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DIRECT EVIDENCE FOR DNA INTRASTRAND CROSS-LINKING BY THE NITROGEN MUSTARD MECHLORETHAMINE IN SYNTHETIC OLIGONUCLEOTIDES

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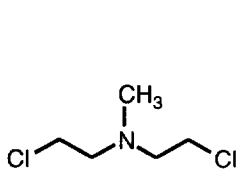
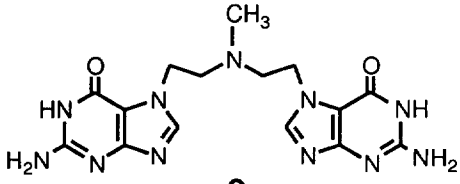
Abstract: Direct evidence for DNA intrastrand cross-link formation by mechlorethamine (**1**) is reported. Following treatment with **1**, duplex and single strand DNAs possessing two or more contiguous dG residues contained intrastrand cross-links, as revealed by the release of **2** following acid hydrolysis.

Mechlorethamine (nitrogen mustard, **1**) is an antitumor agent known to alkylate nucleic acids predominantly at the N^7 position of deoxyguanosine (dG) residues.[1-3] This compound, in principle, could react with two residues residing in the same strand (*intra*strand cross-link) or in opposing strands (*inter*strand cross-link) of the nucleic acid. Indirect evidence for cross-linking of nucleic acids by this agent was provided by the isolation of the adduct **2** from mechlorethamine-treated yeast RNA.[3] Substance **2** was recently shown to be the nucleus of the major mechlorethamine-derived DNA interstrand cross-link,[4] which is formed at 5'-d(GNC) sequences in DNA.[5] Given the proximity of N^7 atoms in adjacent dG residues of DNA, one might predict that mechlorethamine would also form intrastrand cross-links by analogy to the cases of mitomycin C,[6] cisplatin, and transplatin.[7-9] Evidence for the occurrence of intrastrand cross-linking of DNA by mechlorethamine has been obtained by quantitation of released **2** relative to the number of interstrand cross-links,[10] by *in vitro* transcription termination studies on mechlorethamine-treated plasmid DNA,[11] and by comparison of piperidine induced cleavage of 3'- and 5'-end labeled, mechlorethamine-treated DNA.[12]



1



2

We describe herein direct evidence for the formation of dG-to-dG intrastrand cross-links by nitrogen mustard treatment of double stranded and single stranded DNAs containing two or more contiguous dG residues. Digests of such DNAs previously treated with mechlorethamine afforded **2** (MS, UV). A pure, intrastrand cross-linked DNA single strand was isolated, the identity of which was demonstrated by the absence of guanine (<10%) and appearance of ca. 1 equivalent of **2** per single strand following acid hydrolysis and HPLC analysis.

General Methods/Results

The DNAs used in this study were synthesized by the automated phosphoramidite method and are shown in Table 1. They were precipitated two times from ethanol and used without further purification unless otherwise indicated. Each DNA was independently treated with mechlorethamine [0.4 mM mechlorethamine, 40 mM sodium cacodylate buffer (pH 8.0), 37 °C, 3 h]. *Caution: Mechlorethamine is toxic and can be adsorbed through the skin; exercise due care in handling.* The DNA products were recovered by ethanol precipitation, chemically hydrolyzed using aqueous formic acid (98%) and heat (140 °C, 3 h), and analyzed for the presence of **2** by C-18 reverse phase HPLC (RP-HPLC).[13] An authentic sample of **2** was prepared by the method of Brookes and Lawley.[14]

Table 1. DNA Sequences Used in this Study

Sequence (5'→3')	Descriptor
ATACCTTATATATAA**GG**TAT	GG
ATACTCTATATATA**GAG**TAT	GNG
ATACTTCATATAT**GAAG**TAT	GN_2G
ATACTTACTATA**GTAAG**TAT	GN_3G
ATTAATAATCC**GG**ATTATTA	C_2G_2
TTAATAACCC**GGG**TTATTAA	C_3G_3
TTAATACCCC**GGGG**TATTAA	C_4G_4
TATTA**GGGG**ATTA	G_4
TTAATCCCCTAATA	G_0
ATTA**GGG**CATTA	G_3
TTTTTAAT**G**CCCTAATTTTT	G_1
ATCC**GG**AT	C_2G_2-8
AT**GG**CCAT	G_2C_2-8

We initially studied the self-complementary DNA sequences of the form ($\mathbf{GX_nG}$, **n** = 0-3) which lacked the efficient interstrand cross-linking site 5'-d(GNC). RP-HPLC analysis of these digests revealed that only the sequence **n** = 0 returned a detectable quantity of **2**. A comparison of the DNA digests where **n** = 0 and **n** = 1 is shown in Figure 1. The data in Figure 1 for **n** = 1 are representative of that obtained from **n** = 2 and **n** = 3. This result thus provided preliminary evidence for the presence of intrastrand cross-links in mechlorethamine-treated oligonucleotides devoid of the 5'-d(GNC) interstrand cross-linking locus. It was highly unlikely that **2** could have resulted from an interstrand cross-link in the DNA GG, as the guanyl residues on opposite strands are separated by some 35 Å (B-DNA), whereas mechlorethamine can span only about 8 Å.

The extent to which the quantity of **2** varied with the length of the dG run was investigated using a panel of DNAs in which the dG run ranged from 2-4 residues. Self complementary DNAs C_nG_n (**n** = 2-4) were treated with mechlorethamine, acid hydrolyzed, and analyzed by RP-HPLC for the presence of **2**. Each of these digests returned **2**, consistent with the formation of intrastrand cross-links within a run of dG residues. The yield of **2** increased modestly with increasing length of the dG run. The dG residues that were covalently linked within the G-rich sequence were not specifically identified.

dG runs in non-self complementary oligonucleotides G_n (n = 0, 1, 3, 4) were also investigated. Incubation of either single stranded or duplex forms of these DNAs with mechlorethamine followed by acid digestion returned **2**. Compound **2** was undetectable in digests of single strand or duplex DNAs having fewer than two dG residues present. These results constitute indirect evidence for the presence of intrastrand cross-linkages in DNA formed by nitrogen mustard.

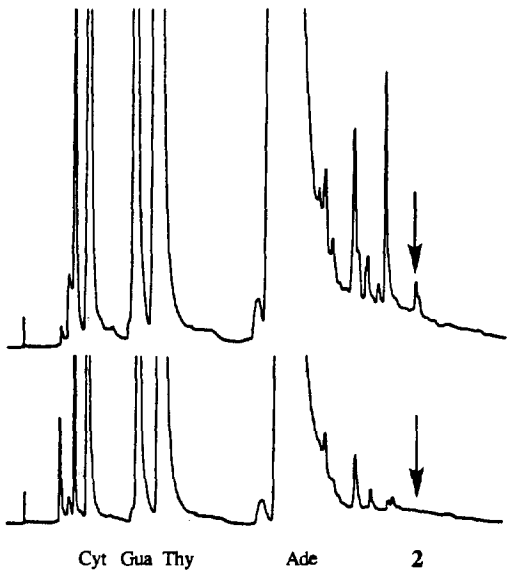


Figure 1. RP-HPLC analysis of mechlorethamine-treated DNAs $\mathbf{GX_nG}$ following acid hydrolysis. Elution order is from left to right. Upper chromatogram: Acid hydrolysate of the sequence GG. Lower chromatogram: Acid hydrolysate of the sequence GNG. Retention time of **2** is approximately 23.2 min.

The intrastrand cross-linking reaction was studied in more detail using the self-complementary DNAs C_2G_2-8 and G_2C_2-8 in 5'-[^{32}P]-phosphate labeled form. These two DNAs were independently treated with mechlorethamine, and the products analyzed by denaturing polyacrylamide gel electrophoresis (DPAGE) (Figure 2). Both DNAs returned predominantly material with the mobility of unalkylated single strands (i.e., starting material). In both cases, new products were found having slightly lower mobility than the starting DNA (labeled "intrastrand cross-linked" in Figure 2, but likely also containing alkylated but not cross-linked DNA) as well as products with very low mobility thus identified as interstrand cross-linked. The yields of these products, as determined by PhosphorImage analysis, are indicated in Table 2, along with their content of **2** per single strand (for intrastrand cross-linked) or duplex (for interstrand cross-linked), as determined by HPLC analysis of the hydrolysate of the DPAGE-purified individual bands. That all of these substances contained less than one mole of **2** per single strand or duplex indicates the structural heterogeneity of the samples from DPAGE. The data suggest that *all* of the **2** present in mechlorethamine-treated C_2G_2-8 has its origin in intrastrand cross-linked DNA; for G_2C_2-8, half of the **2** is intrastrand cross-link derived, with the balance coming from

interstrand cross-linking. The latter is not dissimilar to the 2:1 ratio of intrastrand to interstrand cross-linking determined some 25 years ago.[10]

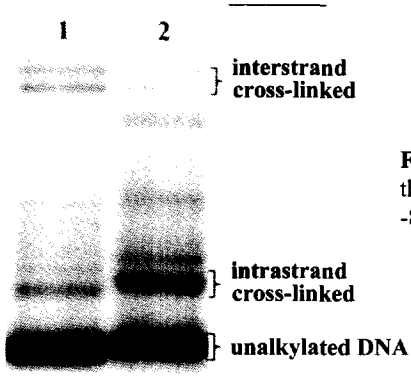


Figure 2. Denaturing PAGE analysis of the mechlorethamine-treated DNAs G_2C_2-8 (lane 1) and C_2G_2-8 (lane 2).[15]

Table 2. Yield of Cross-Links and **2** from mechlorethamine-treated C_2G_2-8 and G_2C_2-8.

DNA	Yield of Interstrand Cross-Link (%)/ Moles **2** per Mole Duplex	Yield of Intrastrand Cross-Link (%)/ Moles **2** per Mole Single Strand
G_2C_2-8	2.2/0.41	3.7/0.21
C_2G_2-8	0.2/0.00	8.7/0.33

Further purification of the intrastrand cross-linked DNA was also achieved. The single strand from C_2G_2-8 was isolated by preparative DPAGE and further subjected to RP-HPLC purification.[16] Hydrolysis of this HPLC-purified DNA returned no guanine (<10%) and instead provided ca. 1 equivalent of **2** per single strand of DNA. This result is consistent with the two guanine residues present in the C_2G_2-8 sequence being covalently tethered together as in **2**.

The identity of **2** from the hydrolysate of the highly purified intrastrand cross-link was further substantiated by spectroscopic comparison to an authentic sample.[14] The UV spectrum for the intrastrand cross-link nucleus gave λ_{min} and λ_{max} at neutral pH that were consistent with the UV data for authentic **2** and with the values reported in the literature.[14] Positive ion electrospray ionization mass spectrometry revealed parent ions m/e 430 ($[M - H + 2Na]^+$), 408 ($[M + Na]^+$) and 386 ($[M + H]^+$), as well as fragment ions 365 ($[M + Na - HNCO]^+$) and 235 ($[M - guanine + H]^+$) (Figure 3). A daughter scan of the parent ion 386 gave fragment ions 235 ($[M - guanine + H]^+$) and 178. These data are identical to those found for the authentic sample of **2**. The **2** from this source was found to co-elute on RP-HPLC with authentic **2**.[13]

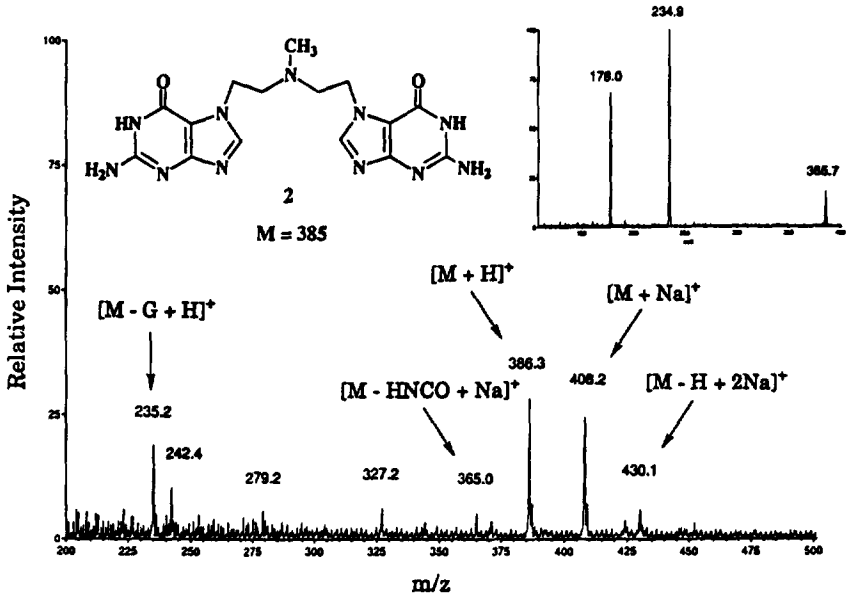


Figure 3. Positive ion electrospray mass spectrum of **2** isolated from the acid hydrolysate of a nitrogen mustard intrastrand cross-linked DNA. Inset: Daughter scan of m/e = 386.

Conclusions/Discussion

We have studied the intrastrand cross-linking of synthetic DNAs by mechlorethamine. The existence of intrastrand cross-links was suggested by the detection of **2** in acid hydrolysates of DNAs only when two or more contiguous deoxyguanosine residues were present in one strand, despite the absence of proximal deoxyguanosine residues on the opposite strand. The relative efficiency of formation of intrastrand and interstrand cross-links was explored using the DNA G_2C_2-8 which could form either of these. DPAGE returned interstrand cross-linked and alkylated DNA in a 1:2 ratio; each of these in turn released about 0.4 moles of **2** per duplex. Thus, some 1/2 of the **2** released from the hydrolysate of G_2C_2-8 treated with mechlorethamine was derived from intrastrand cross-links. A relatively pure sample of the DNA single strand C_2G_2-8 containing a dG-to-dG interstrand cross-link was prepared by HPLC fractionation of the alkylated DNA band from preparative DPAGE. That the dG residues in this sample were cross-linked as in **2** was revealed conclusively by acid hydrolysis and spectroscopic analysis of recovered **2**, isolated by RP-HPLC.

This study unequivocally demonstrates that mechlorethamine forms dG-to-dG intrastrand cross-links at runs of dG residues, as are commonly found in regions of eukaryotic DNA known to be important regulatory elements.[17] The biological relevance of this lesion remains to be established.

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13. HPLC conditions used for analysis of acid hydrolysates and co-elution experiments: Alltech 5 μ C-18, 250 mm x 4.6 mm column, 1.0 mL/min flow rate; solvent A: 10.0 mM ammonium formate; solvent B: 50% (aqueous) MeOH. Gradient: Isocratic 98.2% A for 10 min, a 10 min linear gradient to 8.2% A, isocratic for 7 min, then 5 min linear gradient to initial conditions. Quantitation was based on the peak area ratios at 260 nm obtained from the acid hydrolysis of the native DNAs and were as follows: Cyt (0.9):Gua (1.1):Thy (1.0):Ade (1.9). Acid hydrolysis of native DNA analyzed as 1.8 Cyt:1.8 Gua:2.0 Thy:2.1 Ade (calc'd: 2:2:2:2); highly purified intrastrand cross-linked DNA C_2G_2-8 analyzed as 1.7 Cyt:0.15 Gua:2.0 Thy:2.0 Ade: 0.93 **2** (calc'd: 2:0:2:2:1).
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15. Conditions used to treat C_2G_2-8 and G_2C_2-8 with mechlorethamine: 0.8 mM mechlorethamine, 40 mM sodium cacodylate (pH 8.0), rt, 3 h.
16. HPLC conditions used for purification of intrastrand cross-linked DNA isolated via DPAGE: Hamilton PRP-1, poly(styrene-divinylbenzene), 10 μ spherical 75 Å, 305 mm x 7.0 mm column; 2.0 mL/min flow rate; solvent A: 0.1 M TEAA (pH 7.0), solvent B: 1:1 CH_3CN/A. Gradient used: Isocratic 98.2% A for 10 min, 10 min linear gradient to 1.8% A, isocratic for 5 min, then a 5 min linear gradient to initial conditions.
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