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Tetrahedron Letters
Tetrahedron Letters 47 (2006) 9125–9128

Synthesis of oligonucleotides containing an O^6-G-alkyl-O^6-G interstrand cross-link

Christopher J. Wilds,* Jason D. Booth and Anne M. Noronha

Department of Chemistry and Biochemistry, Concordia University, 7141 Sherbrooke St. West, Montreal, QC, Canada H4B 1R6

Received 14 September 2006; revised 11 October 2006; accepted 12 October 2006
Available online 7 November 2006

Abstract—A methodology to synthesize oligonucleotides containing an alkyl interstrand cross-link between the two O6 atoms of deoxyguanosine has been developed. This cross-link is designed to serve as a stable structural mimic of the lesion formed in duplex DNA with the bifunctional alkylating agent hepsulfam. The O6-alkyl coupling is performed via a Mitsunobu reaction between a nucleoside and mono-protected 1,7-heptanediol. Solid-phase oligonucleotide synthesis using a nucleoside bis-phosphoramidite allows for the assembly of the cross-linked duplex. Sufficient quantities of this cross-linked duplex were obtained for various structural and biological investigations.

Bifunctional alkylating agents encompass a class of chemotherapeutic agents whose antitumor activity has been attributed to their ability to cross-link the strands of duplex DNA.[1] These cross-links can inhibit DNA replication and transcription, leading to cell cycle arrest, apoptosis, and inhibition of tumor growth. A problem presented in cancer treatment is the development of resistance to the effects of antitumor agents, which is caused by enhanced repair of interstrand cross-links.[2] A better understanding of the molecular mechanisms of such repair could lead to more effective chemotherapeutic agents and treatment regimens. In order to conduct the various biological and structural investigations associated with probing DNA repair, methods to produce interstrand cross-linked DNA of well-defined structures in sufficient quantities are required.

One example of a bifunctional alkylating agent used clinically in the treatment of chronic myelogenous leukemia is hepsulfam (1,7-heptanediol disulfamate) (Fig. 1). Mass spectrometry and UV spectroscopy studies by Colvin and co-workers has shown that hepsulfam can form a number of N7 alkylated products with guanosine including 1,7-bis(guanyl)heptane. In addition, studies with model oligonucleotides revealed that hepsulfam forms interstrand cross-links at 5′-GXC-3′ sites.[3] However, the N-7-alkylated bis adducts are unstable with only partial stability imparted to the cross-linked duplex by conversion to the formamidopyrimidyl (FAPY) derivative, which is unstable at room temperature and limits the use of these cross-linked duplexes for structural and biochemical studies to probe DNA repair mechanisms.

This letter describes a methodology to produce interstrand cross-linked DNA, where the alkyl linkage between the two adjacent strands occurs between the O6-positions of deoxyguanosines. The O6 position of deoxyguanosine is a site of alkylation by a number of different agents.[4] It was thought that this may be a structural mimic for the clinically relevant lesion induced in duplex DNA by hepsulfam. In addition, this cross-link should exhibit sufficient chemical stability that would allow for various structural and biochemical investigations related to DNA repair studies. It should be noted that although this O6 cross-linked DNA may serve as a structural mimic of the hepsulfam induced DNA cross-link that occurs at the N7 position of deoxyguanosine, the efficiency and mechanism of DNA repair may be different.

The synthesis of bis-phosphoramidite **6** was accomplished in five steps starting from N^2-phenoxyacetyl-5′-*O*-dimethoxytrityl-3′-*O*-[(allyloxy)carbonyl]-2′-deoxyguanosine **1** (Scheme 1). Although the isobutyryl protecting

Keywords: Oligonucleotides; Interstrand cross-link; DNA repair; Cancer.
* Corresponding author. Tel.: +1 514 848 2424x5798; fax: +1 514 848 2868; e-mail: cwilds@alcor.concordia.ca

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doi:10.1016/j.tetlet.2006.10.074

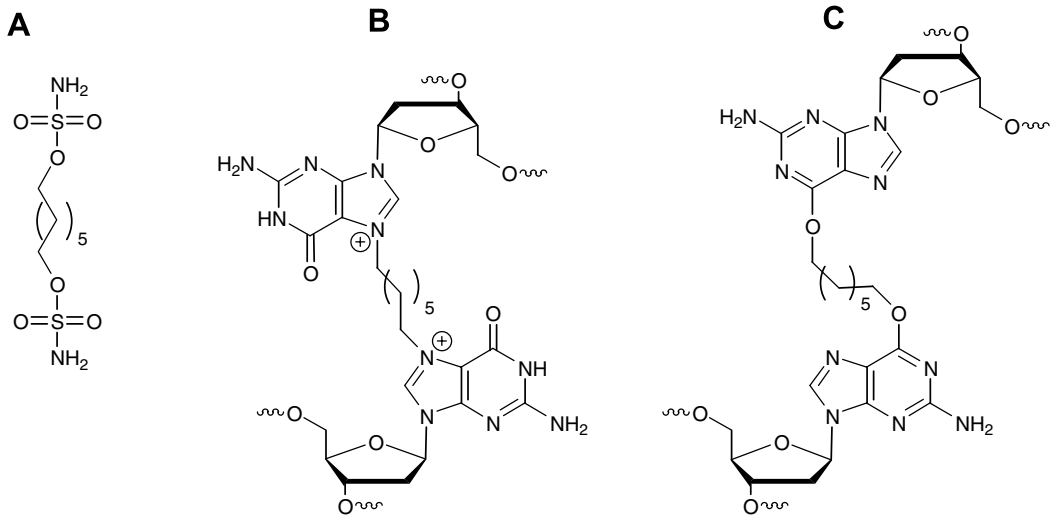
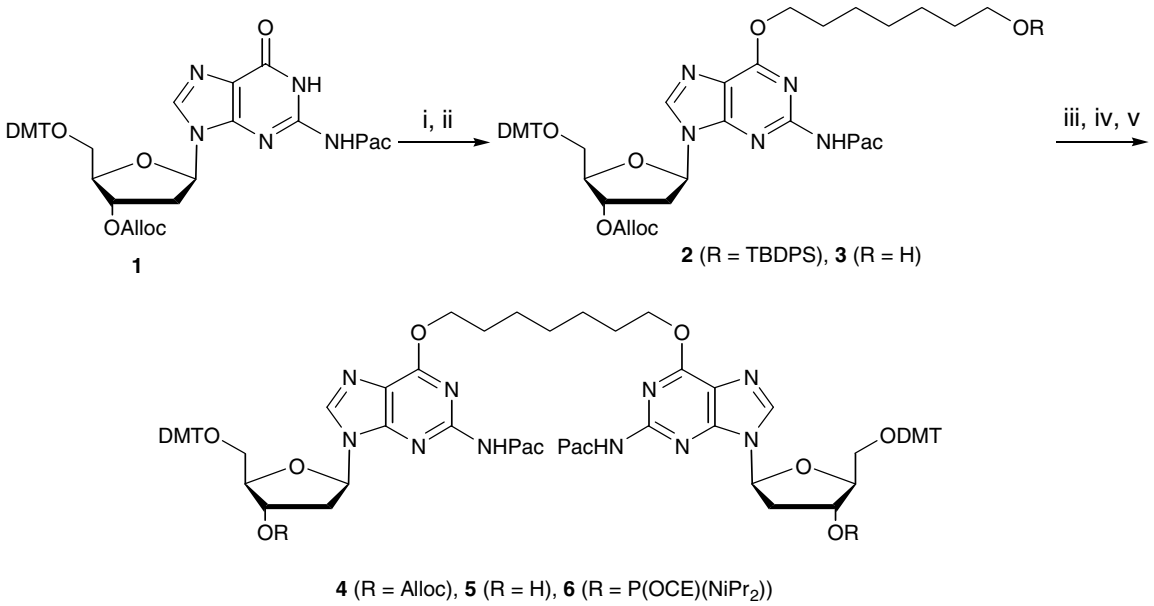


Figure 1. Structure of (A) hepsulfam, (B) the interstrand DNA cross-link induced by hepsulfam and (C) the proposed O6-alkyl-O6 deoxyguanosine interstrand cross-link.

group is one of the more popular protecting groups at the N2 position of deoxyguanosine for solid-phase oligonucleotide synthesis, the phenoxyacetyl group would require milder deprotection conditions thus avoiding any potential side reactions that occur in the synthesis of oligonucleotides containing 6-*O*-alkylguanine bases.[5] Compound **1** and 7-(*tert*-butyldiphenylsiloxy)heptanol were converted to **2** via the Mitsunobu reaction with a 74% yield.[6] The *tert*-butyldiphenylsilyl group was then removed using TBAF (1 M in THF) which gave compound **3** in a quantitative yield. Dimer **4** was produced by a second Mitsunobu reaction between **1** and **3** with a yield of 58%. Both 3′-*O*-(alloxy)carbonyl protecting groups were removed from **4** using a catalytic amount of tetrakis(triphenylphosphine)palladium(0) to give compound **5** with a 72% yield,[7] the two 3′-OH functionalities were then phosphitylated with 3.5 equiv of 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropyl phosphane to give the bis-phosphoramidite **6** in a 70% yield.[8]

Bis-phosphoramidite **6** was used in the assembly of the cross-linked duplex **9**. Bis-phosphoramidites have been used in the synthesis of a number of novel nucleic acid structures including branched nucleic acids and nucleic acid dendrimers.[9] The assembly of the cross-linked oligonucleotide duplex was carried out on a 1 μmol scale using commercially available 3′-*O*-deoxyphosphoramidites (at a concentration of 0.1 M and a coupling time of 120 s). The phenoxyacetyl protecting group at the N2 position of compound **6** makes it necessary to use phenoxyacetic anhydride rather than acetic anhydride as the capping reagent to prevent an undesired

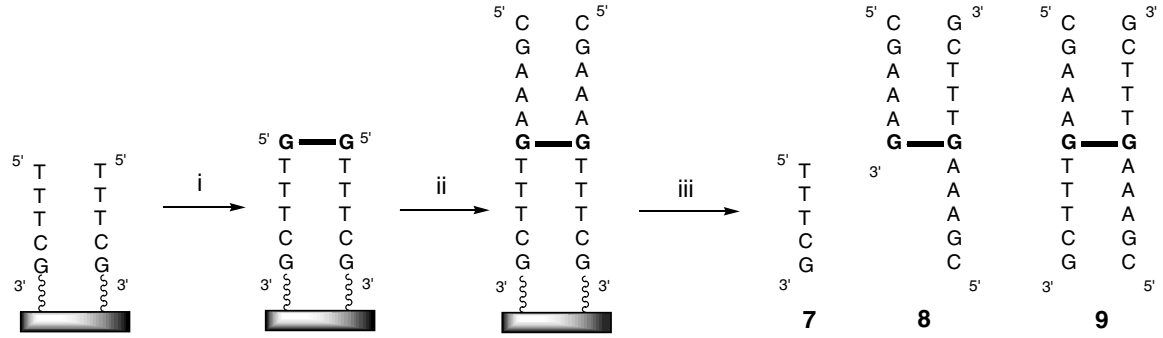


1

2 (R = TBDPS), **3** (R = H)

4 (R = Alloc), **5** (R = H), **6** (R = P(OCE)(NiPr$_2$))

Scheme 1. Synthesis of bis-phosphoramidite **6**, where: (i) 7-(*tert*-butyldiphenylsiloxy) heptanol (1.1 equiv), DIAD (1.1 equiv), triphenylphosphine (1.1 equiv), (ii) TBAF (1 M in THF) (1.1 equiv), (iii) **1**, DIAD, triphenylphosphine, (iv) tetrakis(triphenylphosphine)palladium(0) (0.2 equiv), butylamine (5 equiv), formic acid (5 equiv), (v) 2-cyanoethyl *N*′,*N*′,*N*,*N*-tetraisopropyl phosphane (3.5 equiv), diispropylammonium tetrazolide (3.4 equiv).



Scheme 2. Synthesis of interstrand cross-link duplex **9**. (i) The addition of bis-phosphoramidite **6**, (ii) followed by 3′-*O*-deoxyphosphoramidites, (iii) cleavage from the solid-support and deprotection using 1:3 ethanol/ammonium hydroxide (28%). Nucleoside loading on CPG was 65 μmol/g.

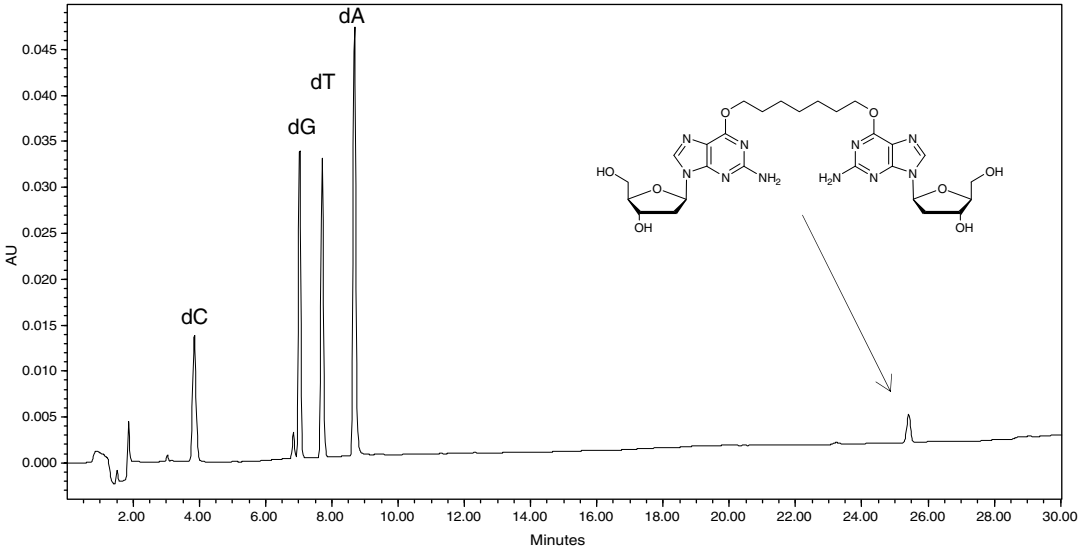


Figure 2. Reversed phase HPLC profiles of the cross-linked oligonucleotide **9** after nuclease digestion. The small peak proceeding dG (at 6.8 min) is deoxyinosine which is the result of deamination due to the presence of deaminase in the snake venom phosphodiesterase (see Table 1).

N-acetylation reaction observed during solid-phase oligonucleotide synthesis employing 'fast-deprotecting' phosphoramidites.[10] The assembly of the cross-linked duplex involves chain growth in the 3′ → 5′ direction with the 3′-*O*-deoxyphosphoramidites, followed by bis-phosphoramidite **6** at the site of the desired cross-link, then continued chain growth with 3′-*O*-deoxyphosphoramidites to give the fully cross-linked duplex **9** (Scheme 2). The crude oligonucleotide was deprotected using 1:3 ethanol/ammonium hydroxide (28%) at 55 °C for 4 h, purified via strong anion exchange (SAX) HPLC and desalted.

In the synthesis of cross-linked duplex **9**, the optimal concentration of bis-phosphoramidite **6** was found to be 0.05 M (coupling time of 600 s). Analytical SAX HPLC runs revealed that when concentrations of **6** below 0.05 M were used more truncated pentamer **7** was present. At concentrations of 0.05 M and above, pentamer **7** decreased and two major products were formed. The first is sequence **8** which is a Y-like 17-mer where only one of the bis-phosphoramidite functionalities coupled to the oligonucleotide chain attached to the solid-support and the other is the fully cross-linked duplex **9**. Both of these products were easily separated by SAX HPLC. The final yield of the cross-linked duplex **9** was 12.6% (27.2 od units from a 1 μmol scale synthesis).

The composition of the cross-linked duplex **9** was confirmed by enzymatic digestion with snake venom phosphodiesterase (SVPDE) and calf intestine phosphatase and analysis of the nucleosides via reversed-phase (C18) HPLC as described previously[11] as well as MALDI-TOF mass spectrometry. Figure 2 shows the HPLC chromatogram, the first four peaks are dC, dG, dT, and dA, respectively. The final peak at 25.4 min coelutes with a completely deprotected standard of dimer **5**. The

Table 1. Nucleoside ratios for the cross-linked duplex **9**

Nucleoside	Peak area	Nucleoside ratios	
		Expected	Observed
dC	101140	1.00	1.00
dG	165006	1.00	1.03
dT	178764	1.50	1.44
dA	272597	1.50	1.39
dG-dG	23911	0.25	0.20

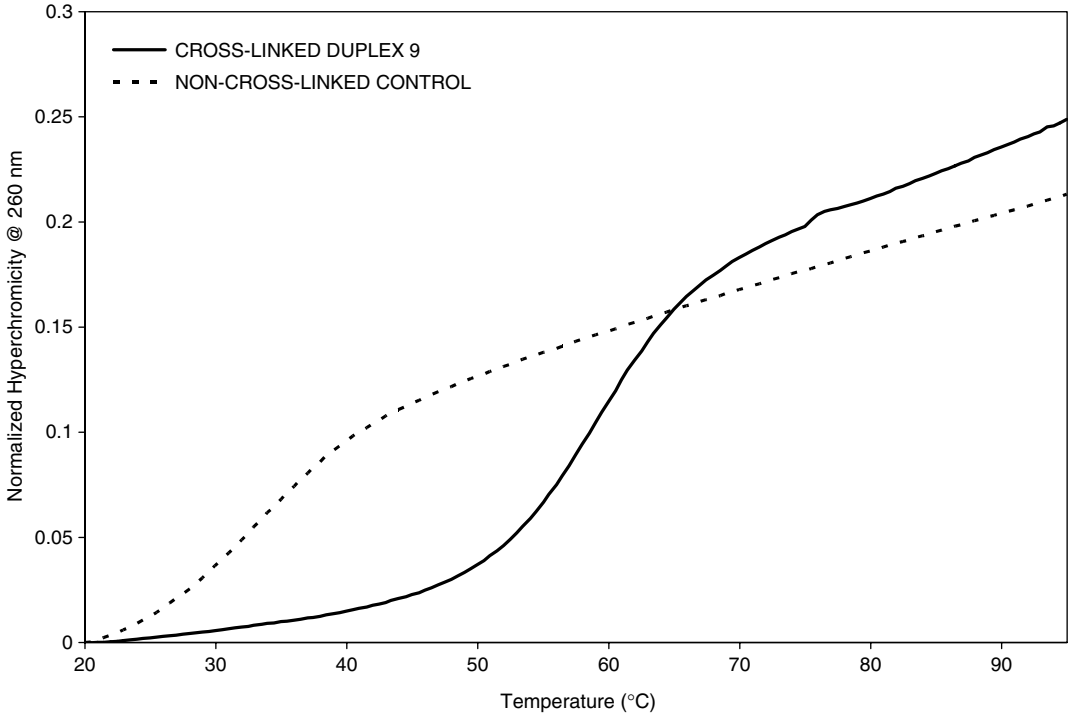


Figure 3. UV thermal denaturation profile of the cross-linked oligonucleotide **9** and non-cross-linked control duplex (5′-dCGAAAGTTTCG)$_2$.

ratio of the peak areas agrees with the nucleotide composition of the cross-linked oligonucleotide **9** (Table 1). MALDI-TOF mass spectral analysis of duplex **9** indicated a molecular weight of 6806.9 (expected 6805.4).

UV thermal denaturation studies were also carried out on the cross-linked duplex **9** (Fig. 3). The cross-linked duplex exhibited a single sigmoidal denaturation profile characteristic of an oligonucleotide duplex with a melting temperature of 58 °C, an increase of 23 °C over the corresponding, non-cross-linked control duplex. Part of this stabilization could be entropic in nature as the two stands are preorganized for complex formation.

In conclusion, a method to synthesize oligonucleotide duplexes that contains an interstrand cross-link between two deoxyguanosines at the O6-positions was developed. Solid-phase oligonucleotide synthesis yields sufficient amounts of material with a high purity that should allow for structural and biological studies.

Acknowledgements

This work was supported by funding from the Natural Sciences and Engineering Research Council of Canada (NSERC), the Canada Foundation for Innovation (CFI) and the Canada Research Chair (CRC) program. We are also indebted to Ms. Margaret Wear and Dr. Paul Miller at Johns Hopkins University for MALDI analysis of the oligonucleotides, Mr. Nadim Saade at McGill University for ESI-MS analysis of the nucleosides and Dr. Sébastien Robidoux at Concordia University for the critical proofreading of this manuscript.

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