Linking DNA

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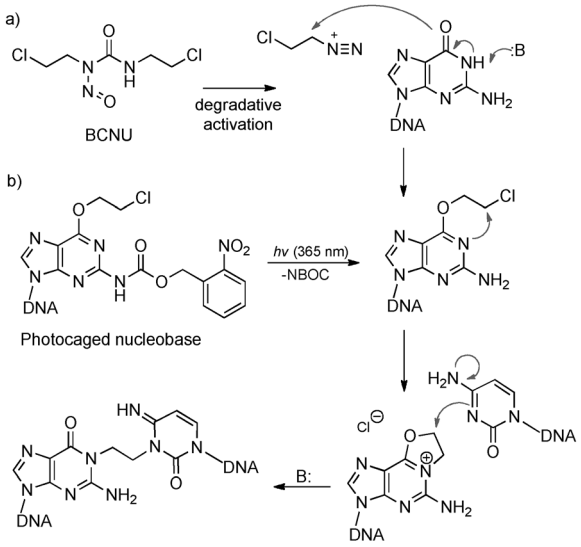
# Synthesis of DNA Interstrand Cross-Links Using a Photocaged Nucleobase**

*Sarah Hentschel, Jawad Alzeer, Todor Angelov, Orlando D. Schärer, and Nathan W. Luedtke**

Bifunctional alkylating agents cause a wide variety of damage to cellular DNA. Among these, DNA–DNA interstrand cross-links (ICLs) are the most cytotoxic, because of their ability to block transcription and replication.[1] ICLs resulting from endogenous (e.g. malondialdehyde) and exogenous (e.g. formaldehyde) agents pose threats to human health.[2–4] ICLs are also generated by anticancer drugs such as cisplatin, nitrogen mustards, and chloro ethyl nitrosoureas.[5]

1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU or "Carmustine") is a widely used chemotherapeutic agent that generates an ethylene bridge between $N^1$ of deoxyguanosine (dG) and $N^3$ of deoxycytidine (dC) in a multistep reaction. The putative mechanism involves chloro alkylation of dG at $O^6$, followed by cyclization to form an $N^1$-$O^6$-ethanoguanine intermediate which undergoes attack by $N^3$ of dC to give a $N^1$(dG)-ethyl-$N^3$(dC) cross-link (Scheme 1).[6,7] Limitations to using BCNU and other nitrosoureas as chemotherapeutics include cellular resistance mechanisms mediated by DNA repair enzymes.[8] Understanding these pathways will lead to improved therapeutic outcomes, but studies aimed at deciphering ICL repair pathways have been limited by the lack of well-defined ICLs to serve as repair substrates.[9] Different approaches have been developed to obtain ICL-containing DNA. In early studies, duplex DNA was exposed to a vast excess of BCNU, but this procedure yields only 1–5% of ICL products that are not amenable to biochemical or high-resolution biophysical studies owing to the presence of extensive monoalkylation.[6,7] A more sophisticated approach is bidirectional DNA synthesis using cross-linked dinucleosides.[10–17] Using this procedure, a $N^1$-deoxyinosine(dI)-ethyl-$N^3$-thymidine(dT) interstrand cross-link was recently reported by Noronha and coworkers as a structural mimic of the dG–dC cross-link caused by BCNU.[16] Drawbacks to this approach include requiring prior knowledge of the exact structure of the desired ICL, as well as complex protecting-group strategies needed for its synthesis. An alternative approach to ICL synthesis involves the incorporation of stable ICL precursors into single-stranded oligonucleotides using standard DNA synthesis.[18–26] Following hybridization to a complementary sequence, ICL formation can be initiated in the modified duplex. Alzeer and Schärer used this approach to generate a $O^6$(dG)-ethyl-$N^3$(dT) ICL by incorporating $N^3$-(2-chloroethyl)thymine into oligonucleotides. Despite such notable progress, no synthesis of a DNA containing the biologically relevant $N^1$(dG)-ethyl-$N^3$(dC) cross-link resulting from BCNU treatment has ever been reported. Such products would provide authentic samples for biochemical, cell biological, and biophysical studies, and the novel methodology used in their synthesis may find other applications in biotechnology.




***Scheme 1.*** a) Reaction pathway for BCNU-mediated ICL formation in DNA; b) Our strategy for the synthesis of DNA–DNA ICLs using a photocaged $O^6$-(2-chloroethyl)-2′-deoxyguanosine. B: represents a general base.

Herein we report a new strategy for ICL synthesis using a photocaged nucleobase as an ICL precursor. Our approach utilizes an $O^6$-(2-chloroethyl)guanine residue containing a photolabile *ortho*-nitrobenzyloxycarbonyl (NBOC) group at the $N^2$ position. NBOC stabilizes the normally reactive $O^6$-chloroethyl guanine by acting as an electron-withdrawing

[*] S. Hentschel, Prof. Dr. N. W. Luedtke
Institute of Organic Chemistry, University of Zurich
Winterthurerstrasse 190, 8057 Zürich (Switzerland)
E-mail: luedtke@oci.uzh.ch
Homepage: http://www.bioorganic-chemistry.com/
Dr. J. Alzeer
Palestine Polytechnic University, Applied Chemistry Department
Hebron (Palestine)
Dr. T. Angelov
Institute of Food, Nutrition and Health, ETH Zürich
Schmelzbergstrasse 9, 8092 Zürich (Switzerland)
Prof. Dr. O. D. Schärer
Department of Pharmacological Sciences and Chemistry
Stony Brook University
New York, 11794 (USA)

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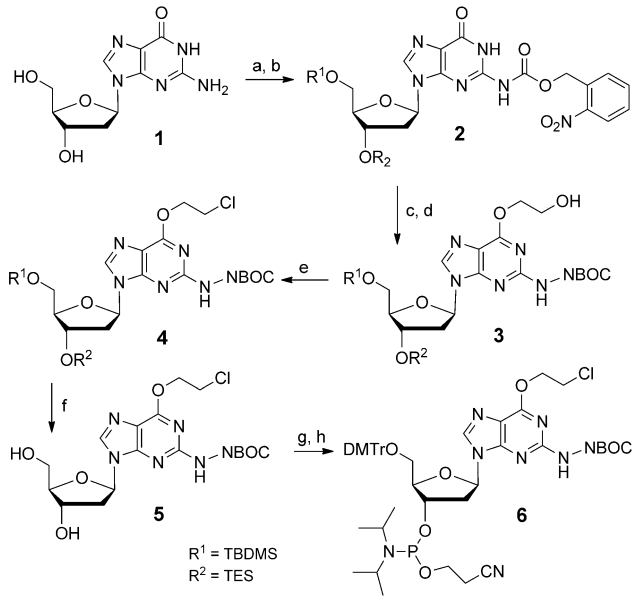
group to the $N^1$ position. This ICL precursor remains stable during and after its synthetic incorporation into duplex DNA until NBOC is selectively removed by irradiation at 365 nm. The resulting free amine at the $N^2$ position activates formation of a $N^1$,$O^6$-ethanoguanine cyclic intermediate by donating electron density to the $N^1$ position which displaces chloride in an intramolecular $S_N2$ reaction. The resulting $N^1$,$O^6$-ethanoguanine cation is a highly reactive intermediate that can alkylate a cytosine residue in the opposite strand to form the ICL in a manner analogous to BCNU itself (Scheme 1b).

The synthesis of phosphoramidite **6** (Scheme 2) commenced with silylation of the 3′-OH and 5′-OH groups of dG (**1**). The TES group was used to protect the 3′-OH, while TBDMS was applied at the 5′-OH position. This strategy circumvented problematic deprotection reactions involving 3′-O-TBDMS intermediates that failed to give the desired nucleoside **5**. The $N^2$ exocyclic amine was protected with a photolyzable group in modest yield by addition of 2-nitrobenzyloxy carbonyl imidazole (NBOC-Im).[27] The $O^6$ position was activated with 2,4,6-triisopropylbenzylsulfonyl chloride and transformed into $O^6$-(2-hydroxyethyl)-2′-deoxyguanosine (**3**) using quinuclidine as a base. After many failed attempts, chlorination was accomplished using a method reported by Wanchai and Warinthorn[28] to furnish the desired $O^6$-(2-chloroethyl)-2′-deoxyguanosine (**4**) in moderate yield.




***Scheme 2.*** Synthesis of a photocaged $O^6$-chloroethyl phosphoramidite **6**: a) TBDMS-Cl, TES-Cl, imidazole, DMF, −10 °C→RT, 20 h, 98%; b) NBOC-Im, [18]crown-6, NaH, THF, RT, 3 h, 55%; c) TiPBS-Cl, $Et_3N$, 4-DMAP, $CH_2Cl_2$, 0 °C, 3 h 81%; d) quinuclidine, ethylene glycol, THF, 0 °C, 4 h, 72%; e) $PPh_3$, $Cl_3CCN$, THF, 0 °C, 20 min, 51%; f) TBAF, *p*-TsOH, MeOH, THF, $H_2O$, 0 °C→RT, 26 h, 53%; g) DMTr-Cl, pyridine, RT, 2 h, 80%; h) $(iPr_2N)_2POC_2H_4CN$, 5-(ethylthio)-1*H*-tetrazole, $CH_2Cl_2$, RT, 20 min, 80%. DMAP = dimethylaminopyridine, TBDMS-Cl = *tert*-butyldimethylsilyl chloride; TES-Cl = chlorotriethyl silane; TiPBS-Cl = 2,4,6-triisopropylbenzene sulfonyl chloride; TBAF = tetra-*n*-butylammonium fluoride; *p*-TsOH = *p*-toluenesulfonic acid; DMTr-Cl = 4,4′-dimethoxytrityl chloride.

Deprotection of the silyl groups at the 3′-OH and 5′-OH by fluoride ions was also problematic, but could be accomplished with a (2:1) mixture of TBAF and *p*-TsOH. The 5′-OH was then protected with 4,4′-dimethoxytrityl chloride and the 3′-OH was activated under standard conditions to give the desired phosphoramidite **6** in an overall yield of 5% over 8 steps.

Phosphoramidite **6** was incorporated into oligonucleotides using "ultra-mild" DNA synthesis according to published procedures.[29,30] Following synthesis, oligonucleotides were cleaved from the solid support and deprotected by treatment with diisopropylamine in methanol (1:10) at room temperature for 15 h. Purification was conducted using HPLC, and the products were analyzed by MALDI-TOF mass spectrometry. The observed molecular weight (7769.3) from the main product of the synthesis was consistent with the calculated molecular weight (7768.3) of oligonucleotide **7** containing an NBOC group at $N^2$ and a chloroethyl group at $O^6$ of the modified G residue.

Following synthesis and purification, oligonucleotide **7** was hybridized to complementary oligonucleotides (**8**–**11**, Figure 1) by heat denaturation and slow cooling in an aqueous phosphate buffer (pH 7.4). The complementary oligonucleotides contained a fluorescent tag (Cy3) on the 5′-end, and variable bases (N = C, T, A, G) opposite to the ICL precursor. The resulting duplexes were irradiated for 3 min with 365 nm laser light to remove the NBOC group.[31,32] Following irradiation, the oligonucleotides were incubated at 37 °C for 17 h, and the products resolved on a 15% denaturating polyacrylamide gel (Figure 1). All four of the irradiated DNA sequences gave single products having reduced electrophoretic mobilities, consistent with the formation of duplex DNAs containing ICLs (Figure 1). Higher yields were obtained when pyrimidines (dT and dC) were positioned opposite to the ICL precursor as compared to purines (dA and dG). To characterize the products generated from

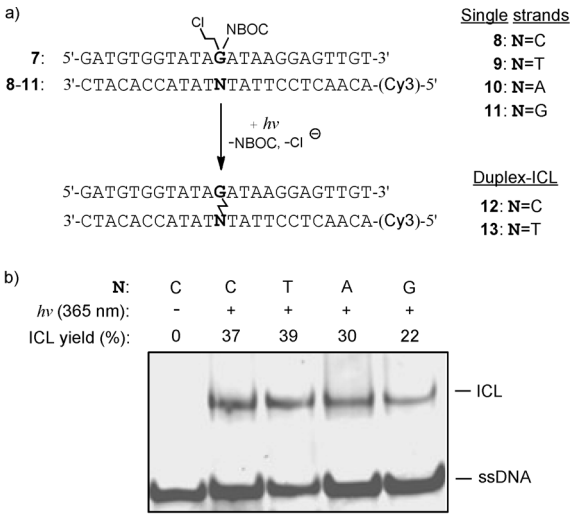



***Figure 1.*** a) Schematic representation of cross-linking reactions. b) Analysis of ICL formation by denaturating polyacrylamide gel electrophoresis (DPAGE). Imaging was conducted using Cy3 fluorescence emission.

reactions involving pyrimidines, HPLC was used to isolate the ICL duplex DNA **12** and **13** (Figure 2). According to MALDI TOF mass spectrometry, the molecular weights of these ICL DNA products (**12**: 15245.0, **13**: 15263.0) were in excellent agreement with the calculated values (**12**: 15245.2, **13**: 15259.0). By contrast, DNA samples that were prepared without irradiation at 365 nm gave no detectable ICL formation (Figure 1). Together these results demonstrate that NBOC removal is a prerequisite for efficient ICL formation.

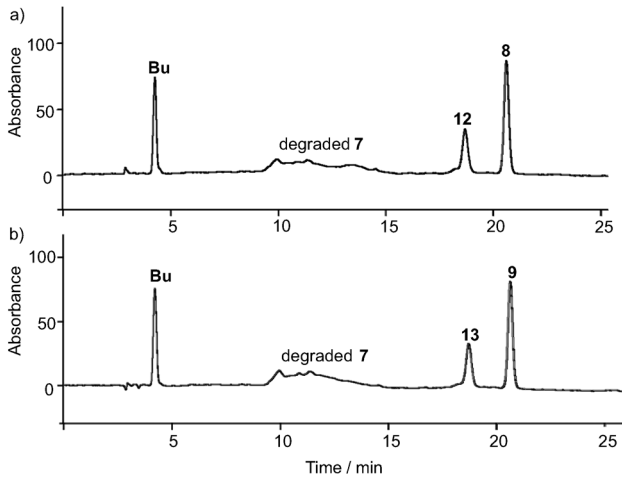



***Figure 2.*** HPLC analysis of cross-link formation following photodeprotection. a) Chromatogram of a crude reaction mixture containing ICL product **12** resulting from oligonucleotides **7** and **8**. b) Chromatogram of a crude reaction mixture containing ICL product **13** and corresponding starting materials **7** and **9**. Bu = buffer components. Wavelength of detection: 260 nm.

To evaluate the structures of cross-links present in **12** and **13**, the purified oligonucleotides were digested with snake venom phosphodiesterase, exonuclease III, and shrimp alkaline phosphatase. Crude products from each reaction mixture were analyzed by HPLC and mass spectrometry (Figure 3). In addition to the four canonical nucleosides, the ICL dinucleotides "**15**" and "**16**" were present in the chromatograms. Dinucleotide **15** exhibited an identical retention time and high-resolution mass spectrum as an authentic sample of $N^1$(dG)-ethyl-$N^3$(dC) that was prepared from $O^6$-(2-fluoroethyl)-2′-deoxyguanosine (Scheme 3). Co-injection of **15** with a crude digestion mixture further verified the structure of the ICL formed (Supporting Information). The high-resolution mass spectrum of **16** was consistent with a G–T cross-link (Supporting Information). Together these results demonstrate that DNA containing a $O^6$-(2-chloroethyl)-2′-deoxyguanosine residue can react with a C, T, A ,or G residue in the opposite strand, and suggest that the putative $N^1$,$O^6$-ethanoguanine cationic intermediate formed after NBOC removal is an extremely reactive and non-specific electrophile.

The proposed mechanism for G–C cross-link formation by BCNU was first proposed by Ludlum and co-workers in 1982 (Scheme 1),[34] but it has never been rigorously demonstrated. Previous mechanistic studies have focused on the analysis of

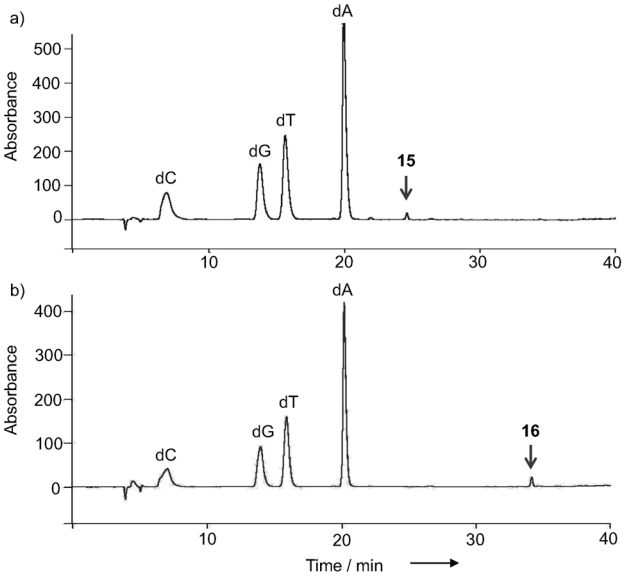
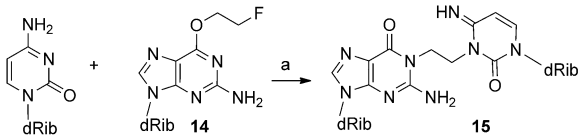



***Figure 3.*** Enzymatic digestion analyses of cross-linked oligonucleotides **12** (a) and **13** (b) by HPLC. Wavelength of detection: 260 nm.




***Scheme 3.*** Synthesis of $N^1$(dG)-ethyl-$N^3$(dC) dinucleoside **15** according to published procedures.[33] a) NaI, DMSO, 55 °C, 11 d, 10%. dRib = 2′-deoxyribose.

complex product mixtures resulting from the addition of BCNU to duplex DNA.[6,7] Our orthogonal strategy for the synthesis ICL DNA utilizes the same mechanistic route as proposed by Ludlum, and it provides the exact same $N^1$(dG)-ethyl-$N^3$(dC) adduct that had been isolated from enzymatic digests of genomic DNA treated with BCNU.[6,7] Our results therefore give direct support for the proposed ICL mechanism while providing the first total synthesis of a DNA containing the biologically relevant $N^1$(dG)-ethyl-$N^3$(dC) cross-link. In stark contrast to BCNU-treatment of duplex DNA, our approach generates homogenous ICL duplex DNA in sufficient quantities for future biophysical and biological analyses.

In conclusion, we have developed a novel strategy for the synthesis of site-specific ICLs using a $N^2$-photocaged-$O^6$-(2-chloroethyl)guanine. While photocaging and release strategies are widely utilized for tuning molecular-recognition interfaces,[32] our results provide, to our knowledge, the first example of a photoinitiated reaction between biological macromolecules accomplished by release of a latent electrophile. This same strategy should be applicable in other situations where highly reactive alkylation intermediates are utilized for site-specific covalent modifications in aqueous solution.

## Experimental Section

DPAGE analysis: Fresh DNA solutions were prepared in 20 mM phosphate buffer (pH 7.4) with 270 mM NaCl and 5.4 mM KCl (total volume 20 μL) containing 1.2 μM of **7**, and 1 μM of **8**, **9**, **10**, or **11**. To anneal the DNA strands, samples were heated for 1 min to 80 °C and slowly cooled to room temperature (2 h). Samples were then irradiated with 365 nm light (3500 mW cm$^{-2}$) using a LED-Pen laser (Abecon AG) for 3 min at room temperature and incubated for 17 h at 37 °C. 5 μL of each sample was diluted with 10 μL of loading buffer (50% formamide, 9 mM Tris/boric acid, 0.2 mM EDTA, 6 M urea, traces of bromphenol blue) and an additional 5 mg urea was added. Samples were heated at 80 °C for 2 min and loaded onto a 15% polyacrylamide gel (19:1 ratio of acylamide to bisacrylamide containing 8 M urea and 14 mM Tris/boric acid, 0.3 mM EDTA) and resolved for 80 min at 100 V. The gels were then imaged using a flatbed scanner equipped for Cy3 detection (Typhoon 9400, GE Healthcare Bioscience-AB).

Preparative-scale synthesis of cross-linked DNA: Oligonucleotide **7** (6.5 μM) was mixed with oligonucleotide **8** or **9** (4 μM) in 20 mM phosphate buffer (pH 7.4) containing 420 mM NaCl, 5.4 mM KCl, and 8 mM NaI (total volume: 1 mL). The samples were annealed and photodeprotected as described above. Products were resolved using Varian Pro Star HPLC system and a C-18 reverse-phase column (YMCbasic, B-22-10P 150 × 10 mm) using a linear gradient of 3–40% $CH_3CN$ in 0.1 M triethylammonium acetate buffer (pH 7) over 26 min. The fractions corresponding to cross-linked DNA **12** and **13** were collected and lyophilized. MALDI-TOF mass spectrometry: 15245.2 (**12**, calcd 15245.2); 15263.0 (**13**, calcd 15259.0).

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