

# Generation of DNA Interstrand Cross-Links by Post-Synthetic Reductive Amination

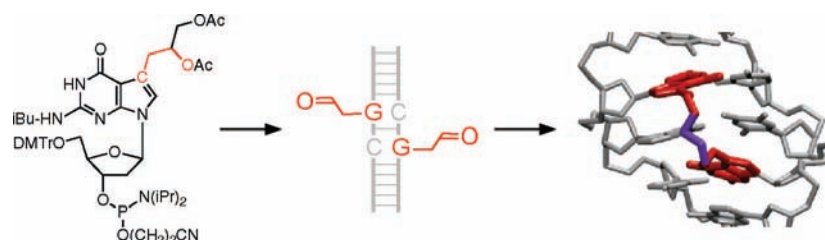
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## ABSTRACT



DNA interstrand cross-links (ICLs) are the clinically most relevant adducts formed by many antitumor agents. To facilitate the study of biological responses triggered by ICLs, we developed a new approach toward the synthesis of mimics of nitrogen mustard ICLs. 7-Deazaguanine residues bearing acetaldehyde groups were incorporated into complementary strands of DNA and cross-link formation induced by double reductive amination. Our strategy enables the synthesis of major groove cross-links in high yields and purity.

A variety of bifunctional electrophilic agents have the ability to covalently link two complementary strands of double-stranded DNA, leading to the formation of DNA interstrand cross-links (ICLs). Due to their ability to efficiently block replication and transcription, ICLs are among the most cytotoxic DNA lesions known. Their cytotoxic potential has found application in anticancer chemotherapy, and cross-linking agents such as the nitrogen mustards, platinum complexes, chloro ethyl nitroso ureas, and mitomycin C are among the most widely used antitumor agents today.<sup>1,2</sup> Many factors contribute to the resistance of tumor cells to treatment with cross-linking agents, and the removal of ICLs from DNA has emerged as one of the most important ones.<sup>3</sup> Repair pathways for ICLs have their evolutionary origin in the

necessity to counteract the threat posed by endogenous and exogenous metabolites, for example, malonic dialdehyde and formaldehyde.<sup>4</sup> ICL repair is an inherently complex process as the two strands need to be unhooked and repaired and no intact template is available for repair synthesis.<sup>2</sup> This complexity provides one explanation why ICL repair pathways remain poorly understood.

A second problem in the study of ICL repair has been the limited availability of defined ICL adducts.<sup>5</sup> ICLs were initially synthesized by the reaction of DNA with cross-linking agents followed by isolation and purification of the ICL.<sup>6–8</sup> This approach yields mixtures of products (mono

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(1) Lawley, P. D.; Phillips, D. H. *Mutat. Res.* **1996**, *355*, 13–40. Rajski, S. R.; Williams, R. M. *Chem. Rev.* **1998**, *98*, 2723–2796.

(2) Noll, D. M.; Mason, T. M.; Miller, P. S. *Chem. Rev.* **2006**, *106*, 277–301.

(3) Panasci, L.; Paiement, J. P.; Christodouloulopoulos, G.; Belenkov, A.; Malapetsa, A.; Aloyz, R. *Clin. Cancer Res.* **2001**, *7*, 454–61. McHugh, P. J.; Spanswick, V. J.; Hartley, J. A. *Lancet Oncol.* **2001**, *2*, 483–90.

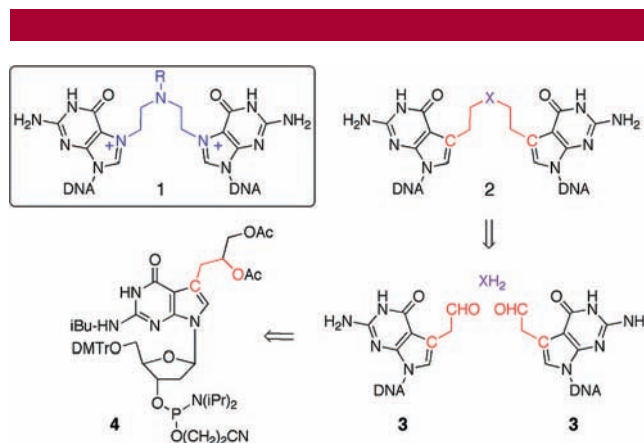
(4) Chaw, Y. F.; Crane, L. E.; Lange, P.; Shapiro, R. *Biochemistry* **1980**, *19*, 5525–31. Stone, M. P.; Cho, Y. J.; Huang, H.; Kim, H. Y.; Kozekov, I. D.; Kozekova, A.; Wang, H.; Minko, I. G.; Lloyd, R. S.; Harris, T. M.; Rizzo, C. J. *Acc. Chem. Res.* **2008**, *41*, 793–804.

(5) Noll, D. M.; Noronha, A. M.; Wilds, C. J.; Miller, P. S. *Front. Biosci.* **2004**, *9*, 421–37. Schärer, O. D. *ChemBioChem* **2005**, *6*, 27–32.

adducts, *intra*- and *interstrand* cross-links) and the desired ICLs usually make up only a small fraction (typically 1–5%) of all the products formed. More efficient approaches to the chemical synthesis of ICLs have subsequently been developed. One was based on the cross-linking of nucleosides outside of DNA followed by incorporation of the cross-linked dimer into DNA using solid-phase synthesis.<sup>9</sup> This approach has been particularly successful in yielding ICLs that connect two bases through their Watson–Crick faces, although the solid-phase synthesis procedures are complicated by the connected nucleoside dimers. A second approach consists of the site-specific incorporation of postsynthetically modifiable cross-link precursors on one or two opposing strands of DNA, annealing of the two single strands and subsequent use of a specific coupling reaction to generate the ICL. This concept has been used in the synthesis of ICLs containing disulfide, psoralen, malondialdehyde, and alkyl linkages.<sup>10,11</sup> With the exception of disulfide ICLs, there has been a lack of efficient syntheses of ICLs formed in the major groove of DNA, where adducts by the clinically most important ICL-forming agents including the nitrogen mustards (NMs) are formed.

NMs preferentially form ICLs between the N7 positions of dG residues in a 5'-GNC sequence context and introduce a slight distortion into the DNA.<sup>6,7,12,13</sup> Following the initial pioneering studies of NM ICLs by the Loechler and Hopkins groups, no further efforts toward high-yielding synthesis of these adducts have been reported.<sup>6</sup>

We designed a strategy for the synthesis of NM ICL mimics **2** based on the incorporation of ICL precursor nucleosides on opposing strands of DNA and the use of a subsequent specific coupling reaction to establish the ICL (Figure 1). We reasoned that alkylamine-containing cross-links may be accessed by a double-amination reaction from two aldehyde groups (**3**) on cDNA strands using an appropriate amine. Since guanine bases alkylated at the 7 position in the native NM ICL **1** are prone to depurination



**Figure 1.** Structures of NM ICLs **1** and strategy for the synthesis of NM ICL analog **2**. NM ICLs (**1**) connect two complementary DNA strands by connecting two guanine bases through the N(7) positions. Our target NM ICL mimic **2** has the nitrogen at the 7 positions replaced with carbon to render the glycosidic bond of the ICL stable for synthesis and functional studies ( $X$  = amine-containing compound). We envisioned that ICL **2** could be obtained via double-reductive amination of an aldehyde **3** with an appropriate amine,  $XH_2$ . **3** in turn could be derived from phosphoramidite **4**, where the aldehyde is masked as a protected diol.

due to the positive charge on nitrogen, we decided to pursue the synthesis of the more stable, isosteric 7-deaza analogues **2**. The increased hydrolytic stability of the 7-deaza compounds should make it possible to incorporate them into DNA using solid-phase synthesis and make them attractive substrates for biological studies. We envisioned that the aldehyde would be introduced into DNA masked as a protected diol **4**,<sup>14</sup> using standard phosphoramidite chemistry (Figure 1).

The synthesis of **4** (Scheme 1) started with 6-chloro-7-deaza-3',5'-di-*O*-*p*-toluoyl-2'-deoxyguanosine **5**,<sup>15</sup> which was protected as an isobutyric amide at the N(2) position and selectively iodinated at C(7).<sup>16</sup> A Stille coupling reaction was then used to introduce the allyl group in **6**. Treatment with pyrimidine-2-carboxaldoxime<sup>17</sup> restored the deazaguanine core, and the toluoyl protecting groups in the carbohydrate moiety were replaced with TBDMS. The allyl group of **7** was then oxidized to corresponding diol (**8**) using osmium tetroxide, and the newly generated hydroxyl groups were protected as acetate esters. Finally, the TBDMS protecting groups were removed, and the sugar moiety was elaborated to the phosphoramidite **4** using standard procedures.

Using solid-phase synthesis, **4** was incorporated into two cDNA strands as a part of a 5'-d(GNC) sequence (**10a–d**, Figure 2A), which has been shown to be the preferred site for NM ICL formation.<sup>7</sup> The two single-stranded oligonucleotides were purified by solid phase extraction using TOP-

(6) Ojwang, J. O.; Grueneberg, D. A.; Loechler, E. L. *Cancer Res.* **1989**, *49*, 6529–37. Millard, J. T.; Raucher, S.; Hopkins, P. B. *J. Am. Chem. Soc.* **1990**, *112*, 2459–2460.

(7) Rink, S. M.; Solomon, M. S.; Taylor, M. J.; Rjur, S.; McLaughlin, L. W.; Hopkins, P. B. *J. Am. Chem. Soc.* **1993**, *115*, 2551–2557.

(8) Warren, A. J.; Hamilton, J. W. *Chem. Res. Toxicol.* **1996**, *9*, 1063–71. Fischhaber, P. L.; Gall, A. S.; Duncan, J. A.; Hopkins, P. B. *Cancer Res.* **1999**, *59*, 4363–8.

(9) Harwood, E. A.; Sigurdsson, S. T.; Edfeldt, N. B. F.; Reid, B. R.; Hopkins, P. B. *J. Am. Chem. Soc.* **1999**, *121*, 5081–5082. Li, H. Y.; Qiu, Y. L.; Moyroud, E.; Kishi, Y. *Angew. Chem., Int. Ed. Engl.* **2001**, *40*, 1471–1475. Noll, D. M.; Noronha, A. M.; Miller, P. S. *J. Am. Chem. Soc.* **2001**, *123*, 3405–11. Wilds, C. J.; Noronha, A. M.; Robidoux, S.; Miller, P. S. *J. Am. Chem. Soc.* **2004**, *126*, 9257–9265. Wilds, C. J.; Xu, F.; Noronha, A. M. *Chem. Res. Toxicol.* **2008**, *21*, 686–95.

(10) Ferentz, A. E.; Keating, T. E.; Verdine, G. L. *J. Am. Chem. Soc.* **1993**, *115*, 9006–9014. Erlanson, D. A.; Chen, L.; Verdine, G. L. *J. Am. Chem. Soc.* **1993**, *115*, 12583–12584. Kobertz, W. R.; Essigmann, J. M. *J. Am. Chem. Soc.* **1997**, *119*, 5960–5961. Dooley, P. A.; Tsarouhtsis, D.; Korbel, G. A.; Nechev, L. V.; Shearer, J.; Zegar, I. S.; Harris, C. M.; Stone, M. P.; Harris, T. M. *J. Am. Chem. Soc.* **2001**, *123*, 1730–9. Hong, I. S.; Greenberg, M. M. *J. Am. Chem. Soc.* **2005**, *127*, 10510–1. Hong, I. S.; Greenberg, M. M. *J. Am. Chem. Soc.* **2005**, *127*, 3692–3.

(11) Alzeer, J.; Schäfer, O. D. *Nucleic Acids Res.* **2006**, *34*, 4458–66.

(12) Rink, S. M.; Hopkins, P. B. *Biochemistry* **1995**, *34*, 1439–45. Dong, Q.; Barsky, D.; Colvin, M. E.; Melius, C. F.; Ludeman, S. M.; Moravsek, J. F.; Colvin, O. M.; Bigner, D. D.; Modrich, P.; Friedman, H. S. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 12170–4.

(13) Fan, Y.-H.; Gold, B. *J. Am. Chem. Soc.* **1999**, *121*, 11942–11946.

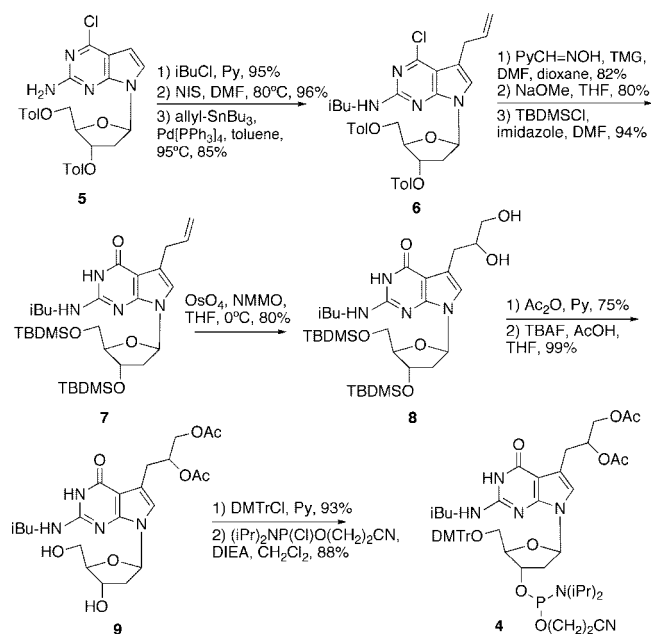
(14) Khullar, S.; Varaprasad, C. V.; Johnson, F. *J. Med. Chem.* **1999**, *42*, 947–50.

(15) Seela, F.; Westermann, B.; Binding, U. *J. Chem. Soc., Perkin Trans. I* **1988**, 697–702.

(16) Ramzaeva, N.; Seela, F. *Helv. Chim. Acta* **1995**, *78*, 1083–1090.

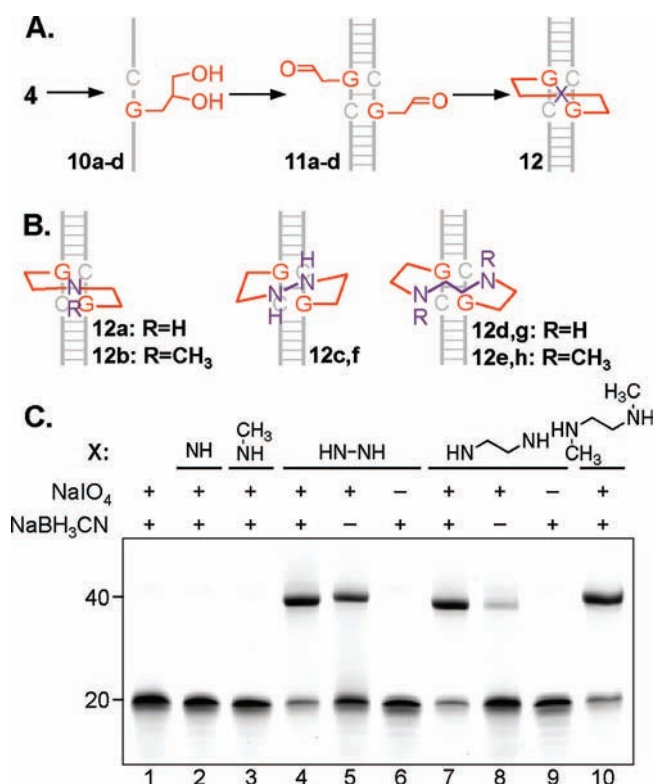
(17) Buhr, C. A.; Wagner, R. W.; Grant, D.; Froehler, B. C. *Nucleic Acids Res.* **1996**, *24*, 2974–80.

### Scheme 1. Synthesis of the ICL Precursor Phosphoramidite 4



cartridges (Varian), and the incorporation and integrity of the diol was verified by ESI-MS (Table 1, Supporting Information). Exploratory experiments revealed that the diol-containing DNA could be oxidized to the aldehyde and derivatized with an aldehyde-reactive semicarbazide fluorescent dye (data not shown), validating our approach for the generation of the aldehyde precursor.

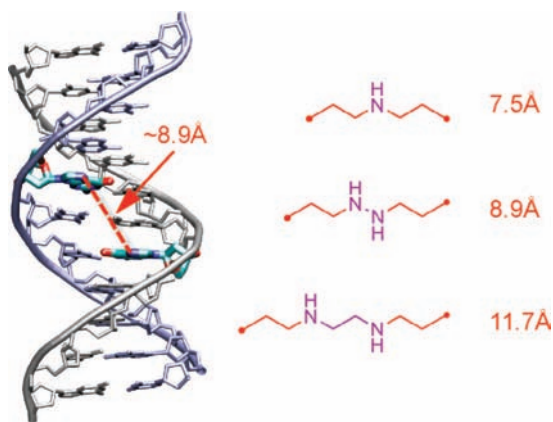
With the aldehyde-containing single-stranded oligonucleotides in hand, we investigated the use of these building blocks in ICL formation. To circumvent the need for lengthy manipulations of the potentially unstable aldehyde functionalities, the two diol-containing strands were first annealed and subsequently oxidized with sodium periodate. Excess oxidizing reagent was quenched by addition of sodium sulfite, and the aldehyde-containing oligonucleotides were treated with a variety of amines and  $\text{NaBH}_3\text{CN}$ . Unexpectedly, incubation with  $\text{NH}_4\text{OAc}$  or methylamine did not lead to any detectable formation of ICLs **12a** or **12b**, as only a band corresponding to the 20-mer single stranded oligonucleotide substrates was visible on a denaturing polyacrylamide gel (Figure 2C, lanes 2 and 3). By contrast, treatment with hydrazine, ethylenediamine, or *N,N'*-dimethylethylenediamine resulted in the formation of a new major band with mobility corresponding to a size roughly double that of the starting 20-mers, indicating that the formation of ICLs **12c**, **12d**, and **12e** had occurred (Figure 2B, C, lanes 4, 7, and 10). While the formation of ICLs by the ethylenediamines was dependent on the presence of  $\text{NaBH}_3\text{CN}$ , hydrazine ICLs were also formed in the absence of any reducing agent, which could be explained by the formation of a stable hydrazone adduct (Figure 2C, compare lanes 4 and 5). Treatment with excess hydrazine and formaldehyde readily reversed of the nonreduced form of hydrazine ICL, but not the reduced form of **12c** (data not shown), indicating that the hydrazone linkage



**Figure 2.** Synthesis of DNA ICLs through reductive amination. (A) Phosphoramidite **4** was incorporated into complementary strands of DNA and deprotected (**10a-d**). Following annealing and oxidation with  $\text{NaIO}_4$  to aldehydes **11a-d**, reaction with ammonium chloride, methylamine, hydrazine, ethylenediamine, or dimethylethylenediamine followed by reduction with  $\text{NaBH}_3\text{CN}$  was attempted to generate ICLs **12**. (B) Structures of the target ICLs **12a-h**. (C) Denaturing PAGE analysis of the reductive amination reaction of the two aldehyde containing 20-mers. The amine group X resulting from the reaction, as well as the presence or absence of  $\text{NaIO}_4$  and  $\text{NaBH}_3\text{CN}$  in the reaction mixture, are indicated above the gel. The positions of marker 20-mer (for ssDNA) and 40-mer (for ICL containing DNA) oligomers are indicated. The gel was stained with methylene blue.

was indeed reduced by  $\text{NaBH}_3\text{CN}$ . The formation of an ICL was dependent on the presence of aldehyde reactive groups on both strands of DNA, as no slower migrating band was formed if the aldehyde functionalized guanine residues were absent or present only on one of the two strands (data not shown). The ICL-containing oligonucleotides were purified by reverse-phase HPLC and subjected to MS and nucleoside composition analysis. ESI-MS analysis of the cross-linked 20-mer oligonucleotides revealed good agreement between the calculated and measured mass, but were not accurate enough to ascertain that the ICLs was present with two reduced amine bonds (Table 1, Supporting Information). To obtain more precise mass determinations, we repeated the synthesis to obtain the shorter 11-mer ICL-containing oligonucleotides **12f-h**. In this case, the molecular weight could be determined for all the ICL-containing oligonucleotides with a precision of  $\pm$  one mass unit (Table 1, Supporting Information) consistent with ICL formation and reduction of the imine and hydrazone linkages. We further





**Figure 3.** Structure of B-form DNA highlighting the distance linked by a NM ICL. The distance between the two cross-linked N7 atoms of guanine is marked by a red dotted line. The nominal distances of an amine, hydrazine, and ethylenediamine ICLs are indicated in the right panel.

attempted to analyze the ICL-containing oligonucleotides by nucleoside composition analysis and incubated them with phosphodiesterase I, exonuclease III and calf intestine phosphatase, conditions we had previously employed in the analysis of ICLs.<sup>11</sup> HPLC analysis of the digestion of the dimethylethylenediamine ICL **12e** yielded the expected peaks for the four native nucleosides dA, dC, dG, and T and an additional slower eluting peak (Figure 1, Supporting Information). This peak was identified by MS as the expected cross-linked nucleoside dimer. Unexpectedly, digestion of the hydrazine or ethylenediamine ICLs did not result in the formation of a peak for the cross-linked dimers, as we observed either incomplete digestion or decomposition at the peak in the area of the dimethyl ethylenediamine dimer (data not shown). Nonetheless, the data from MS analyses and digestion of the dimethyl ethylenediamine ICL as well as the difference of stability of reduced and nonreduced ICLs are only consistent with structures **12c–h**.

The lack of ICL formation in the reductive amination reaction with ammonia and methylamine deserves some comment. It has previously been shown that since the distance between the N7-dG sites in 5'-d(GNC) sequences ( $\sim 8.9$  Å) is longer than the NM linkage ( $\sim 7.5$  Å), the NM ICLs must induce a bend into DNA (Figure 3).<sup>13</sup> In our case, the intrinsically reversible initial imine formation step of the reductive amination reaction apparently does not provide enough strength to lead to the formation of ICLs **12a** and **12b** with these two amines. To exclude that the inability to form ICL **12a** was due to reductive amination of both

aldehyde groups with ammonia prior to ICL formation, we also attempted to generate ICL **12a** by reaction between a 7-(2-oxoethyl)-7-deazaguanine and a 7-(2-aminoethyl)-7-deazaguanine residue on opposing strands in a d(GNC) sequence. This approach also did not lead to formation of ICL **12a** (T.A. and O.D.S., data not shown), suggesting that indeed distance and reactivity constraints were responsible for the inability to complete the reductive amination with ammonia and methylamine. The formation of hydrazine ICLs (**12c**) and ethylenediamine ICLs (**12d**, **12e**) on the other hand would not require the introduction of a bend in the DNA due to the longer linkage (8.9 Å for hydrazine, 11.7 Å for ethylenediamine), thereby facilitating ICL formation. An open question is how much the higher reactivity of hydrazine compared to an amine contributes to successful ICL formation in the case of **12c**. Upon reaction with the aldehyde, hydrazine forms a hydrazone, which is much more stable than an imine and more reactive toward the aldehyde in the second reaction. Our observations that the reaction of the dialdehyde can lead to efficient ICL formation with hydrazine in the absence of a reducing agent supports this notion (Figure 2C, lane 5).

In conclusion, we have developed synthetic methodology for the preparation of pure oligonucleotides containing site-specific ICLs in the major groove using a double reductive amination reaction of aldehyde functionalities in two complementary DNA strands at a scale of  $>10$  nmol. This method should be readily scalable by a factor of 10 or 100 to generate ICLs in quantities that will enable detailed structural studies. These defined ICLs have already been proven to be valuable tools for the study of ICL repair mechanisms.<sup>18</sup>

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**Supporting Information Available:** Supplementary Table 1, supplementary Figure 1, experimental procedures, <sup>1</sup>H, <sup>13</sup>C, and <sup>31</sup>P NMR spectra of all new compounds, and ESI-MS spectra of oligonucleotides **10a–d** and **12c–h**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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(18) Raschle, M.; Knipsheer, P.; Enoiu, M.; Angelov, T.; Sun, J.; Griffith, J. D.; Ellenberger, T. E.; Schärer, O. D.; Walter, J. C. *Cell* **2008**, *134*, 969–80.