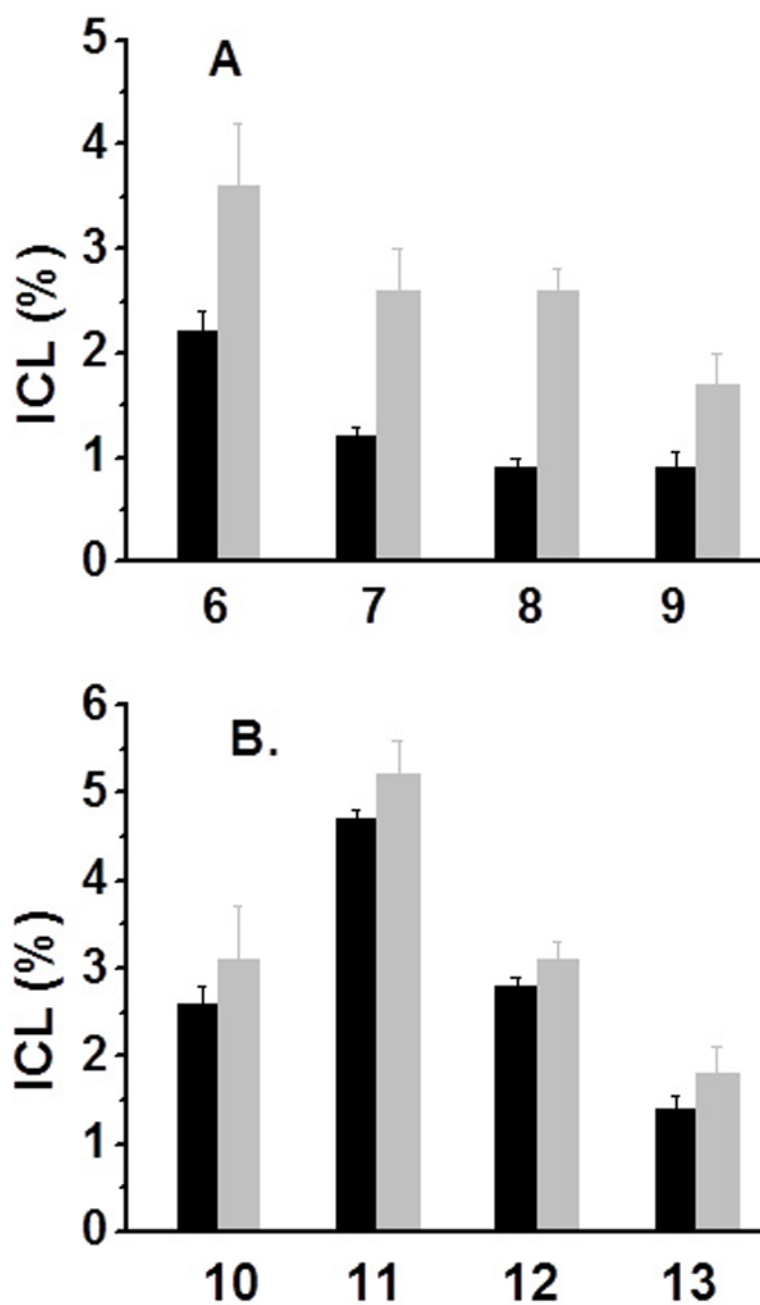


Figure 2. Cross-linking efficiency of compounds **1a** and **1b** under aerobic and anaerobic conditions: (A) Duplexes containing **1a** (**6–9**) were irradiated by UV for 2 h; (B) Duplexes containing **1b** (**10–13**) were UV-irradiated for 2 h [black bar -aerobic conditions (air); grey bar - anaerobic conditions (Ar₂)].

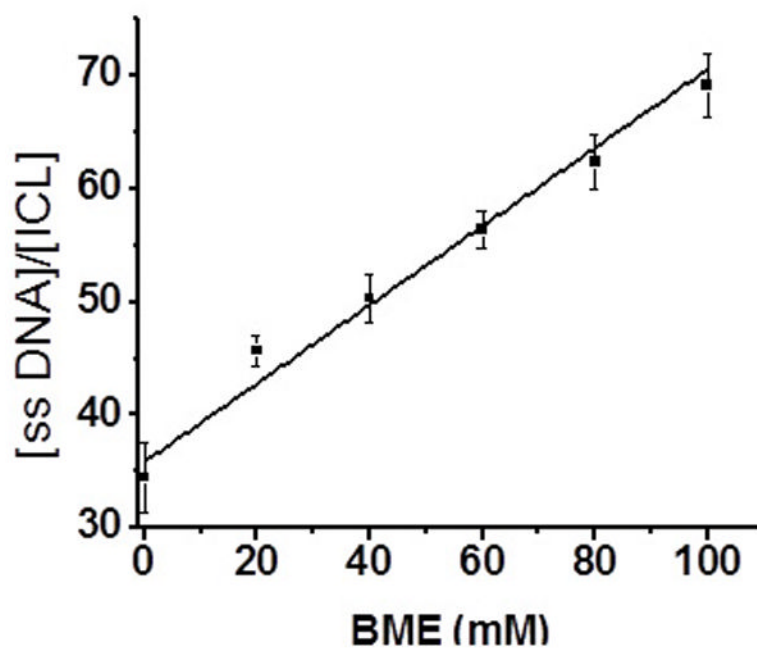
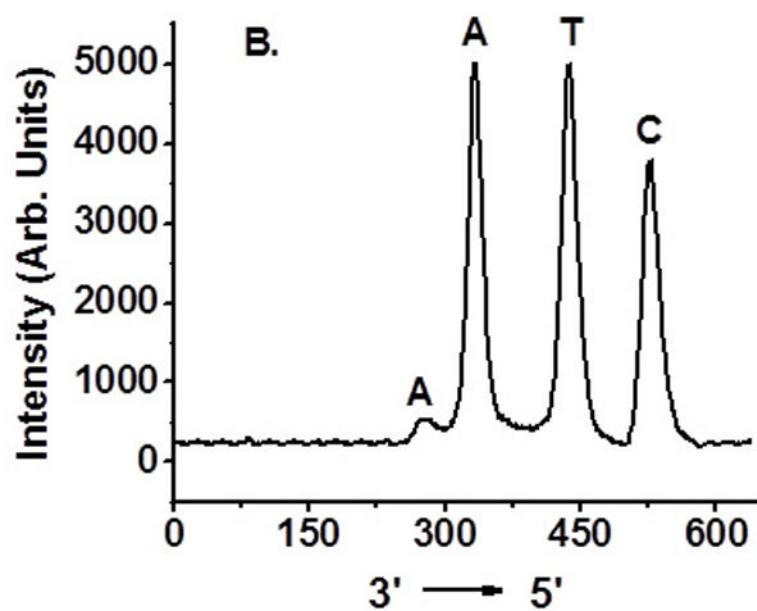
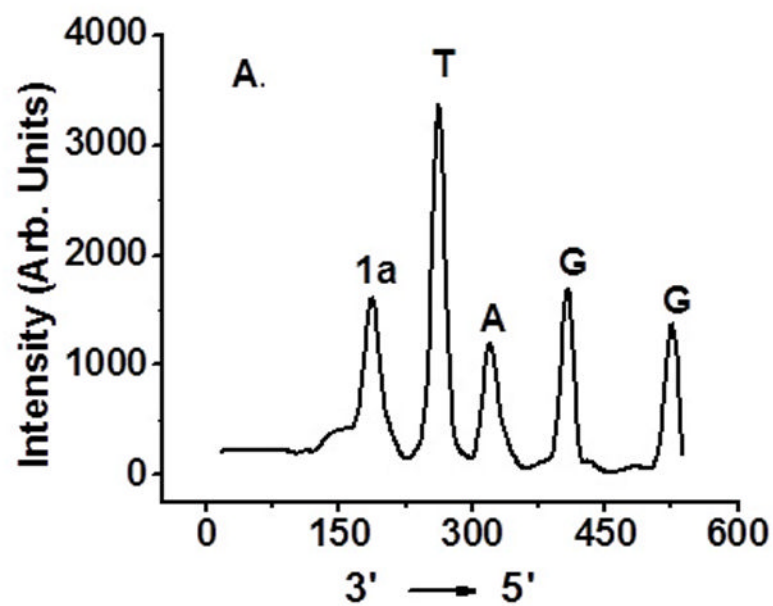


Figure 3. Ratio of ssDNA:ICL produced from **6** as a function of BME concentration (ssDNA: single stranded DNA; ICL: interstrand cross-links).



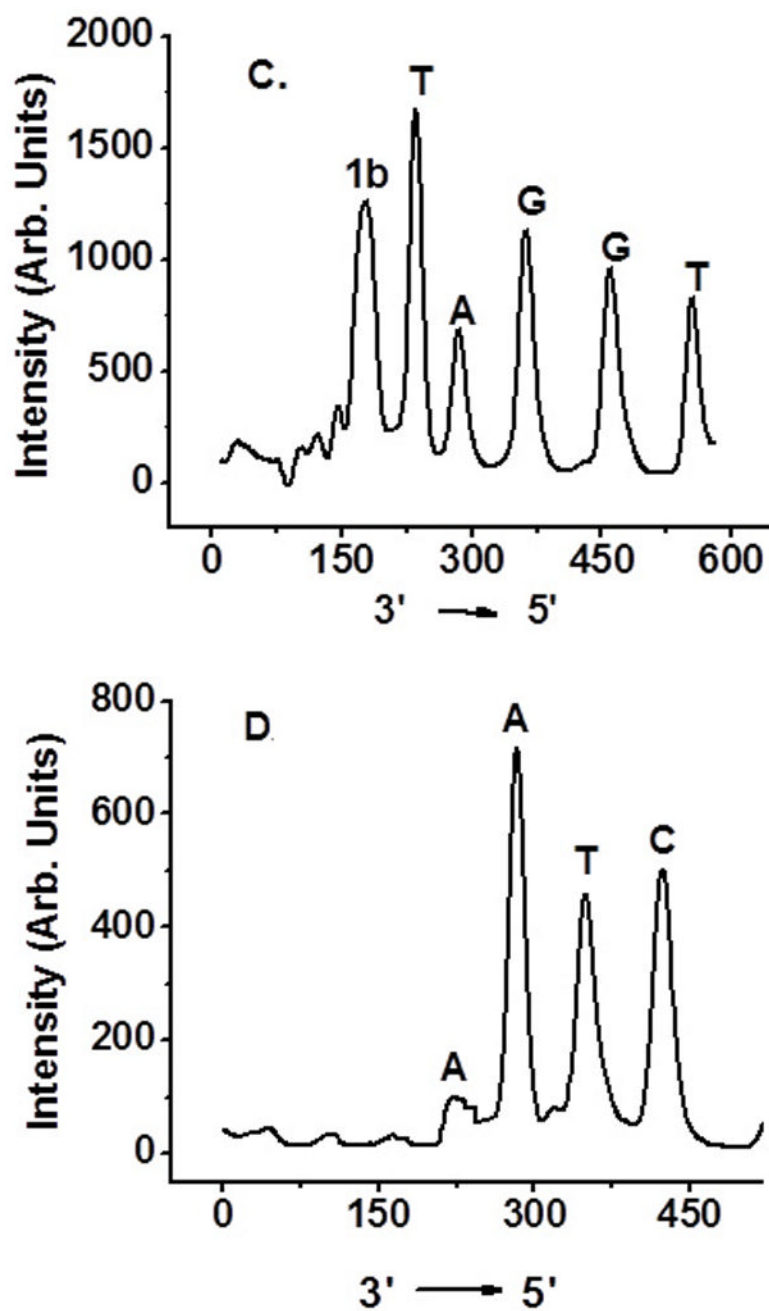
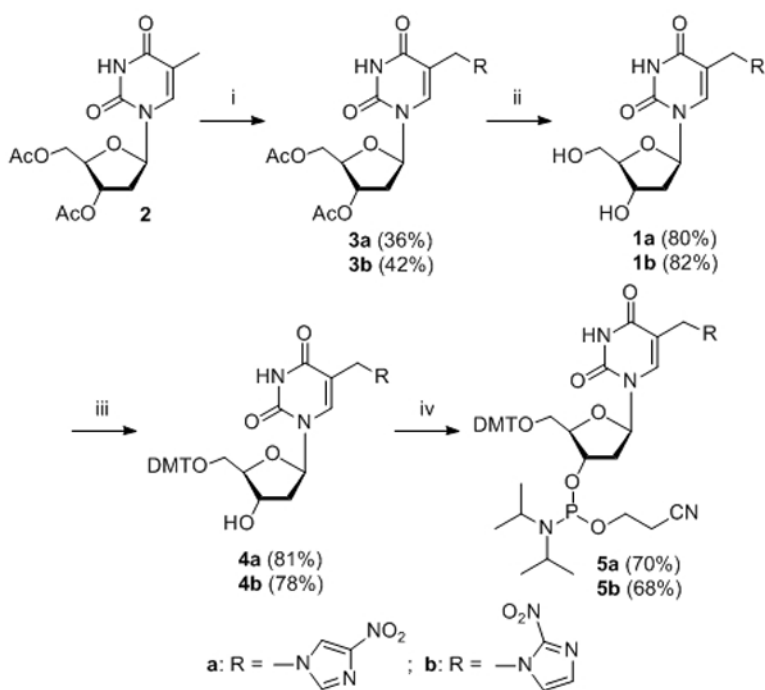
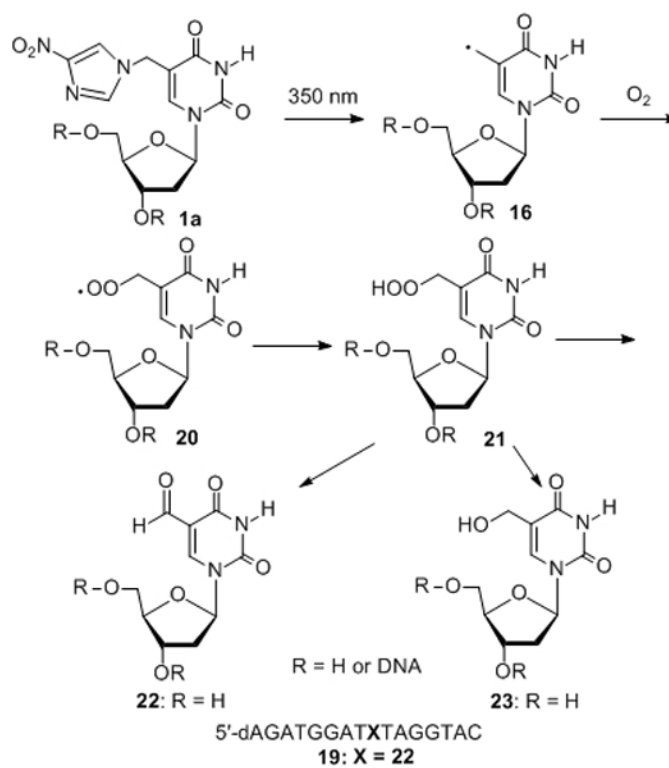


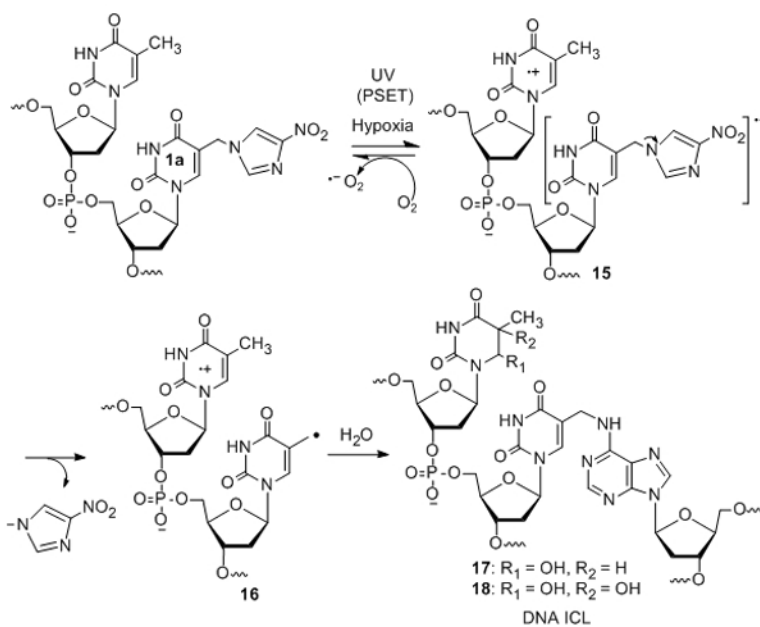
Figure 4. Determination of interstrand cross-linking position via $\text{OH}\cdot$ cleavage of UV treated **6** and **10**: (A) $5'$ - ^{32}P -**6a**, 4-nitroimidazole containing strand labeled; (B) $5'$ - ^{32}P -**6b**, complementary strand labeled; (C) $5'$ - ^{32}P -**10a**, 2-nitroimidazole containing strand labeled; (D) $5'$ - ^{32}P -**10b**, complementary strand labeled.

**Scheme 1.**

Synthesis of compound **1a** and **1b**. Reagents: i. a) NBS, benzene, b) NaH, nitroimidazole, DMF; ii) aq. NH₃, 1, 4-dioxane; iii) DMTCl, pyridine; iv) 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite, *N,N*-diisopropylethylamine, CH₂Cl₂.



Scheme 2.
 Products generated from **1a** upon UV-photolysis.



Scheme 3.
Proposed mechanism for the ICL formation.

Table 1

Melting temperatures [in °C] of duplexes 5'-dAGATGGATXTAGGTAC (complementary strand 3'-dTCTACCTAYATCCATG).

X•Y	T_m (ΔT_m)	X•Y	T_m (ΔT_m)
dT•dA (14)	52.1		
1a•dA (6)	48.8	1b•dA (10)	47.7
1a•dC	38.0 (-10.8)	1b•dC	39.7 (-8.0)
1a•dG	41.1 (-7.7)	1b•dG	42.0 (-5.7)
1a•dT	38.4 (-10.4)	1b•dT	39.1 (-8.6)