Chemical Synthesis and Preliminary Structural Characterization of a Nitrous Acid Interstrand Cross-Linked Duplex DNA

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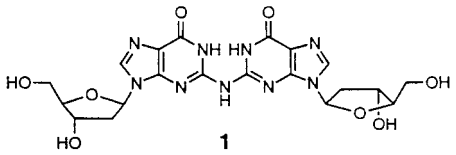
Nitrous acid is a mutagenic substance; it converts the exocyclic amino groups of DNA to carbonyl groups[1−6] and forms interstrand cross-links in duplex DNA.[7] There is considerable interest in these reactions due to the dietary and environmental exposure of humans to oxides of nitrogen which can initiate these reactions. For example, sodium nitrite is used extensively in the preparation of cured meats.[8,9] The reactions of nitrous acid with DNA are believed to proceed by the diazotization of an exocyclic amino group, followed by displacement by a nucleophile, such as water, or an amino group of another nucleotide.

Nitrous acid-induced DNA interstrand cross-links form preferentially between two deoxyguanosine (dG) residues at the sequence [5′-d(CG)]$_2$, forming a cross-link lesion in which the guanines share a common N2 atom (as in **1**).[7,10,11] It has been



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proposed that this sequence preference is due to the close proximity of an exocyclic amine of dG on one DNA strand to a diazonium ion intermediate on the other strand.[10,12,13] Molecular modeling studies suggest that the resulting cross-link lesion can be accommodated with minimal structural reorganization in B-form DNA, despite a severe propeller twist of the cross-link lesion.[10,12] A plausible alternative structure for this cross-link would involve extrusion of the partner dC residues at the cross-link, as has recently been seen in cisplatin interstrand cross-linked DNA.[14] We desired to obtain a homogeneous sample of nitrous acid cross-linked DNA to determine experimentally its structure and to investigate repair of these lesions. We report here two new methods for the chemical synthesis of interstrand cross-linked duplex DNA, applied to the synthesis of nitrous acid interstrand cross-linked DNA, and show preliminary structural characterization of this DNA by solution NMR spectroscopy. The method complements that recently reported by Harris and co-workers.[15]

Exposure of duplex DNA to aqueous nitrous acid is not a viable route for the preparation of structurally homogeneous interstrand cross-linked DNA due to concomitant deamination reactions. Instead, we focused on the synthesis of cross-link lesion **1**, found in small quantities in enzymatic digests of cross-linked DNA, and its incorporation into DNA by chemical synthesis. Several unsuccessful attempts were made to prepare **1** by the displacement of a leaving group in the 2-position of an inosine derivative by the exocyclic amino group of dG derivatives. However, Pd-catalyzed reaction of **2** with **3** proceeded to give **4** in good yield after removal of the silyl groups (Scheme 1).[16−18] Hydrogenolysis of compound **4** removed the benzyl-protecting groups to yield **1**, which was compared to an authentic sample obtained from nitrous acid interstrand cross-linked DNA.[17] However, due to the low solubility of **1**, the benzyl-protecting groups of **4** were removed *after* incorporation into DNA.

Two strategies allowed the incorporation of the cross-link lesion **1** into duplex DNA (Scheme 2). In the first, phosphoramidite **6** was synthesized (Scheme 1) and coupled to the growing oligomer in a standard solid-phase synthesis protocol. Subsequent removal of the DMT groups by acid treatment and continuation of oligomer synthesis yielded a three-armed DNA oligomer (Scheme 2A). The 5′-alcohols were capped with acetic anhydride and the allyl carbonate was removed from the 3′-end with a palladium catalyst in the presence of butylamine.[19,20] The last arm of the cross-linked DNA was synthesized using inverted phosphoramidites, in which the 3′-hydroxyl group is protected with a DMT group and the 5′-alcohol has been converted into a phosphoramidite.[21] Deprotection with aqueous ammonia and purification by denaturing polyacrylamide gel electrophoresis (DPAGE) gave the cross-linked oligomer in 10% overall yield.

The second strategy utilized symmetrical phosphoramidite **7** and a more heavily loaded solid support[22] (Scheme 2B). Indeed, we obtained the cross-linked DNA in 10% overall yield after ammonia deprotection and purification by DPAGE. This approach is simpler than that illustrated in Scheme 2A; phosphoramidite **7** is synthesized in fewer steps and in significantly higher yield than **6**, and less **7** was required. This method was used to prepare samples for structural characterization.

A method for removing the benzyl-protecting groups in DNA was needed. Incubation with hydrogen gas in the presence of palladium on carbon, however, appeared to be inapplicable, because it has been reported that the double bond in thymidine can be reduced under these conditions.[23] The benzyl groups were quantitatively removed by transfer hydrogenolysis by means of palladium black in 35% formamide, 35% ethanol, 10% ethyl

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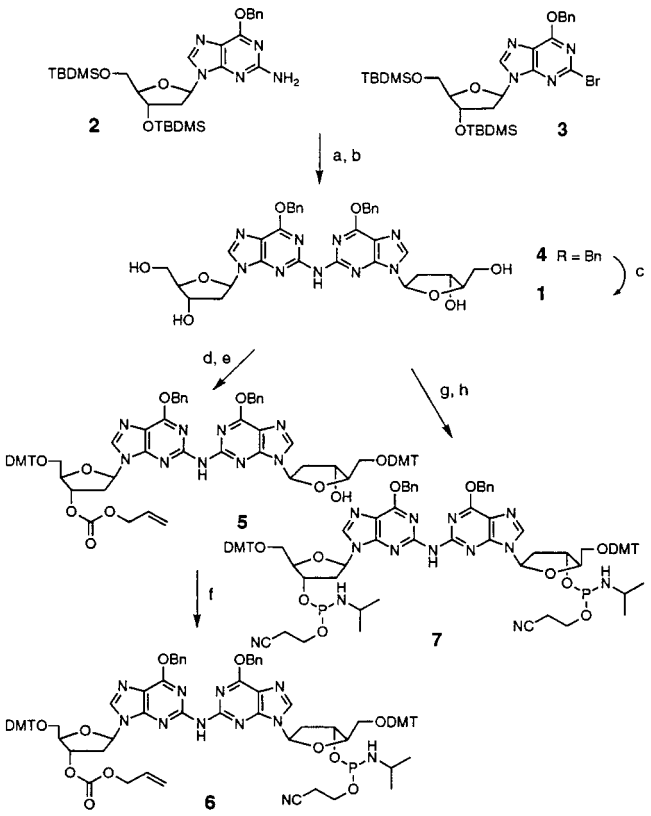
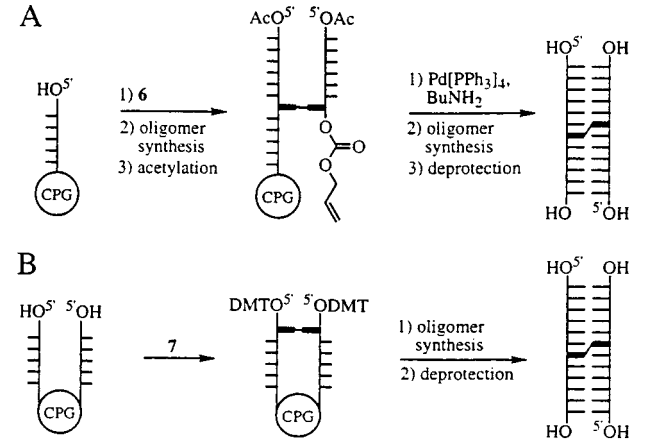
Scheme 1. Preparation of Phosphoramidite Derivatives of Compound **1** for Synthesis of Nitrous Acid Interstrand Cross-Linked Duplex DNA[a]



[a] (a) Tris(dibenzylideneacetone)-dipalladium(0) (Pd_2dba_3), (R)(+)-2,2′-bis(diphenylphosphino)-1,1′-binaphthyl (BINAP), sodium *tert*-butoxide, toluene, 40%, (b) TBAF, THF, 85%, (c) Pd black, 1,4-cyclohexadiene, formamide, EtOH, MeOH, EtOAc, 63%, (d) DMT-Cl, pyridine, 79%, (e) allyl 1-benzotriazolyl carbonate, pyridine, 30%, (f) *O*-(2-cyanoethyl) *N*,*N*,*N*′,*N*′-tetraisopropylphosphoramidite, diisopropylammonium tetrazolide, CH_2Cl_2, 67% (g) DMT-Cl, pyridine, 79%, (h) *O*-(2-cyanoethyl) *N*,*N*,*N*′,*N*′-tetraisopropylphosphoramidite, diisopropylammonium tetrazolide, CH_2Cl_2, 65%.

Scheme 2. Two Strategies for the Synthesis of Nitrous Acid Interstrand Cross-Linked Duplex DNA[a]



[a] CPG stands for controlled pore glass.

acetate, and 20% 1,4-cyclohexadiene. HPLC analysis of enzymatic digests of the phosphodiester backbone revealed cross-link lesion **1** in the expected amount[24] along with the appropriate ratio of all four nucleosides (see Supporting Information). The mass of DNA I as determined by electrospray mass spectrometry was 7278.3 g/mol (calcd 7276.4).

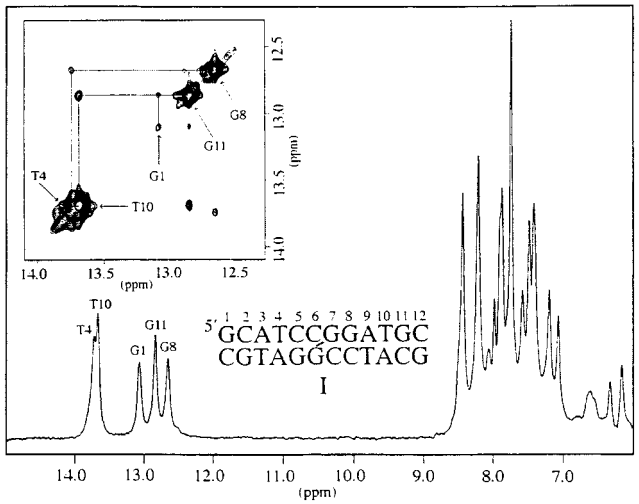


Figure 1. The 750 MHz ^{1}H NMR spectrum of cross-linked oligomer I (in 90%H_2O/10%D_2O, 200 mM NaCl), recorded at 0 °C. The inset shows the 2D NOESY spectrum (150-ms mixing time) of the imino region. The intra- and interstrand imino proton connectivities are indicated. Note the absence of imino protons for the cross-linked guanines, in both the 1D and 2D spectra.

The 1D ^{1}H NMR spectrum of a 0.4 μmol sample of DNA I,[25] prepared as illustrated in Scheme 2B, was recorded in water and the region from 6 to 15 ppm is shown in Figure **1**. Due to the symmetry of the duplex, six imino resonances were expected. However, inspection of the region between 12 and 14 ppm reveals only five imino resonances, corresponding to the two AT base pairs and three CG base pairs. Inspection of cross-peaks in the imino region of the 2D NOESY spectrum (Figure 1, inset) enables an "imino-walk" to be made from G1 to G11 to T10 to T4 to G8. The absence of imino protons at the site of the cross-link (G7) indicates that this lesion is unlikely to contain base pairing. The complete assignment of the ^{1}H NMR spectrum and structural determination are in progress and will be reported in due course.

The method described in Scheme 2B has been used to prepare several different sequences flanking the lesion site but is restricted to the preparation of self-complementary sequences. However, Scheme 2A allows preparation of non-self-complementary cross-linked duplex DNAs by extending the reverse synthesis to produce one 3′-overhang which allows ligation to another duplex DNA of any sequence.

In summary, we have described a chemical synthesis of a nitrous acid cross-linked DNA and have shown evidence for the formation of at least 10 stable base pairs in a 12-nucleotide long cross-linked duplex. This synthetic strategy should be applicable for other cross-linked oligonucleotides as long as the cross-link lesion is stable under the standard conditions of solid-phase synthesis.

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Supporting Information Available: Syntheses of compounds **2**−**7**, preparation and purification of nitrous acid interstrand cross-linked DNA, HPLC traces of enzymatic digests of interstrand cross-linked DNA and co-injections with an authentic sample of compound **1**, negative ion mode electrospray mass spectra of cross-linked DNAs (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(24) Identity verified by co-injection of a sample obtained from nitrous acid cross-linked DNA.; for quantification, using 16 800 M^{-1} cm^{-1} as the molar extinction coefficient.

(25) This sample was prepared by 12 syntheses using the aforementioned solid support. The cross-linked product was purified sequentially by DPAGE and HPLC.