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Stereospecific Formation of the (*R*)- γ -Hydroxytrimethylene Interstrand *N*²-dG:*N*²-dG Cross-Link Arising from the γ -OH-1,*N*²-Propano-2'-deoxyguanosine Adduct in the 5'-CpG-3' DNA Sequence

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Abstract

Acrolein reacts with dG to form hydroxylated 1,*N*²-propanodeoxyguanosine (OH-PdG) adducts. Most abundant are the epimeric 3-(2-deoxy- β -D-*erythro*-pentofuranosyl)-5,6,7,8-tetrahydro-8-hydroxypyrimido[1,2a] purin-10(3*H*)-ones, commonly referred to as the γ -OH-PdG adduct. When placed complementary to deoxycytosine in duplex DNA, these undergo rearrangement to the *N*²-(3-oxopropyl)-dG aldehyde. The latter forms diastereomeric interstrand *N*²-dG:*N*²-dG cross-links in the 5'-CpG-3' sequence. Here we report the structure of the stereochemically favored (*R*)- γ -hydroxytrimethylene *N*²-dG:*N*²-dG interstrand DNA cross-link in 5'-d(G¹C²T³A⁴G⁵C⁶X⁷A⁸G⁹T¹⁰C¹¹C¹²)-3'•5'-d(G¹³G¹⁴A¹⁵C¹⁶T¹⁷C¹⁸Y¹⁹C²⁰T²¹A²²G²³C²⁴)-3' (X⁷ is the dG adjacent to the α -carbon of the carbinolamine linkage and Y¹⁹ is the dG adjacent to the γ -carbon of the carbinolamine linkage; the cross-link is in the 5'-CpG-3' sequence). The structure was characterized using isotope-edited ¹⁵N NOESY-HSQC NMR, in which the exocyclic amines at X⁷ or Y¹⁹ were ¹⁵N-labeled. Analyses of NOE intensities involving Y¹⁹ *N*²H indicated that the (*R*)- γ -hydroxytrimethylene linkage was the major cross-link species, constituting 80–90% of the cross-link. The X⁷ and Y¹⁹ imino resonances were observed at 65 °C. Additionally, for the 5'-neighbor base pair G⁵•C²⁰, the G⁵ imino resonance remained sharp at 55 °C, but broadened at 65 °C. In contrast, for the 3'-neighbor A⁸•T¹⁷ base pair, the T¹⁷ imino resonance was severely broadened at 55 °C. Structural refinement using NOE distance restraints obtained from isotope-edited ¹⁵N NOESY HSQC data indicated that the (*R*)- γ -hydroxytrimethylene linkage maintained the C⁶•Y¹⁹ and X⁷•C¹⁸ base pairs with minimal structural perturbations. The (*R*)- γ -hydroxytrimethylene linkage was located in the minor groove. The X⁷ *N*² and Y¹⁹ *N*² atoms were in the *gauche*-conformation with respect to the linkage, which maintained Watson-Crick hydrogen bonding of the cross-linked base pairs. The anti conformation of the hydroxyl group with respect to C ^{α} of the tether minimized steric interaction, and more importantly, allowed the formation of a hydrogen bond between the hydroxyl group and C²⁰ O² located in the 5'-neighboring base pair G⁵•C²⁰. The formation of this hydrogen bond may, in part, explain the thermal stability of this carbinolamine interstrand cross-link, and the stereochemical preference for the (*R*)-configuration of the cross-link.

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Supporting Information Available

Table S1, NOEs used in the rMD simulations and the corresponding atom distances in the average refined structure; complete references 56 and 57. This material is available free of charge via Internet at <http://pubs.acs.org>.

Introduction

DNA interstrand cross-links represent a severe form of DNA damage due to their ability to block of DNA replication and transcription.^{1,2} The nucleotide excision repair (NER) pathway is involved in the repair of the interstrand cross-link lesion.³ Many chemicals cause interstrand cross-links, including bifunctional alkylating reagents, platinum compounds, and psoralen.³ α,β -Unsaturated aldehydes (enals) such as acrolein,^{4–6} crotonaldehyde,⁵ and *trans*-4-hydroxynonenal (HNE)⁷ represent a group of chemicals that produce DNA interstrand cross-links. They are produced from the incomplete combustion of organic matter such as fuels, wood, and tobacco;^{8,9} enals are also produced endogenously as a byproduct of lipid peroxidation.^{10–17}

Enals are mutagenic.^{18–22} They are bis-electrophiles and undergo sequential reaction with two nucleophilic groups of DNA bases. Reaction of deoxyguanosine with enals produce exocyclic 1,*N*²-deoxyguanosine adduct **1** through an initial Michael addition.^{23–27} When placed opposite deoxycytosine in duplex DNA, these exocyclic 1,*N*²-dG adducts undergo ring-opening to *N*²-(3-oxopropyl) adduct **2**, unveiling a reactive aldehyde functionality; it exists in equilibrium with the corresponding aldehydols **3** or related species (Scheme 1).^{28–31} We have demonstrated that the *N*²-amino group of a neighboring deoxyguanosine on the complementary strand can react with the ring-opened aldehyde to form an interstrand *N*²-dG:*N*²-dG cross-links.^{4–7,32} The formation of the DNA interstrand cross-links by the exocyclic 1,*N*²-deoxyguanosine adducts is sequence dependent, forming in a local 5'-CpG-3' sequence context but not in the 5'-GpC-3' sequence context.⁵ This is attributed to the longer distance between the *N*²-dG atoms in the 3'-direction than in the 5'-direction. The formation of a 3-carbon tether in the 3'-direction greatly perturbs the cross-linked base pairs and destabilizes the DNA duplex.^{33,34} The formation of interstrand cross-links is also influenced by the stereochemistry of the exocyclic 1,*N*²-dG adducts derived from enal with longer carbon chains.^{5,7} The crotonaldehyde derived 1,*N*²-deoxyguanosine adduct of (6*R*) configuration induces interstrand cross-linking more efficiently than does the (6*S*) stereoisomer.⁵ Structure and modeling studies reveal that the orientations of the aldehyde group in the ring-opened (*R*)- and (*S*)-crotonaldehyde derived 1,*N*²-dG adducts are different.²⁸ Steric interactions between the methyl group of the cross-link of the (6*S*) stereoisomer with the neighboring base pairs result in perturbations of the DNA duplex;^{28,35} these unfavorable steric interactions are not present in the (6*R*) crosslink. Similar considerations apply to the HNE-derived 1,*N*²-dG adducts,³⁶ of which only the (6*S*,8*R*,11*S*) configuration forms interstrand cross-links in the 5'-CpG-3' sequence.⁷

Possible structures for the DNA interstrand cross-links include carbinolamine **4**, imine **5**, and the ring-closed pyrimidopurinone **6**, or possibly an equilibrium mixture of all three (Scheme 1). Pyrimidopurinone **6** is observed upon enzymatic digestion of the cross-linked DNA duplex^{5,7} but is undetectable in the duplex oligodeoxynucleotides.^{28,29,37} Although the existence of imine **5** in duplex DNA is surmised by the observation that the interstrand cross-link can be reduced by NaCNBH₃,^{5,7} the interstrand cross-links involving the acrolein and crotonaldehyde derived 1,*N*²-dG adducts exist primarily as diastereomeric γ -hydroxytrimethylene (carbinolamine **4**) tethers at *N*²-dG:*N*²-dG positions (Scheme 2).^{28,29,37} Both imine **2** or pyrimidopurinone **3** are predicted to perturb the Watson-Crick hydrogen bonding and stacking of the cross-linked base pairs, whereas the formation of the carbinolamine linkage has a minimum impact.^{28,29}

While structures of reduced models of these cross-links, in which a trimethylene chain links the *N*²-positions have been reported,^{33,34} the native diastereomeric carbinolamine cross-links have not been examined. The observation of thermally stable carbinolamine interstrand cross-links in duplex DNA in aqueous solution^{28,29} suggests that additional interactions might stabilize the linkage. Presently, we show that the acrolein-derived diastereomeric (*R*)- γ -

hydroxytrimethylene N^2 -dG: N^2 -dG interstrand DNA cross-link is favored in 5'-d ($G^1C^2T^3A^4G^5C^6X^7A^8G^9T^{10}C^{11}C^{12}$)-3'•5'-d ($G^{13}G^{14}A^{15}C^{16}T^{17}C^{18}Y^{19}C^{20}T^{21}A^{22}G^{23}C^{24}$)-3' (X^7 is the dG adjacent to the α -carbon of the carbinolamine linkage and Y^{19} is the dG adjacent to the γ -carbon of the carbinolamine linkage; the cross-link is in the 5'-CpG-3' sequence). The structure has been characterized using isotope-edited ^{15}N NOESY-HSQC NMR. Analyses of NOE intensities involving the ^{15}N -labeled $Y^{19} N^2H$ indicate that the (*R*)- γ -hydroxytrimethylene linkage is the major interstrand cross-link species, constituting 80–90% of the total cross-link. Structural refinement using NOE distance restraints obtained from isotope-edited ^{15}N NOESY HSQC data indicates that the (*R*)- γ -hydroxytrimethylene linkage maintains the $C^6 \bullet Y^{19}$ and $X^7 \bullet C^{18}$ base pairs with minimal structural perturbations. The $X^7 N^2$ and $Y^{19} N^2$ atoms are in the gauche-conformation with respect to the linkage, which maintains Watson-Crick hydrogen bonding of the cross-linked base pairs. The anti conformation of the hydroxyl group with respect to C^α of the tether minimizes steric interactions and allows the formation of a hydrogen bond between the hydroxyl group and $C^{20} O^2$ located in the 5'-neighboring base pair $G^5 \bullet C^{20}$. The formation of this hydrogen bond may, in part, explain the thermal stability of this carbinolamine interstrand cross-link, and the stereochemical preference for the (*R*)-configuration of the cross-link.

Results

Stereochemistry of the Carbinolamine Cross-Link

The carbinolamine interstrand cross-links of the acrolein adduct of dG were prepared as reported,⁵ and occurred as a mixture of (*R*) and (*S*) diastereomers at the carbinolamine carbon. An expansion of ^{15}N HSQC filtered NOESY spectrum of the $Y^{19} N^2$ ^{15}N -labeled interstrand cross-link is shown in Figure 1A. For the major diastereomeric cross-linked species, a complete set of NOEs between $Y^{19} N^2H$ and the γ -hydroxymethylene tether was observed. The $Y^{19} N^2H \rightarrow H^\gamma$ NOE was weaker than the $Y^{19} N^2H \rightarrow H^{\beta 2}$ NOE and only slightly stronger than the two diastereotopic $Y^{19} N^2H \rightarrow H^\alpha$ NOEs. This was consistent with modeling, in which the (*R*)- γ -hydroxytrimethylene tether placed $Y^{19} N^2H$ and H^γ at a distance of 2.9–3.1 Å. In contrast, the modeling suggested that the (*S*)- γ -hydroxytrimethylene tether placed $Y^{19} N^2H$ and H^γ at a closer distance of 2.2–2.4 Å, which would have resulted in a stronger NOE. In addition, a weak $Y^{19} H1' \rightarrow H^\gamma$ NOE was observed (Figure 1B), suggesting H^γ was oriented toward Y^{19} . These data indicated that the (*R*)- γ -hydroxytrimethylene linkage (Scheme 2) was the major interstrand cross-link species. The relative peak intensities in the ^{15}N HSQC spectrum suggested that the (*R*)- γ -hydroxytrimethylene cross-link constituted 80–90% of the overall interstrand cross-links.

Spectral Assignments of the (*R*)- γ -Hydroxytrimethylene Cross-Link

By comparison of the non-isotope-edited NOESY spectrum with the ^{15}N NOESY-HSQC spectrum of the $X^7 N^2$ ^{15}N -labeled interstrand cross-link, the G^5 , X^7 , T^{17} , and Y^{19} imino protons were assigned (Figure 2). The $G^5 N1H \rightarrow C^{20} N^4Hs$, $X^7 N1H \rightarrow C^{18} N^4Hs$, $T^{17} N3H \rightarrow A^8 H2$, and $Y^{19} N1H \rightarrow C^6 N^4Hs$ NOE interactions arising from Watson-Crick hydrogen bonding were observed, indicating the Watson-Crick hydrogen bonds at the cross-linked base pairs were not significantly perturbed.

The base aromatic protons and the deoxyribose $H1'$ protons were assigned by comparing non-isotopically edited NOESY spectra of the modified duplex before and after cross-linking (Figure 3). For the freshly prepared duplex, aldehydrol **3** was the predominant species present in neutral solution.^{29,30} The major NOE cross peaks in Figures 3A and 3B, which were sequentially connected,^{38,39} were therefore assigned to the correlations of aromatic $H6/H8$ protons with $H1'$ protons of aldehydrol **3**. The sample was then incubated for a week at 37 °C, during which time the interstrand cross-link **4** formed, and was eventually present at 50% of

the duplex^{5,29} with the (*R*)- γ -hydroxytrimethylene predominant. Figures 3C and 3D display the same region of the NOESY spectrum of the cross-linked sample. Except for the nucleotides close to the ends of the duplex, two sets of NOEs were sequentially assigned. The first set of NOEs were the same as those in Figures 3A and 3B (not demonstrated in Figures 3C and 3D) and arose from aldehydrol **3**, whereas the second set of NOEs were assigned to the (*R*)- γ -hydroxytrimethylene of cross-link **4**. These NOEs arising from cross-link **4** were sequentially connected with interruptions from C⁶ to X⁷ and from C¹⁸ to Y¹⁹ (Figures 3C and 3D), indicating the (*R*)- γ -hydroxytrimethylene interstrand cross-link maintained a B-DNA geometry. The X⁷ H8→C⁶ H1' and Y¹⁹ H8→C¹⁸ H1' correlations were missing and the A⁸ H8→X⁷ H1' interaction was stronger than corresponding others, indicating structural perturbation at the cross-linked base pairs. The assignments were supported by other NOEs such as H6/H8→H3', H6/H8→H2'(*'*), H1'→H3', and H1'→H2'(*'*) *etc.*

Stabilities of the Base Pairs for the (*R*)- γ -Hydroxytrimethylene Interstrand Cross-link

The resonances of pyrimidine N3H and purine N1H imino protons at different temperatures are shown in Figure 4. The melting temperature (T_m) of the DNA interstrand cross-links was 91 °C in 1.0 M NaCl buffer.⁵ The X⁷ and Y¹⁹ imino resonances were still observable at 65 °C, although starting to broaden, indicating the cross-linked base pairs were stabilized; this observation is consistent with a high melting structure. For the 5'-neighbor base pair G⁵•C²⁰, the G⁵ imino resonance remained sharp at 55 °C, but was broadened at 65 °C. At the 3'-neighbor A⁸•T¹⁷ base pair, the T¹⁷ imino resonance was severely broadened at 55 °C. For the corresponding unmodified duplex these imino resonances were all broadened at 40 °C.

Chemical Shift Perturbations of the (*R*)- γ -Hydroxytrimethylene Cross-Link

The chemical shifts were compared with the corresponding unmodified duplex (Figure 5). Small chemical shift perturbations were observed for most of the nucleotides. The X⁷•C¹⁸ base pair was minimally perturbed, and the C¹⁸ H6 and C¹⁸ H1' resonances shifted upfield 0.11 and 0.32 ppm, respectively. The chemical shift perturbations for C⁶•Y¹⁹ base pair were not remarkable. The C⁶ H6, C⁶ H1', Y¹⁹ H8, and Y¹⁹ H1' resonances shifted -0.03, -0.13, 0.06, and 0.09 ppm, respectively. Significantly, the 5'-neighboring C²⁰ H6 had the most significant perturbation among the aromatic protons, 0.18 ppm downfield from the related proton in the unmodified sequence. C²⁰ H5 and C²⁰ H1' also shifted downfield, 0.22 and 0.13 ppm, respectively, indicating the neighboring C²⁰ was involved in the cross-link formation.

The backbone conformation was evaluated by the distribution of phosphorus resonances. The assignments of the C⁶ and C¹⁸ H3' protons of the cross-link were based upon their H1'→H3' and H6→H3' NOEs, respectively. They exhibited ³¹P→H3' correlations in the HMBC spectrum. Compared with other nucleotides, the C⁶ and C¹⁸ phosphates were shifted downfield, indicating that the backbone at the cross-linked base pairs was perturbed from ideal B-DNA.

Structural Refinement of the (*R*)- γ -hydroxytrimethylene Cross-Link

The cross-link was simulated in explicit water at 300 K. The nineteen NOE cross peaks in the ¹⁵N NOESY-HSQC spectra assigned to X⁷ N²H and Y¹⁹ N²H were converted to distance restraints to refine the cross-link tether. In addition, 52 empirical distance restraints and 200 empirical torsion angle restraints were used to maintain Watson-Crick hydrogen bonding and B-type DNA, respectively.

A fully solvated molecular dynamics simulation of 8 ns was carried out starting from both A- and B-type DNAs. The all-atom mass-weighted root-mean-square deviations (RMSDs) referenced to the starting structures were used to determine the time for the simulations to reach equilibrium, and in turn, the range of the trajectories to be used for the sequence analysis. The

simulation equilibrated within 500–600 ps starting from A-type DNA, and 200–300 ps starting from B-type DNA.

The rMD trajectories after simulating for 1 ns were used to analyze the potential hydrogen bonding of the carbinol hydroxyl group of the tether. Its occupancy by hydrogen bond acceptors in the rMD trajectories revealed that it was likely to form a hydrogen bond in solution. Starting from both A- and B-type DNA, the simulations predicted the same hydrogen bond trends (Table 1). The carbinol hydroxyl group did not form a hydrogen bond with the solvent throughout the simulations. The duplex tended to adopt a conformation in which the hydroxyl group formed a hydrogen bond with C²⁰ O², although the potential for a hydrogen bond to C²⁰ O4' was also observed. No hydrogen bonds with C⁶ O² or Y¹⁹ N3 were formed.

The trajectories of the molecular dynamics simulations from 1–8 ns starting from A- and B-type DNAs were averaged. The RMSD value of these two refined structures was 0.54 Å, reflecting the similarities of the converged structures. The atom distances agreed with the NOE intensities (Table 1) and suggested that the refined structures obtained from the molecular dynamics simulations were accurate. Significantly, for the refined structures, the Y¹⁹ N²H→H^γ distance was 3.0 Å, which corroborated the observation of a weak NOE between Y¹⁹ N²H and H^γ. The NOEs in Figures 3C and 3D were not used as the distance restraints. The distances of X⁷ H8→C⁶ H1', A⁸ H8→X⁷ H1' and Y¹⁹ H8→C¹⁸ H1' in the average structure were 6.0, 2.9, and 5.5 Å, respectively. Consistently, the X⁷ H8→C⁶ H1' and Y¹⁹ H8→C¹⁸ H1' NOEs were missing and A⁸ H8→X⁷ H1' NOE were stronger than other NOEs of aromatic protons with the 5'-neighbor H1' protons (Figures 3C and 3D). Other isolated NOEs of the cross-link in Figures 3C and 3D also agreed with the average structure. These indicated that the average structure provided an accurate depiction of the NMR data.

Refined Structure of the (*R*)- γ -hydroxytrimethylene Cross-Link

Starting from either A- or B-type DNA, the cross-link converged to B-type with the tether located in the minor groove. Figure 6A provides an expanded view of the average structure observed from the minor groove. Consistent with Y¹⁹ N1H→C⁶ N⁴Hs and X⁷ N1H→C¹⁸ N⁴Hs NOEs (Figure 2), the C⁶•Y¹⁹ and X⁷•C¹⁸ base pairs conserved Watson-Crick hydrogen bonding with small distortions. They were closer to each other as compared to other base pairs. The backbone torsion angles of C⁶, X⁷, C¹⁸, and Y¹⁹ were not ideal B-form geometry and agreed with the downfield shifts of C⁶ and C¹⁸ phosphates.

The (*R*)- γ -hydroxytrimethylene tether was located in the minor groove (Figure 6A). The β -carbon oriented towards C⁶ and X⁷. Figure 7 provides Newman projections of the tether viewed along the C ^{α} -C ^{β} and C ^{β} -C ^{γ} bonds. Both X⁷ N² and Y¹⁹ N² were in the gauche-conformation with respect to the (*R*)- γ -hydroxytrimethylene tether, which facilitated the Watson-Crick hydrogen bonding of the C⁶•Y¹⁹ and X⁷•C¹⁸ base pairs. The trans-configuration of the hydroxyl group with C ^{α} minimized their steric interaction.

As shown in Figure 6A, the hydroxyl group of the (*R*)- γ -hydroxytrimethylene tether oriented towards C²⁰ O² and formed a hydrogen bond. The distance and angle of the carbinol OH→C²⁰ O² in the average structure were 2.1 Å and 177°, respectively (Table 2). No hydrogen bond interaction existed between the hydroxyl group and C⁶ O², Y¹⁹ N3, or C²⁰ O4'. The orientation of the hydroxyl group towards C²⁰ O² agreed with its occupancy by the hydrogen bond acceptors in the trajectory analysis (Table 1). The hydrogen bond was also consistent with the NMR data. The G⁵•C²⁰ base pair was stabilized by the cross-link (Figure 4). Chemical shift perturbations of C²⁰ H5, H6, and H1' were observed (Figure 5). In contrast, chemical shifts of the corresponding cytosine H5, H6, and H1' protons in the interstrand cross-links bridged by either trimethylene³⁴ or α -methyltrimethylene tether,³⁵ in which a hydrogen bond is not possible, underwent small perturbations.

Molecular Dynamics Simulations of the (*S*)- γ -Hydroxytrimethylene Tether

It was not possible to obtain NOE-based distance restraints for the (*S*)- γ -hydroxytrimethylene tether, which was present in solution at only low levels (Figure 1). Consequently, to explore the conformational possibilities for this stereoisomeric of the cross-link, non-restrained molecular dynamics simulations were carried out in explicit solvent, starting from either A- or B-DNAs, respectively. Figures 6B and 6C show expanded views of the refined structures starting from A- and B-type DNAs, respectively. In both cases, the (*S*)- γ -hydroxytrimethylene tether converged to similar structures, but which were different from the refined structures of the (*R*)- γ -hydroxymethylene tether. For the (*S*)- γ -hydroxytrimethylene tether, the calculations predicted that the carbinol hydroxyl might form a hydrogen bond with Y¹⁹ N3 (Figure 6B) or a 3-point hydrogen bond with Y¹⁹ N3 and C²⁰ O4' (Figure 6C). The distance for the Y¹⁹ N²H to the H ^{γ} for the (*S*)- γ -hydroxytrimethylene tether, was 2.2 or 2.3 Å, respectively.

Discussion

Interstrand DNA cross-links block DNA replication and transcription, leading to cytotoxic and mutagenic effects if not repaired.^{1,2} A DNA interstrand cross-link that covalently connects both strands represents a challenge for the cell and an elaborate mechanism is required for its repair.³ The DNA interstrand cross-link induced by the exocyclic 1,*N*²-dG adduct **1** exists as an equilibrium mixture of carbinolamine **4**, imine **5**, and pyrimidopurinone **6**. We have demonstrated that carbinolamine **4** is the only observable cross-linked species in duplex DNA.²⁹ Imine **5** and pyrimidopurinone **6** are predicted to cause significant perturbations to the DNA duplex and are thereby disfavored. Carbinolamine **4** might be anticipated to be readily reversible,^{40–42} yet it stabilizes the DNA duplex with respect to thermal denaturation ($T_m > 90^\circ \text{C}$); thus, the forces involved in stabilizing these stereoisomeric carbinolamine interstrand cross-links are of interest.

(*R*)- γ -Hydroxytrimethylene Linkage as the Major Interstrand Cross-Link

The weak Y¹⁹ N²H \rightarrow H ^{γ} and Y¹⁹ H1' \rightarrow H ^{γ} NOE interactions (Figure 1) suggest that the (*R*)- γ -hydroxytrimethylene linkage is the major form of the interstrand cross-link. The (*R*)- γ -hydroxytrimethylene interstrand cross-link conserves B-type DNA geometry. The (*R*)- γ -hydroxytrimethylene tether has a minor groove orientation and adopts the U-conformation.³⁴ Watson-Crick hydrogen bonding of the cross-linked C⁶•Y¹⁹ and X⁷•C¹⁸ base pairs is conserved (Figure 6A). X⁷ N² and Y¹⁹ N² are in the gauche-orientation with respect to the α - or γ -carbons, respectively, in the extended chain conformation (Figure 7). The C⁶•Y¹⁹ and X⁷•C¹⁸ base pairs are placed closer as compared to others.

Role of Hydrogen Bonding in Interstrand Cross-Link Formation

The orientation of the (*R*)- γ -hydroxytrimethylene tether towards C⁶ and X⁷ minimizes the steric interaction of the bulky substituents (Figure 7) and more importantly, facilitates the formation of a hydrogen bond between the hydroxyl group and C²⁰ O² (Figure 6A), such as has been observed for the active sites of type I aldolases in which carbinolamine is stabilized in the active site.^{43–46} Thermal denaturation of the DNA duplex results in the loss of this hydrogen bond, allowing the (*R*)- γ -hydroxytrimethylene tether to quickly dissociate.⁵ The formation of this hydrogen bond to C²⁰ O² augments the Watson-Crick hydrogen bonding (Figure 4) and base stacking of the cross-linked base pairs.

The presence of this hydrogen bond to C²⁰ O² may explain the favored (*R*) stereochemistry of the carbinolamine linkage. These calculations suggested that for the (*S*) stereochemistry, the hydroxyl group might form a hydrogen bond with Y¹⁹ N3 (Figure 6B) or a 3-point hydrogen bond with Y¹⁹ N3 and C²⁰ O4' (Figure 6C), neither of which would be expected to stabilize the duplex in the same manner as the cross-link to C²⁰ O². Overall, we suggest that formation

of the carbinolamine linkage favors the (*R*) stereochemistry because of the favorable energetics associated with hydrogen bonding to $C^{20} O^2$.

Comparison to Reduced Trimethylene Cross-link Structures

Dooley et al.^{33,34} cross-linked DNA duplexes with a trimethylene tether to simulate the DNA interstrand cross-links induced by the malondialdehyde and acrolein derived 1,*N*²-dG adducts. In contrast to the present results, they concluded that the reduced interstrand cross-link badly perturbed the Watson-Crick hydrogen bonding of the cross-linked base pairs.³⁴ In their structures, the trimethylene tether adopted an extended chain *W*-conformation and was accommodated by unwinding of the duplex at the cross-linking base pairs to produce a bulge. In the extended chain conformation of the tether, the exocyclic nitrogens of each deoxyguanosine were in the anti-orientation with respect to the α - or γ -carbons of the reduced tether, respectively.

Crotonaldehyde-Derived DNA Interstrand Cross-links

The α -methyl group of the crotonaldehyde lesion can assume either the *R* or *S* configuration. In this 5'-CpG-3' sequence the crotonaldehyde derived 1,*N*²-dG adduct with the *R* configuration of the α -methyl group induces DNA interstrand cross-links more efficiently than does the adduct with the *S*-configuration of the α -methyl group. The *R* configuration of the α -methyl group orients the reactive aldehyde proximal to the *N*²-dG cross-linking site in the complementary strand, and, moreover, the resulting carbinolamine cross-link results in less structural perturbation to the DNA duplex.^{28,35}

The stereochemistry of the carbinolamine carbon of the native carbinolamine cross-links is not available. The orientations of the stereoisomeric α -methyltrimethylene tethers of the reduced analogs are different.³⁵ For both, the steric interaction of the methyl group was minimized by the anti-conformation of the methyl group with respect to C^γ . The orientation of the (*S*)- α -methyltrimethylene tether also reduced the steric interference of the α -methyl group with the neighbor A⁸•T¹⁷ base pair. Extrapolating this structural information to the native cross-link suggests that the orientation of the (*R*)-stereochemistry of the carbinolamine would not be positioned to form the same hydrogen bond with $C^{20} O^2$ at the 5'-neighbor base pair as presently observed for the acrolein-derived 1,*N*²-dG exocyclic adduct.³⁵ Instead, formation of a hydrogen bond to $C^{20} O^2$ by the (*R*)- γ -hydroxytrimethylene linkage arising from the crotonaldehyde-derived *R*- α -methyltrimethylene tether would require re-orientation of the α -CH₃ group and C^γ into the gauche-conformation, which would be predicted to be less stable than the conformation of the (*R*)- γ -hydroxytrimethylene tether induced by acrolein derived 1,*N*²-dG adduct. This might explain, in part, why the crotonaldehyde-derived 1,*N*²-dG lesion having the *R* configuration of the α -methyl group ultimately produces less of the interstrand cross-links in the 5'-CpG-3' sequence than does the acrolein-derived 1,*N*²-dG adduct.⁵

Summary

When placed opposite deoxycytosine in the 5'-CpG-3'duplex, the acrolein-derived exocyclic 1,*N*²-dG adduct induces stereoisomeric carbinolamine interstrand cross-links. The *R*-configuration of the carbinolamine linkage is favored. The formation of a hydrogen bond between the hydroxyl group and $C^{20} O^2$ located in the 5'-neighboring base pair G⁵•C²⁰, may in part explain the stereochemical preference for the formation of the (*R*)- γ -hydroxytrimethylene linkage and its thermal stability in duplex DNA. The cross-link causes minimal structural perturbations to the DNA duplex, and allows maintenance of Watson-Crick hydrogen bonding and base stacking at the tandem cross-linked base pairs.

Experimental Section

Materials

The oligodeoxynucleotide 5'-d(GGACTCGCTAGC)-3' was synthesized and purified by anion-exchange chromatography by the Midland Certified Reagent Co. (Midland, TX). The G¹⁹ N² ¹⁵N-labeled 5'-d(GGACTCGCTAGC)-3' and the oligodeoxynucleotides containing the acrolein-derived exocyclic 1,N²-dG adduct in the dodecamer 5'-d(GCTAGCXAGTCC)-3' (X=1,N²-dG adduct **1**, whether N² ¹⁵N-labeled or not), were synthesized, purified, and characterized as reported.^{29,47} The purities of the oligodeoxynucleotides were assessed by capillary gel electrophoresis, HPLC, and MALDI-TOF mass spectroscopy. Oligodeoxynucleotides were desalted by chromatography on Sephadex G-25. The stereoisomeric carbinolamine interstrand cross-links were prepared at pH 7.0 at 37°C, as reported.⁵ Reaction was monitored by ¹⁵N HSQC experiments.

NMR

Samples for the NMR experiments were at 1.0 mM strand concentration. Samples for the non-exchangeable protons were dissolved in 280 μ L of a buffer containing 10 mM NaH₂PO₄, 100 mM NaCl, 50 μ M Na₂EDTA (pH 7.0). They were exchanged with D₂O and suspended in 280 μ L 99.996% D₂O. The pH was adjusted to 7.0 with dilute DCl or NaOD solutions. Samples for the observation of exchangeable protons were dissolved in 280 μ L of 10 mM NaH₂PO₄, 100 mM NaCl, 50 μ M EDTA, (pH 7.0) containing 9:1 H₂O/D₂O (v/v) and the pH was adjusted to 7.0. ³¹P experiments were carried out on a Bruker Avance 600 spectrometer. Other NMR experiments were performed on a Bruker Avance 500 spectrometer with a cryogenic probe (Bruker Instruments). The temperature was 25 °C for observation of the non-exchangeable protons and 5 °C for observation of the exchangeable protons. Chemical shifts for ¹H were referenced to water, and chemical shifts for ¹³C, ¹⁵N and ³¹P were referenced indirectly. Data were processed using FELIX 2000 (Accelrys Inc.). For all NMR experiments, a relaxation delay of 1.5 s was used. Two-dimensional homonuclear NMR spectra were recorded with 512 real data in the t₂ dimension and 2048 real data in the t₁ dimension. NOESY spectra were zero-filled during processing to create a matrix of 1024 \times 1024 real points, other spectra were zero-filled to create a matrix of 1024 \times 512 real points. For assignment of exchangeable protons, NOESY experiments used the WATERGATE sequence.⁴⁸ The mixing time was 250 ms. For assignment of non-exchangeable protons and the derivation of distance restraints, NOESY experiments used TPPI quadrature detection and a mixing time of 250 ms were used. 2D ¹⁵N NOESY-HSQC pulse programs were modified from corresponding 3D pulse program^{49,50} with 128 points centered at 81ppm. The water signal was suppressed with the WATERGATE method. The mixing time was 250 ms. The spectrum was recorded with 512 real data in the t₂ dimension and 2048 real data in the t₁ dimension and was zero-filled during process to create 1024 \times 512 matrix.

Experimental Restraints

NOE-derived distance restraints used for the refinement of the (*R*)- γ -hydroxytrimethylene structure were calculated from NOE cross-peak volumes in the ¹⁵N NOESY-HSQC spectra using MARDIGRAS v3.2.^{51,52} Isotropic correlation times 2, 3 and 4 ns were used. The relaxation time for the analyses was 1.5 s. The volume error was one-half the volume of the smallest peak. Volumes were normalized based on all the peak intensities. The RANDMARDI algorithm carried out 50 iterations for each set of data, randomizing peak volumes within limits specified by the input noise level.⁵³ The distances were averaged. The standard deviation in a particular distance served as the error bond for the distance. Empirical restraints preserving Watson-Crick hydrogen bonding and preventing propeller twisting between basepairs were used.⁵⁴ The backbone and sugar pucker torsion angle restraints were also derived using empirical data derived from B-DNA.⁵⁵

Structural Refinement of the (R)- γ -Hydroxytrimethylene Cross-Link

Molecular dynamics simulations used the AMBER force field (Vol. 9, parm99 force field).⁵⁶ Partial charges of the carbinolamine linkage were obtained by DFT calculation at B3LYP/6-31G* level with Gaussian 03.⁵⁷ Starting structures were constructed from A- and B-type DNA using Insight II. The duplexes were energy minimized by conjugate gradients for 1,000 iterations without experimental and empirical restraints. Molecular dynamics simulations were carried out in explicit water using the particle mesh Ewald (PME) method. DNA duplexes were placed in a 10 Å cubic TIP3P water box. 22 Na⁺ ions were added to neutralize the DNA interstrand cross-link. The cutoff radius for nonbonding interactions was 10 Å. The restraint energy function contained terms describing distance and torsion angle restraints, both in the form of square well potentials. Bond lengths involving hydrogens were fixed with the SHAKE algorithm.⁵⁸

Firstly, a 1,000-step energy minimization was performed with an integrator time of 1 fs with the heavy atoms of the cross-link restrained. A 10,000-iteration simulation was carried out with constant volume at 300 K. The heavy atoms of the cross-link were restrained. Then, molecular dynamics simulation at constant pressure was performed at 300 K for 8 ns with an integrator time of 1 fs. Experimental and empirical distance and torsion angle restraints with force constants of 32.0 kcal·mol⁻¹·Å⁻¹ were applied. One trajectory was saved in each 1,000-iteration during simulation. The trajectories after reaching simulation equilibrium were averaged. The mean structures were filtered out from water box and energy-minimized for 250 iterations without experimental and empirical restraints.

The PTRAJ program from AMBER 9 package was used to analyze the trajectories. The RMSD values of the trajectories were referenced to the starting structures. A distance of less than 3.5 Å, and an angle of greater than 120° between the potential hydrogen donor and acceptor, were used as criteria for hydrogen bond formation. The backbone conformation of the average structure was analyzed with the program 3DNA.⁵⁹

Structural Refinement of the (S)- γ -Hydroxytrimethylene Cross-Link

Similar molecular dynamics simulations were performed to refine the structure of the (S)- γ -hydroxytrimethylene cross-link. Because this was the minor species in solution, it was not possible to obtain experimental NOEs, and thus molecular dynamics simulations were carried out without experimental distance restraints.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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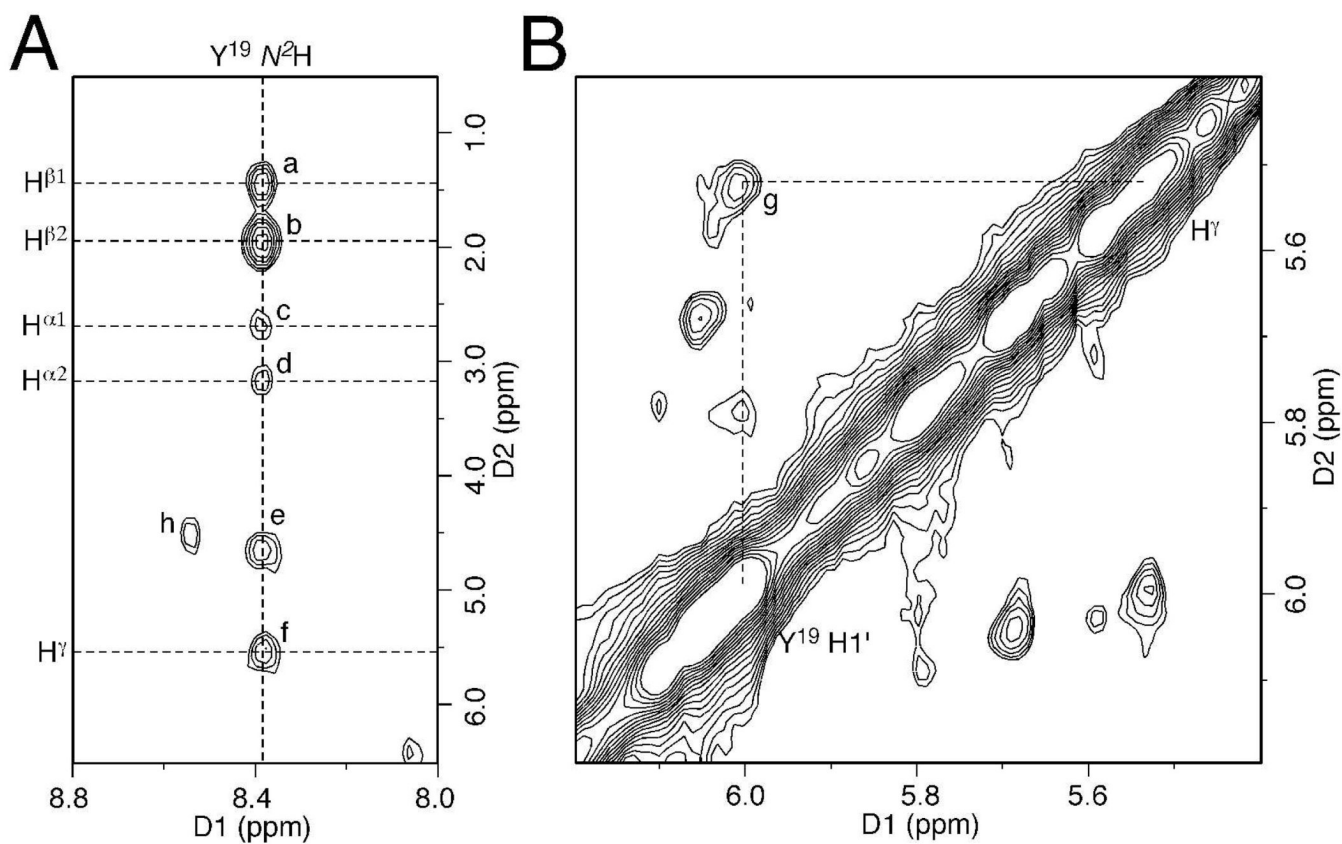


Figure 1. NOE cross peaks to identify the stereochemistry of the DNA interstrand cross-link. (A) ^{15}N NOESY-HSQC of the $\text{Y}^{19} \text{N}^2$ ^{15}N -labeled sample. (B) Non-isotope-edited NOESY. NOEs for the major diastereomer of the cross-link were assigned as: (a) $\text{Y}^{19} \text{N}^2\text{H} \rightarrow \text{H}^{\beta 1}$; (b) $\text{Y}^{19} \text{N}^2\text{H} \rightarrow \text{H}^{\beta 2}$; (c) $\text{Y}^{19} \text{N}^2\text{H} \rightarrow \text{H}^{\alpha 1}$; (d) $\text{Y}^{19} \text{N}^2\text{H} \rightarrow \text{H}^{\alpha 2}$; (e) water exchange peak of $\text{Y}^{19} \text{N}^2\text{H}$; (f) $\text{Y}^{19} \text{N}^2\text{H} \rightarrow \text{H}^{\gamma}$; (g) $\text{Y}^{19} \text{H}1' \rightarrow \text{H}^{\gamma}$; (h), $\text{Y}^{19} \text{N}^2\text{H} \rightarrow \text{H}^{\gamma}$ of the minor diastereomer.

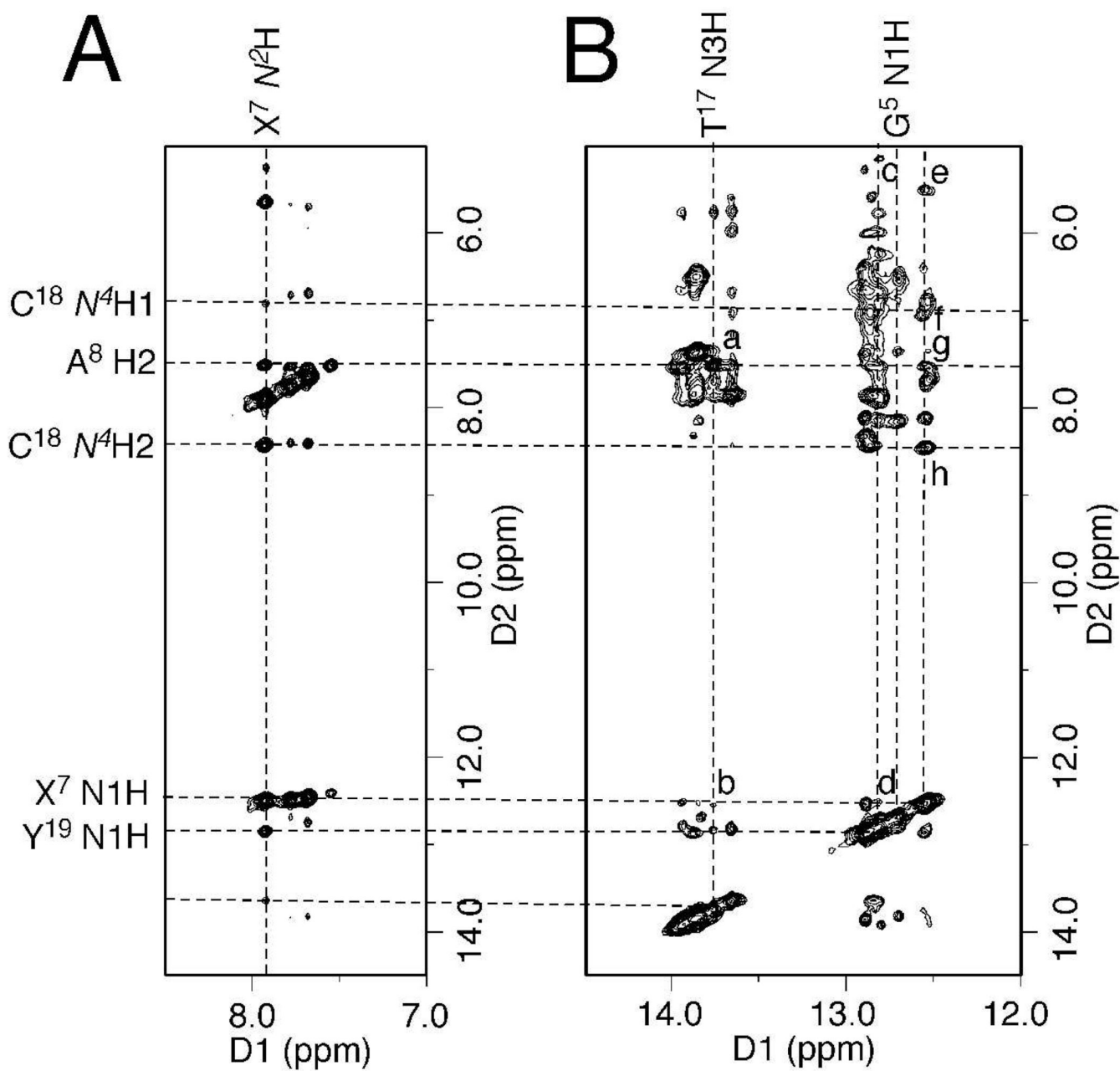


Figure 2.

Expansions of NOESY spectra indicating G⁵•C²⁰, C⁶•Y¹⁹, X⁷•C¹⁸ and A⁸•T¹⁷ base pairs of the (*R*)- γ -hydroxytrimethylene cross-link maintained Watson-Crick base pairing. (A) ¹⁵N NOESY-HSQC of the X⁷ N² ¹⁵N-labeled sample. (B) The non-isotopically edited NOESY spectrum. NOEs were assigned as: (a) T¹⁷ N3H→A⁸ H2; (b) T¹⁷ N3H→X⁷ N1H; (c) Y¹⁹ N1H→C⁶ H5; (d) Y¹⁹ N1H→X⁷ N1H; (e) X⁷ N1H→C¹⁸ H5; (f) X⁷ N1H→C¹⁸ N⁴H1; (g) X⁷ N1H→A⁸ H2; (h) X⁷ N1H→C¹⁸ N⁴H2.

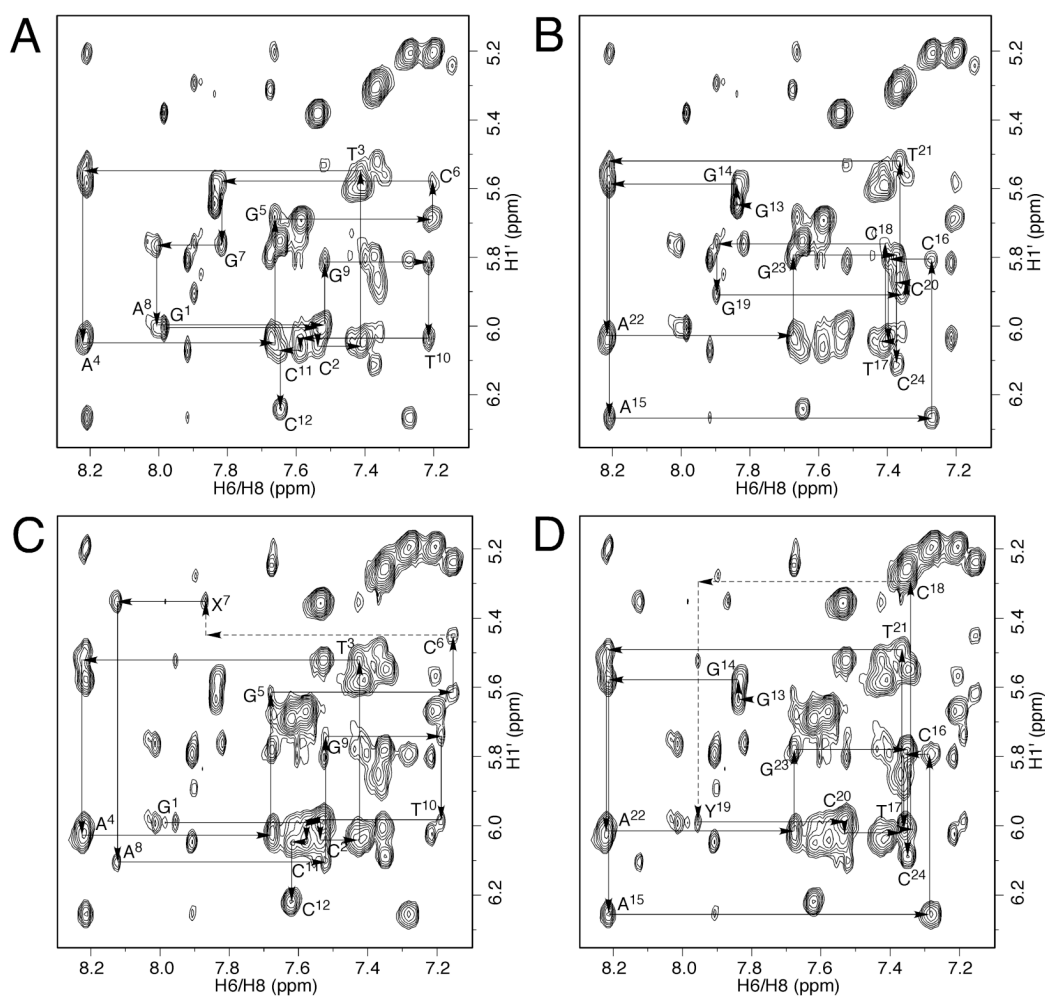


Figure 3.

NOESY spectra of the duplex containing the acrolein-derived 1,*N*²-dG exocyclic adduct and the resulting interstrand cross-links. (A, B) Duplex before cross-linking. The major NOEs, which were sequentially connected by solid lines, were assigned to the aldehyde **3**. (C, D) Duplex after cross-linking. Two sets of NOE connectivities were assigned. One set arose from aldehyde **3**, the other was assigned to (*R*)-γ-hydroxytrimethylene cross-link **4**. The NOEs of the (*R*)-γ-hydroxytrimethylene cross-link **4** were sequentially connected (solid and dashed lines). The X⁷ H8→C⁶ H1' and Y¹⁹ H8→C¹⁸ H1' NOEs were missing and the A⁸ H8→X⁷ H1' correlation was stronger than corresponding others.

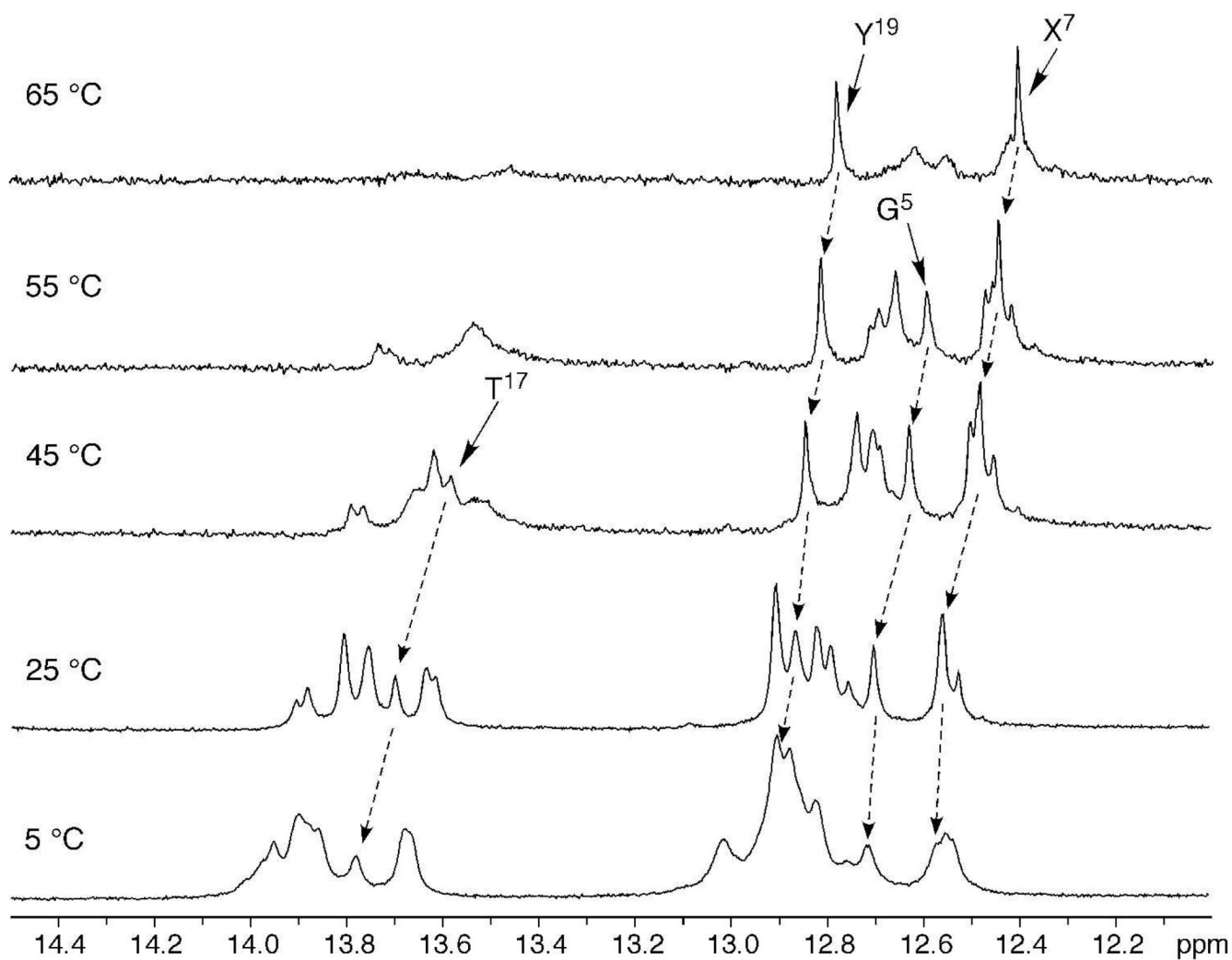


Figure 4. NMR melting studies of the (*R*)- γ -hydroxytrimethylene interstrand cross-link showing basepairs G⁵•C²⁰, C⁶•Y¹⁹, and X⁷•C¹⁸ at the cross-linked region were stabilized.

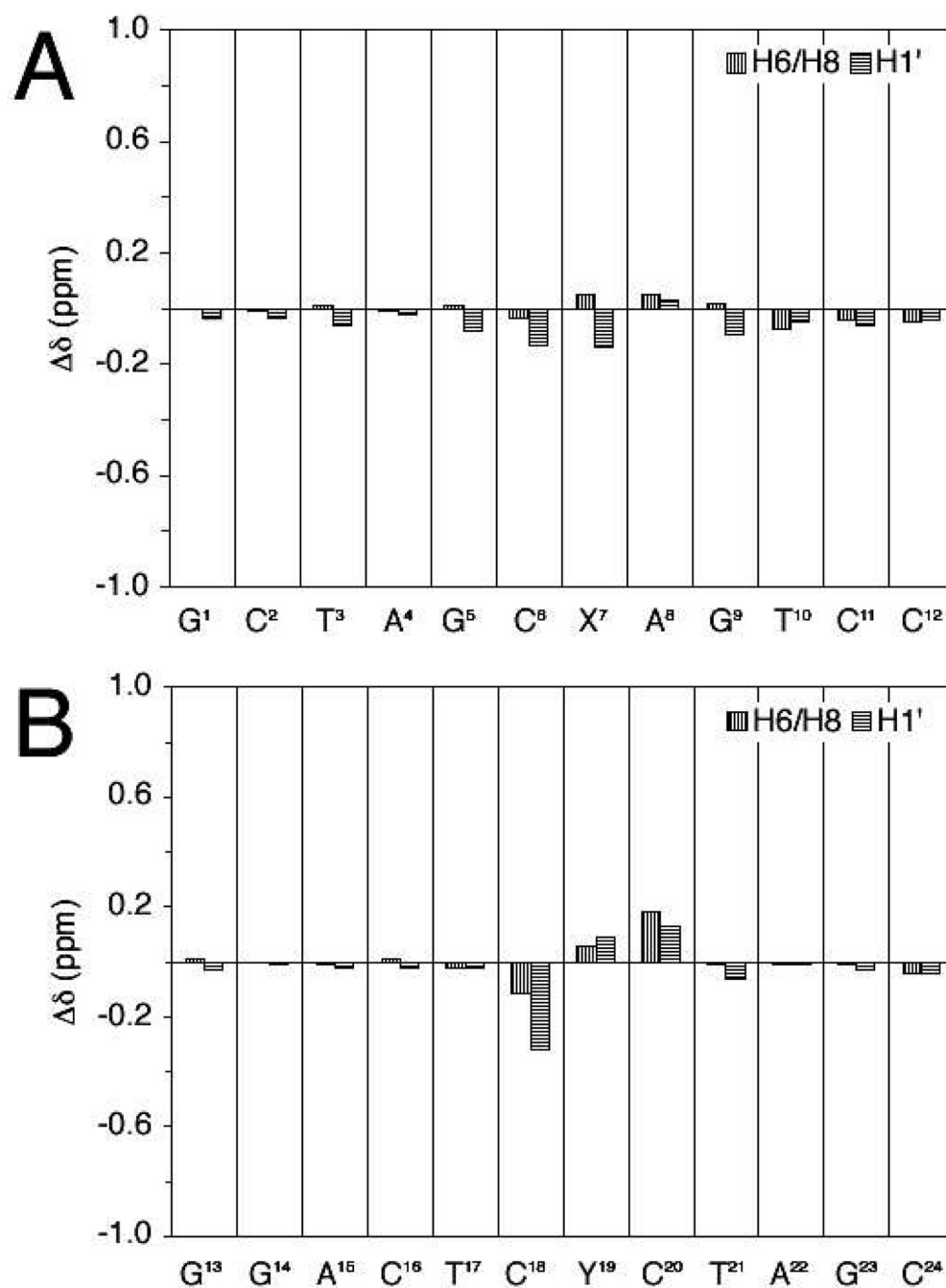


Figure 5. Chemical shift perturbations of the (*R*)- γ -hydroxytrimethylene interstrand cross-link compared to the unmodified duplex.

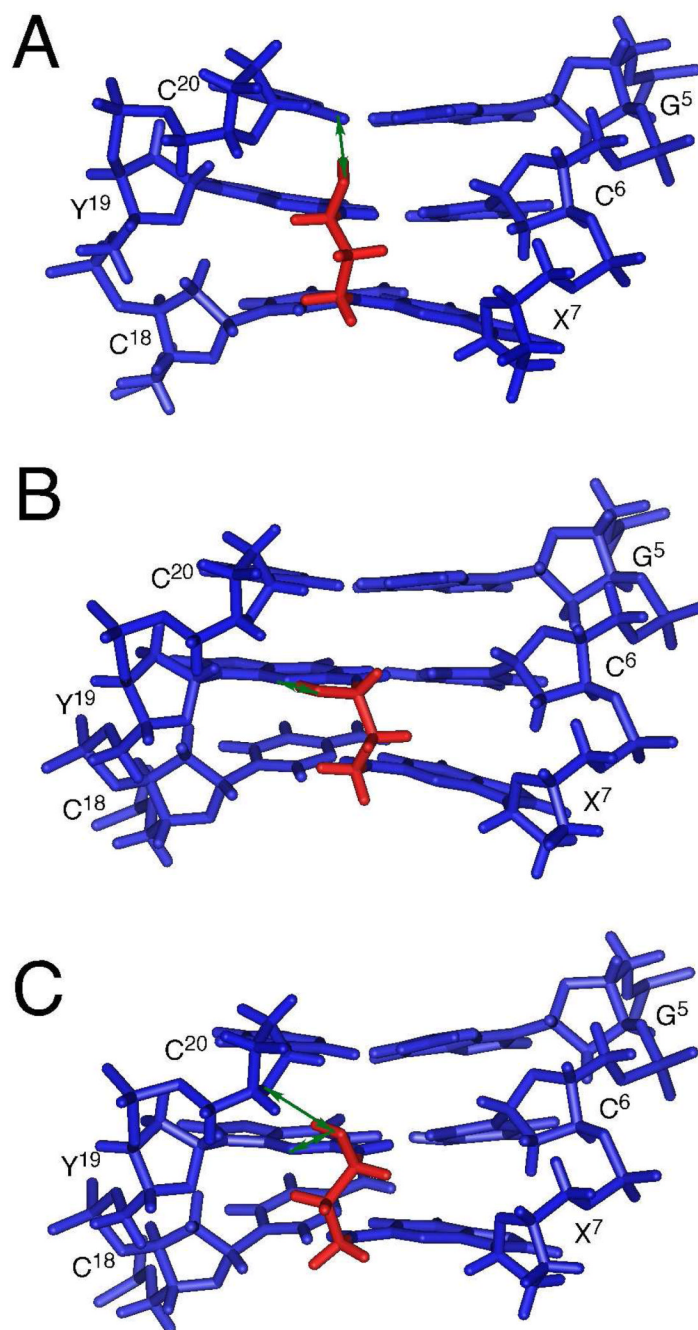


Figure 6. Expanded views, observed from the minor groove, of the diastereomeric carbinolamine interstrand cross-links. (A) Average structure of the (*R*)- γ -hydroxytrimethylene cross-link. (B) Predicted structure of the (*S*)- γ -hydroxytrimethylene cross-link from molecular dynamics simulations starting from A-DNA. (C) Predicted structure of the (*S*)- γ -hydroxytrimethylene cross-link from molecular dynamics simulations starting from B-DNA. Blue and red sticks represent nucleotides and the γ -hydroxytrimethylene tether, respectively. The green arrows indicate putative hydrogen bonds involving the hydroxyl groups.

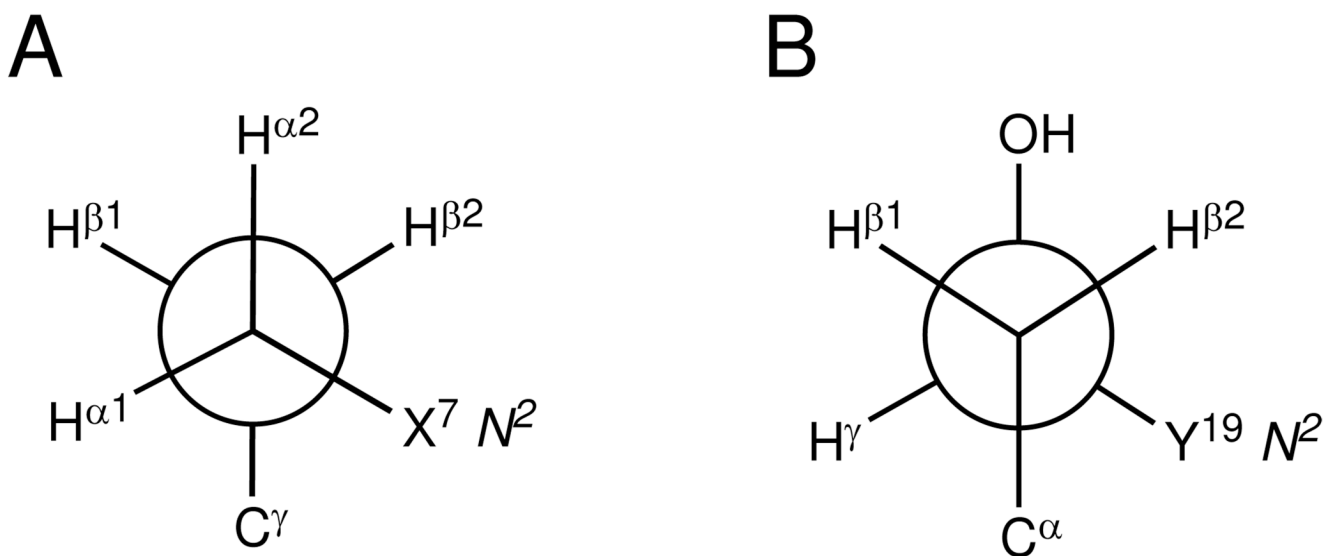
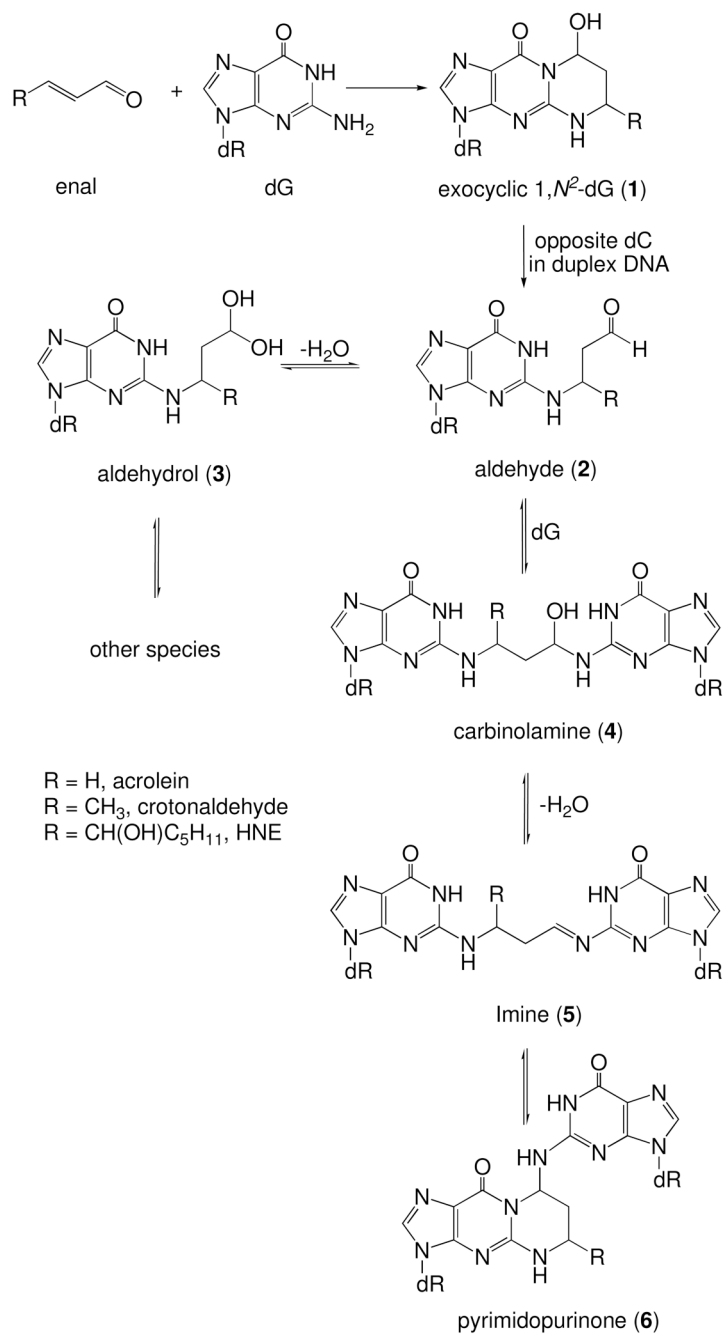
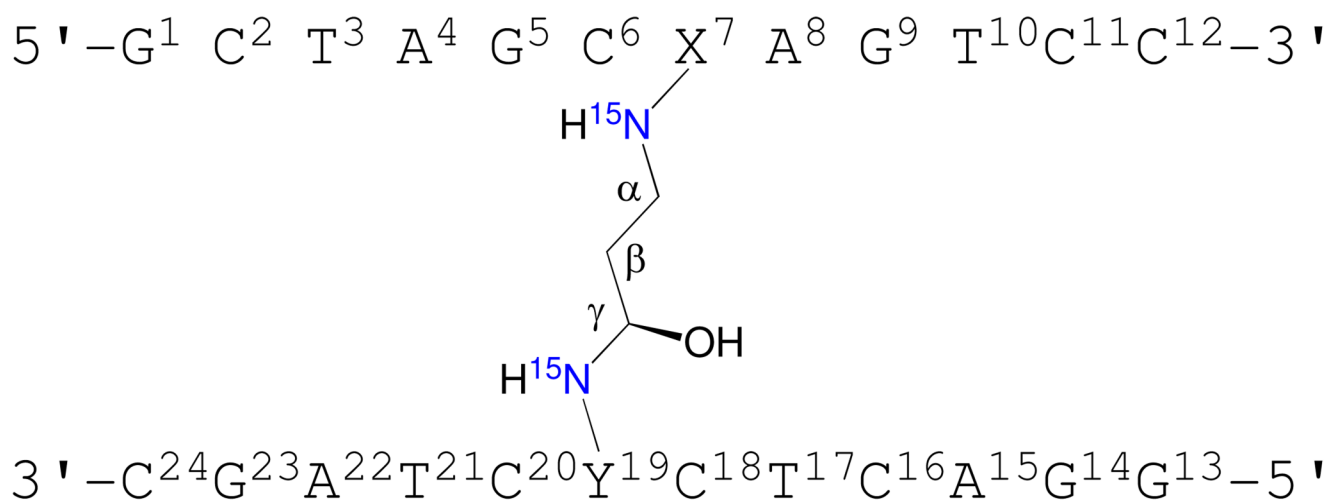


Figure 7. Newman projections showing conformation of the (*R*)- γ -hydroxytrimethylene tether. (A) Viewed along C^α - C^β bond. (B) Viewed along C^β - C^γ bond. Both $X^7 N^2$ and $Y^{19} N^2$ were in the gauche-conformation with the tether to maintain the Watson-Crick hydrogen bonding. The trans-conformation of hydroxyl group with C^α minimizes the steric interaction and allows the formation of the carbinol $\text{OH} \rightarrow \text{C}^{20} \text{O}^2$ hydrogen bond.



Scheme 1.
Chemistry of Enal-Derived Exocyclic 1,N²-Deoxyguanosine Adduct in the 5'-CpG-3' Sequence When Placed Opposite Deoxycytosine in Duplex DNA

**Scheme 2.**

Numbering scheme of the oligodeoxynucleotides containing the (*R*)- γ -hydroxytrimethylene interstrand cross-link in the 5'-CpG-3' sequence. The two ¹⁵N isotopic labeling sites are indicated in blue.

Table 1

Occupancy of the Hydroxyl Group by the Hydrogen Bond Acceptors During Molecular Dynamics Simulations, and the Distances and Angles of the Corresponding Atoms in the Average Structure of the (*R*)- γ -Hydroxytrimethylene Cross-link.

potential hydrogen bond	occupancy of OH (%) ^a		distance (Å)	angle (°)
	from A-DNA	from B-DNA		
Carbinol OH→C ²⁰ O ²	62	74	2.1	177
Carbinol OH→C ²⁰ O4'	22	14	3.0	97
Carbinol OH→Y ¹⁹ N3	2.7	1.7	3.2	103
Carbinol OH→C ⁶ O ²	1.0	0.70	3.6	93
Carbinol OH→solvent	0.20	0		

^aThe criteria for a hydrogen bond formation: distance ≤ 3.5 Å and angle $\geq 120^\circ$.