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Structure of the 1,4-Bis(2'-deoxyadenosin-*N*⁶-yl)-2*S*,3*S*-butanediol Intra-strand DNA Cross-link Arising from Butadiene Diepoxide in the Human *N-ras* Codon 61 Sequence

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Abstract

The 1,4-bis(2'-deoxyadenosin-*N*⁶-yl)-2*S*,3*S*-butanediol intra-strand DNA cross-link arises from bis-alkylation of tandem *N*⁶-dA sites in DNA by *R,R*-butadiene diepoxide (BDO₂). The oligodeoxynucleotide 5'-d(C¹G²G³A⁴C⁵X⁶Y⁷G⁸A⁹A¹⁰G¹¹)-3'-5'-d(C¹²T¹³T¹⁴C¹⁵T¹⁶T¹⁷G¹⁸T¹⁹C²⁰C²¹G²²)-3' contains the BDO₂ cross-link between the second and third adenines of the codon 61 sequence (underlined) of the human *N-ras* protooncogene and is named the (*S,S*)-BD-(61-2,3) cross-link (X,Y = cross-linked adenines). NMR analysis reveals that the cross-link is oriented in the major groove of duplex DNA. Watson-Crick base pairing is perturbed at base pair X⁶·T¹⁷, whereas base pairing is intact at base pair Y⁷·T¹⁶. The cross-link appears to exist in two conformations, in rapid exchange on the NMR time scale. In the first conformation, the β-OH is predicted to form a hydrogen bond with T¹⁶ O⁴, whereas in the second, the β-OH is predicted to form a hydrogen bond with T¹⁷ O⁴. In contrast to the (*R,R*)-BD-(61-2,3) cross-link in the same sequence [Merritt, W.K., Nechev, L.V., Scholdberg, T.A., Dean, S.M., Kiehna, S.E., Chang, J.C., Harris, T.M., Harris, C.M., Lloyd, R.S., and Stone, M.P. (2005) *Biochemistry* 44, 10081–10092], the *anti* conformation of the two hydroxyl groups at C_β and C_γ with respect to the C_β-C_γ bond results in a decreased twist between base pairs X⁶·T¹⁷ and Y⁷·T¹⁶, and an approximate 10° bending of the duplex. These conformational differences may account for the differential mutagenicity of the (*S,S*)- and (*R,R*)-BD-(61-2,3) cross-links, and suggest that stereochemistry plays a role in modulating biological responses to these cross-links [Kanuri, M., Nechev, L. V., Tamura, P. J., Harris, C. M., Harris, T. M., and Lloyd, R. S. (2002) *Chem. Res. Toxicol.* 15, 1572–1580].

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¹Abbreviations: BD, butadiene; BDO, butadiene monoepoxide (1,2-epoxy-3-butene); BDO₂, butadiene diepoxide (1,2:3,4-diepoxibutane); BDE, 3,4-dihydroxy-1,2-epoxybutanes; SBR, styrene-butadiene rubber; MALDI-TOF, matrix assisted laser desorption ionization time-of-flight; TOCSY, total correlation spectroscopy, DQF-COSY, double-quantum filtered correlation spectroscopy; TPPI, time-proportional phase increment; CORMA, complete relaxation matrix analysis; rMD, restrained molecular dynamics; rmsd, root mean square deviation; R₁^X, sixth root residual.

²The definitions of the prochiral protons C_α and C_δ of the *N*⁶,*N*⁶-dA cross-link are based upon the Cahn, Ingold and Prelog nomenclature. The proton H_α is defined as the pro-*R* proton at C_α; H_{α'} is defined as the pro-*S* proton at C_α. Likewise, H_δ is the pro-*R* proton at C_δ; H_{δ'} is the pro-*S* proton at C_δ.

Introduction

1,3-Butadiene (CAS RN 106-99-0) (BD)ⁱ is used in the manufacture of styrene-butadiene rubber (SBR) (1,2); several billion lbs/yr are produced in the United States. It is a combustion product from automobile emissions (3) and cigarette smoke (4). BD is genotoxic and is a carcinogen in rodents, particularly in mice (5–7) and also in rats (8). BD is classified by the United States Environmental Protection Agency as "carcinogenic to humans by inhalation" (9). The International Agency for Cancer Research (IARC) lists BD as a "probable human carcinogen" (Group 2A) (10–12). Chronic human exposure in the SBR industry may induce genotoxic effects (13–15) and is correlated with increased risk for leukemia (1,16–24).

BD is epoxidized primarily by cytochrome P450 2E1, but also by cytochrome P450 2A6, to form 1,2-epoxy-3-butenes (BDO) (Scheme 1) (25,26). These may be further oxidized by cytochrome P450 2E1 or 3A4 to form 1,2:3,4-diepoxibutenes (BDO₂) (25,27–31). Hydrolysis of BDO mediated by epoxide hydrolase forms 1,2-dihydroxy-3-butenes (29,32,33), which are metabolized by cytochrome P450 to hydroxymethylvinylketone (HMK) (34). Either BDO₂ or the 1,2-dihydroxy-3-butenes may undergo cytochrome P450-mediated oxidation to form 1,2-dihydroxy-3,4-epoxibutenes (BDE) (29,32,35). Thus, proximate electrophiles arising from BD metabolism include BDO, BDO₂, and BDE, and potentially, HMK (36).

All three optical isomers of BDO₂ are genotoxic (2,10,37), and *S,S*-BDO₂ is the most cytotoxic and genotoxic (38,39); it is believed to be the active form of the anti-tumor agent L-threitol-1,4-bismethanesulfonate (treosulfan) (40–42). The genotoxicity is probably due to its potential to form DNA-DNA (43–46) and DNA-protein cross-links (47,48), the latter having been observed in mice (49,50). Mice possess greater sensitivity to butadiene exposure than rats, and this is attributed to their efficient oxidation of BD to BDO₂ (51,52), presumably facilitating DNA cross-linking. Butadiene genotoxicity is further enhanced in knockout mice lacking a functional microsomal epoxide hydrolase gene (53). Polymorphisms in the human epoxide hydrolase gene may also contribute to differences in BD genotoxicity within the human population (54,55).

The predominant DNA cross-link induced by BDO₂, isolated from DNA and characterized by mass spectrometry (46), involves inter-strand bis-alkylation at N7-dG, particularly in 5'-GNC-3' sequences (45). The formation of N7,N7-dG cross-links is dependent upon stereochemistry of the BDO₂; with *S,S* BDO₂ > *R,R* BDO₂ > *meso* BDO₂ (56,57). Treatment of DNA with *meso* BDO₂ yields comparable numbers of 1,2-intra-strand and 1,3-inter-strand bis-N7-dG cross-links, while *S,S* BDO₂ produces few intra-strand cross-links. Treatment of DNA with *R,R*-BDO₂ yields mostly 1,3-interstrand bis-N7-dG cross-links, with smaller amounts of 1,2-interstrand and 1,2-intrastrand cross-links. The differential cross-linking specificities of BDO₂ stereoisomers may be related to the differential relative configurations of the hydroxyl and remaining oxirane groups in the mono-adducted N7-dG intermediate, with differential hydrogen bonding interactions involving the hydroxyl group mediating the consequent orientation of the oxirane in the major groove (56). Ultimately, these structural differences may be related to biological processing (56).

The structural characterization of bis-N7-dG cross-links remains elusive, in part because N7-dG alkylation products are chemically unstable. In contrast, the synthetically accessible and stable *N*⁶,*N*⁶-dA intra-strand cross-links (58) provide a model system with which to probe the role of hydroxyl stereochemistry in modulating the structure of BDO₂-induced cross-links in the major groove of DNA, and their consequent biological processing. With regard to occurrence in DNA, asymmetrical *N*⁶-dA:N7-dG but not *N*⁶,*N*⁶-dA cross-links are

characterized; the N^6 -dA:N7-dG cross-links are less abundant than are bis-N7-dG cross-links, but may contribute to butadiene genotoxicity (59). The prevailing hypothesis posits that the less abundant N^6 alkylation products of butadiene epoxides result from initial alkylation at the N1 position, followed by Dimroth rearrangement (60,61). This probably explains why N^6,N^6 -dA intra-strand cross-links occur infrequently in duplex DNA (N. Tretyakova, personal communication). However, the BDT N1-dA mono adduct was identified in humans exposed occupationally to BD (62), and the N^6 -dA BDT mono adduct was identified in Chinese Hamster ovary cells (63), suggesting that N^6,N^6 -dA intra-strand cross-links might form in duplex DNA, albeit at low levels.

Replication of DNAs containing the 1,4-bis(2'-deoxyadenosin- N^6 -yl)-2*R*,3*R*-butanediol cross-link in COS-7 cells yields 59% of single base substitutions at the 3' adducted base, while 19% mutagenesis is detected using the 1,4-bis(2'-deoxyadenosin- N^6 -yl)-2*S*,3*S*-butanediol cross-link. The primary mutations are A→G transitions observed at the 3' adenine of the cross-link. However, the level of mutations observed in the COS-7 cells is greater than is observed in the bacterial cells. Because N^6,N^6 -dA cross-links (58) are significantly more mutagenic than are N^6 -dA monoadducts arising from BDO or BDE (64,65), their potential as rare but biologically significant lesions cannot be discounted.

Since both of the stereoisomeric cross-links are readily replicated by COS-7 cells (58), it was proposed that these cross-links are accommodated within the major groove, similar to several N^6 -dA adducts of styrene oxide, which also are readily replicated in site-specific mutagenesis experiments (66,67). This is in contrast to the corresponding N^2,N^2 -dG (*R,R*) and (*S,S*) intrastrand cross-links, which are severe replication blocks to cells (68). The structure of the 1,4-bis(2'-deoxyadenosin- N^6 -yl)-2*R*,3*R*-butanediol cross-link, the (*R,R*)-BD-(61-2,3) cross-link, which arises from *S,S*-BDO₂, in the oligodeoxynucleotide 5'-d(CGGACXYGAAG)-3'·5'-d(CTTCTTGTC CG)-3' (X,Y = cross-linked adenines) (69) confirms that the cross-linked moiety is accommodated in the major groove of the DNA. Watson-Crick base pairing is disrupted at position X⁶·T¹⁷, the 5'-side of the intrastrand cross-link. Helicoidal analysis suggests an opening of this base pair. A proposed hydrogen bond between the β-hydroxyl group of the cross-link, and T¹⁷ O⁴, the nucleotide complementary to X⁶, shields the T¹⁷ imino hydrogen from exchange with solvent. In contrast, Watson-Crick base pairing is intact at position Y⁷·T¹⁶, the 3'-side of the cross-link.

Presently, we report that the 1,4-bis(2'-deoxyadenosin- N^6 -yl)-2*S*,3*S*-butanediol cross-link, the (*S,S*)-BD-(61-2,3) cross-link, which arises from *R,R*-BDO₂, is also oriented in the major groove of duplex DNA. Watson-Crick base pairing is perturbed at base pair X⁶·T¹⁷, whereas base pairing is intact at base pair Y⁷·T¹⁶. The major conformational difference as compared to the (*R,R*)-BD-(61-2,3) cross-link (69) regards the conformation of the four-carbon butadiene chain. The *anti* conformation of the two hydroxyl groups at C_β and C_γ with respect to the C_β-C_γ bond in the (*S,S*)-BD-(61-2,3) cross-link requires greater structural perturbation to the DNA duplex, resulting in a decreased twist between base pairs X⁶·T¹⁷ and Y⁷·T¹⁶, and an approximate 10° bending of the cross-linked duplex. The (*S,S*)-BD-(61-2,3) cross-link appears to exist in two conformations, in rapid exchange on the NMR time scale. The two conformations are predicted to exhibit differential hydrogen bonding involving the β-OH moiety of the butadiene cross-link. In the first, the β-OH is predicted to form a hydrogen bond with T¹⁶ O⁴, whereas in the second conformation, the β-OH is predicted to form a hydrogen bond with T¹⁷ O⁴.

Materials and Methods

Sample Preparation

The oligodeoxynucleotide 5'-d(CTTCTTGTC CG)-3' was synthesized by the Midland Certified Reagent Co. (Midland, TX) and purified by anion-exchange chromatography. The

butadiene-cross-linked oligodeoxynucleotide 5'-d(CGGACXYGAAG)-3' was synthesized as described (58,70). The concentrations of the single-stranded oligodeoxynucleotides were determined from the extinction coefficients at 260 nm (71). The modified oligodeoxynucleotide and its complementary strand were annealed in a buffer solution consisting of 10 mM NaH₂PO₄, 0.1 M NaCl, and 50 μ M Na₂EDTA at pH 7.0. The annealed duplex DNA was eluted from a column containing DNA Grade Biogel hydroxylapatite (Bio-Rad Laboratories, Richmond, CA) with a gradient from 10 to 200 mM NaH₂PO₄ at pH 7.0. It was lyophilized, resuspended in 1 mL of H₂O, and desalted using Sephadex G-25. In order to determine that both the correct modified and complementary strands were in solution, MALDI-TOF mass spectra were obtained on a Voyager-DE (PerSeptive Biosystems, Inc.) instrument in negative reflector mode. The matrix used in each of these studies contained 0.5 M 3-hydroxypicolinic acid and 0.1 M ammonium citrate. NMR samples were prepared in 10 mM NaH₂PO₄, 0.1 M NaCl, and 50 μ M Na₂EDTA at pH 7.0. For observation of non-exchangeable protons, the samples were exchanged three times with 99.9% D₂O and dissolved in 0.5 mL of 99.99% D₂O. For observation of exchangeable protons, the samples were dissolved in 0.5 mL of 9:1 H₂O/D₂O.

Thermal Melting Experiments

The melting temperatures were measured on a Varian Cary 4E spectrophotometer at 260 nm in a buffer consisting of 10 mM sodium phosphate, 50 μ M Na₂EDTA, and 1M NaCl at pH 7.0, at a temperature increasing rate of 1.0 °C/min from 10 to 80 °C. The melting temperatures of the unmodified and modified oligodeoxynucleotides were obtained by the midpoints of the melting curves from the first-order derivatives.

Nuclear Magnetic Resonance

¹H NMR spectra were recorded at 600.13 MHz and 800.23 MHz. The non-exchangeable protons were monitored at 25 °C; the exchangeable protons were monitored at 10 °C. Chemical shifts were referenced to the water resonance. NMR data were processed using FELIX2000 (Accelrys, Inc., San Diego, CA). NOESY spectra of the non-exchangeable protons were recorded using TPPI phase cycling with mixing times of 150, 200, and 250 ms. These spectra were acquired sequentially without removing the sample from the magnet. Spectra for the exchangeable protons were recorded using a 200 ms mixing time. These experiments were recorded with 1024 real data points in the d1 dimension and 2048 real data points in the d2 dimension. A relaxation delay of 2.0 s was used. Water suppression was performed using the WATERGATE sequence (72). TOCSY experiments were performed with mixing times of 90 and 150 ms, utilizing the homonuclear Hartman-Hahn transfer with the MLEV17 sequence for mixing. DQF-COSY spectra were zero-filled to give a matrix of 1024 $\times$ 2048 real points. A skewed sine-bell square apodization function with a 90° phase shift and a skew factor of 1.0 was used in both dimensions.

Experimental Restraints

(a) Distance Restraints—Footprints were drawn around cross peaks obtained at a mixing time of 200 ms using FELIX2000. Identical footprints were transferred and fit to the crosspeaks obtained at the other two mixing times. The intensities of these cross peaks were determined by volume integration. These were combined as necessary with intensities generated from complete relaxation matrix analysis of a starting DNA structure to generate a hybrid intensity matrix (73–75). MARDIGRAS (76–78) was used to iteratively refine the hybrid intensity matrix and to optimize the agreement between the calculated and experimental NOE intensities. Calculations were initiated using isotropic correlation times of 2, 3, and 4 ns, and with both IniA and IniB starting structures and the three mixing times, yielding eighteen sets of distances. Analysis of this data yielded the experimental distance restraints used in subsequent restrained

molecular dynamics calculations, and the corresponding standard deviations for the distance restraints. The distance restraints were divided into five classes, reflecting the confidence level in the experimental data.

(b) Torsion Angle Restraints—Deoxyribose pseudorotation (57) was determined graphically using the sums of $^3J^{\text{H}}$ coupling constants (58), measured from DQF-COSY spectra. Discrete $J_{1/2''}$ and $J_{1/2'}$ couplings were measured from active and passive couplings, respectively, of the H2'' (d2) to H1' (d1) spectral region. The data were fit to curves relating the coupling constants to the deoxyribose sugar pseudo rotation angle (P), sugar pucker amplitude (ϕ), and the percentage S-type conformation. The sugar pseudo rotation angle and amplitude ranges were converted to the five dihedral angles ν_0 to ν_4 . Coupling constants measured from ^1H - ^{31}P HMBC spectra were applied (79,80 1991 1991) to the Karplus relationship (81) to determine the backbone dihedral angle ϵ (C4'-C3'-O3'-P), related to the H3'-C3'-O3'-P angle by a 120° shift. The ζ (C3'-O3'-P-O5') backbone angles were calculated from the correlation between ϵ and ζ in B-DNA (60).

Restrained Molecular Dynamics Calculations

Classical A-DNA and B-DNA were used as reference structures to create starting structures for the refinement (82). The butadiene adduct was constructed at A⁶ using the BUILDER module of INSIGHT II (Accelrys, Inc., San Diego, CA). A-form and B-form structures of the appropriate sequence were energy-minimized by the conjugate gradients method for 200 iterations using the AMBER 7.0 force field (83) without experimental restraints to give starting IniA and IniB used for the subsequent relaxation matrix analysis and molecular dynamics calculations. The restraint energy function was comprised of terms describing distances and dihedral restraints, both of which were in the form of a standard square-well potential (84). Bond lengths involving hydrogens were fixed with the SHAKE algorithm (85). The generalized Born approach was used to model solvation (86,87). The calculations utilized a salt concentration of 0.2 mM. A series of randomly seeded rMD calculations were performed over a time course of 40 ps. These used the SANDER module of AMBER 7.0, and the Cornell *et al.* force field (88), including the Parm94.dat parameter set. Restrained molecular dynamics calculations were performed using AMBER in implicit solvent. The simulated annealing protocol utilized a starting temperature of 25 K. In the first 1 ps the temperature was increased to 600 K and this target temperature is maintained for 4 ps, followed by cooling to 298 K over 15 ps. During the final 20 ps the temperature was reduced to 0 K. Temperature was controlled by coupling the molecules to a temperature bath. The first 1 ps of heating used a coupling of 0.4 ps, followed by 4 ps of constant temperature dynamics with a coupling of 1.0 ps. In the following 15 ps of cooling, a heat coupling of 1.0 ps was used, followed by a value of 0.5 ps for the next 15 ps. During the final 5 ps of cooling, the coupling was ramped down to 0.01 ps. In the first 1 ps of heating, the experimental force constants were amplified by factors that ranged from 0.5 to 1.00. During the 4 ps of constant temperature dynamics and the first 15 ps of cooling, the amplification factor was increased to 1.75. In the final 20 ps of cooling, the amplification factor was reduced to a value of 1.00. Structure coordinates extracted from the final 4 ps of each rMD calculation were averaged and energy-minimized for 200 iterations using the conjugate gradient algorithm.

Back-calculation of ^1H NOE data was performed using CORMA (v. 4.0) (75). Helicoidal parameters were examined using 3DNA (89) and Curves (90,91).

Results

Sample Properties

The duplex oligodeoxynucleotide 5'-d(C¹G²G³A⁴C⁵A⁶G⁷A⁸A⁹G¹⁰G¹¹)-3' • 5'-d(C¹²T¹³T¹⁴C¹⁵T¹⁶T¹⁷G¹⁸T¹⁹C²⁰C²¹G²²)-3' utilized in the present studies contains the coding sequences for codons 60, 61 (underlined), and 62 of the human *N-ras* proto-oncogene, and was engineered to be inserted into bacterial and mammalian site-specific mutagenesis systems. The *ras* oncogene was discovered in the Harvey and Kirsten murine sarcoma viruses (92). Mutations in codon 61 activate the oncogene. Mutations in *ras* are frequently detected alteration in human tumors (92–97). The identity of the duplex was verified using MALDI-TOF mass spectrometry. Mass measurement showed two signals corresponding to mass units of 3485 and 3274, which originated from the cross-linked adduct strand 5'-d(CGGACXYGAAG)-3' and the complementary strand 5'-d(CTTCTTGTC CG)-3', respectively. Melting temperature of the (S,S)-BD-(61-2,3) duplex was 43 °C, lower than the 57 °C melting temperature of the unmodified *ras*61 duplex and the 50 °C of the (R,R)-BD-(61-2,3) duplex (69). The cross-linked sample yielded excellent NMR data in the temperature range of 10 °C–25 °C.

DNA ¹H Resonance Assignments

(a) Non-exchangeable Protons—The sequential assignment for the (S,S)-BD-(61-2,3) cross-linked duplex was accomplished using standard protocols (98,99). The sequential NOEs between the aromatic and anomeric protons of the (S,S)-BD-(61-2,3) cross-linked oligodeoxynucleotide duplex are displayed in Figure 1. For the modified strand, weak NOE connectivities were observed between X⁶ H1' to Y⁷ H8 and Y⁷ H1' to G⁸ H8. In the complementary strand, the connectivity between T¹⁶ H1' and T¹⁷ H6 was interrupted. The complete assignments of the deoxyribose H2', H2'' and H3' protons were achieved and partial assignments were made for the deoxyribose H4', H5' and H5'' protons due to heavy overlap.

(b) Exchangeable Protons—A plot of the region ranging from 11 to 15 ppm of the NOESY experiment is shown in Figure 2. The imino cross peaks located in this region were well-resolved, with the exception of T¹⁴ and T¹⁶, which were overlapped. The assignments of the thymine imino resonances were confirmed by the identification of cross peaks to adenosine H2 protons of each A·T pair, as shown in Figure 2B. Cross peaks arising from the Y⁷ H2 to T¹⁶ N3H and A⁹ H2 to T¹⁴ N3H NOEs confirmed that both T¹⁴ N3H and T¹⁶ N3H resonated at 13.8 ppm. The sequential connectivities of the imino protons were obtained from base pairs G²·C²¹→C⁵·G¹⁸ and Y⁷·T¹⁶→A¹⁰·T¹³, with breaks in connectivity observed between base pairs C⁵·G¹⁸→X⁶·C¹⁷ and between X⁶·C¹⁷→Y⁷·T¹⁶. The NOE between T¹⁷ N3H and X⁶ H2 was of reduced intensity. The NOE between T¹⁶ N3H and Y⁷ H2 was also weak. The imino resonances from the terminal base pairs C¹·G²² and G¹¹·C¹² were missing, which presumably reflected the effects of strand fraying and resulting rapid exchange of this proton with solvent.

Butadiene Protons

The butadiene cross-link protons were assigned by the combined use of DQF-COSY, TOCSY and NOESY spectra. As shown in Figure 3, at a mixing time of 250 ms, NOEs were observed between the butadiene cross-link protons. The H_α proton at 2.87 ppm showed strong NOEs to H_{α'} at 3.32 ppm and H_β at 3.89 ppm, along with a weak NOE to H_γ at 3.95 ppm. The H_β proton showed NOEs to H_α, H_{α'} and H_γ, along with a weak NOE to H_δ at 3.01 ppm, H_{δ'} at 3.71 ppm. The H_γ proton exhibited NOEs to H_δ and H_{δ'}, along with weak NOEs to H_α and H_{α'}.

Butadiene-DNA NOEs

A total of 5 NOEs were observed between the BD protons and the DNA. Some of these are shown in Figure 4. The H_α and H_{α'} protons exhibited cross peaks to 5' direction neighboring

base C⁵ H₅. NOEs were also observed between butadiene protons H_α, H_{α'}, H_β and the exchangeable proton T¹⁷ N₃H. These NOEs established the orientation of the butadiene cross-link in the major groove.

Torsion Angle Measurements

Analysis of DQF-COSY data suggested that all deoxyribose pseudorotation angles remained in the C2'-endo sugar ring conformation anticipated for B-family DNA. The glycosyl torsion angle conformations were evaluated by inspection of ¹H NOESY data. The weak NOEs between the purine H₈ or pyrimidine H₆ protons and the anomeric H1' protons of the attached deoxyribose sugars were consistent with glycosyl torsion angles in the normal anti conformational range. Measurements for the phosphodiester backbone torsion angles ε and ζ were obtained from a heteronuclear ¹H-³¹P correlation experiment. The ³¹P chemical shifts were observed in the expected chemical shift range for B-DNA. There were also no unusual ³J ¹H-³¹P couplings observed in the ³¹P spectrum. Data obtained from ³J ¹H-³¹P experiments indicated that the torsion angles associated with the backbone phosphodiester linkages were not significantly perturbed by the presence of the cross-link.

Chemical shift effects

The ¹H spectrum of the cross-linked duplex exhibited significant chemical shift differences at the modified base X⁶ and its complementary base T¹⁷ as compared to that of the unmodified *ras61* oligodeoxynucleotide (Figure 5). An upfield chemical shift of 0.36 ppm was observed for X⁶ H1', while a downfield shift of 0.29 ppm was observed for T¹⁷ H₆. For the imino protons, the greatest downfield shift of 0.46 ppm was observed for T¹⁷ N₃H. Other smaller chemical shifts were observed for a number of protons near the adduction sites.

Structural Refinement

The structural refinement utilized a simulated annealing protocol for the molecular dynamics calculations. The Generalized Born model was utilized to approximate charge-charge interactions in the presence of a continuum solvent (86,87). The molecular dynamics calculations were restrained by 413 experimental distance restraints, including the 5 NOEs between the BD protons and the DNA. These were evenly distributed over the length of the oligodeoxynucleotide. In addition to the NOE-based distance restraints, 90 deoxyribose pseudorotation restraints, 104 empirical phosphodiester backbone angle measurements and 39 hydrogen bonding restraints were included in the calculations.

Sets of randomly seeded rMD calculations were carried out from both B-form DNA and A-form DNA starting structures. These starting structures exhibited a root mean square deviation (rmsd) of 6.26 Å. The structures that emerged following 40 ps of rMD were subjected to potential energy minimization and evaluated as to pairwise rmsd. Irrespective of starting structure, two ensembles of structures emerged from the calculations. In the first, designated conformation I, the butadiene C_β-hydroxyl proton was within hydrogen bonding distance of T¹⁶ O⁴. The maximum pairwise rmsd for this ensemble of structures was 0.87 Å. In the second, designated conformation II, the butadiene β-hydroxyl proton was within hydrogen bonding distance of T¹⁷ O⁴. The maximum pairwise rmsd for this ensemble of structures was 0.85 Å (Table 1). Stereoviews of the two different energy minimized structures that emerged from the randomly-seeded rMD calculations are shown in Figure 6.

The two sets of converged structures, conformations I and II, that emerged from the rMD calculations were assessed by complete relaxation matrix calculations, using the program CORMA (v. 4.0) (75). These calculations yielded sixth root residuals (R₁^x values) between the NOE intensities predicted by the refined structures and the experimentally measured NOE intensities. Figure 7 shows R₁^x values as a function of nucleotide position. For both

conformations I and II, the calculations revealed reasonable agreement between calculated intensities and experimental NOE data. This reflected the fact that the two conformations differed only in the orientation of the butadiene C β -hydroxyl group, and the magnitudes of most or all of the 5 butadiene-DNA NOEs did not differ between the two potential conformations. The residual values were consistent over the length of the modified oligodeoxynucleotide, ranging from 0.017 to 0.152. It was concluded that either conformations I or II, or an equilibrium mixture of the two, could be consistent with the available NOE data, and most likely, the observed spectral data reflected the presence of an equilibrium mixture of both conformations, in rapid exchange on the NMR time scale.

Structure of the (S,S)-BD-(61-2,3) Cross-linked Adduct

The (S,S)-BD-(61-2,3) butadiene cross-link was oriented in the major groove, as shown in Figure 8. In the four carbon butadiene-derived cross-link the two hydroxyl groups at C β and C γ were in the *anti* conformation with respect to the C β -C γ bond. This allowed the C β -OH group to insert between T¹⁶ and T¹⁷ in the complementary strand, while allowing the C γ -OH group to orient into the major groove and away from the DNA helix. It resulted in distortions of base pairs X⁶·T¹⁷ and Y⁷·T¹⁶ (Figure 9). Nevertheless, base pairs X⁶·T¹⁷ and Y⁷·T¹⁶ remained inserted into the DNA duplex, and base stacking (Figure 10) was minimally perturbed, as compared to the corresponding stacking interactions between A⁶ and A⁷ in the unmodified *ras*61 oligodeoxynucleotide (100). Helicoidal analysis suggested a 10° decrease of the twist angle between base pairs X⁶·T¹⁷ and Y⁷·T¹⁶, resulting in an unwinding of the duplex (89). An approximate 10° bending of the cross-linked duplex was calculated using the program CURVES (90,91), probably a consequence of placing the two hydroxyl groups at C β and C γ in the *anti* conformation with respect to the C β -C γ bond, which did not allow the four carbon cross-link to exist in the extended chain conformation. The presence of the C β -OH group inserted between T¹⁶ and T¹⁷ in the complementary strand allowed it to swivel either towards T¹⁶ O⁴ or T¹⁷ O⁴. Thus, two potential conformations of the (S,S)-BD-(61-2,3) cross-linked adduct in the *ras*61 oligodeoxynucleotide differed in hydrogen bonding interactions between the C β -OH group of the cross-link and T¹⁷ O⁴ (conformation I) or T¹⁶ O⁴ (conformation II).

Discussion

The genotoxicity of butadiene may be related to its ability to form cross-links in DNA via its diepoxide oxidation product. Animal studies revealed species differences between mice and rats with regard to BD genotoxicity, which mice exhibiting greater sensitivity (7,8,101). This was explained by the observation that in mice the conversion of BD to BDO₂ was more efficient than in rats, an observation which also pointed to BDO₂ as the key proximate electrophile in BD-mediated genotoxicity (52). This suspicion was confirmed by studies showing that BDO₂ was indeed considerably more mutagenic than the mono-epoxide EB (37)—probably due to its cross-linking ability. The primary evidence suggesting the presence of BDO₂-induced DNA cross-links in vivo comes from studies in which human lymphoblastoid TK6 and splenic T cells were exposed to low levels of BDO₂ (37,102,103). These experiments revealed transitions and transversions at both G•C and A•T sites, suggesting the presence of both dG and dA adducts arising from BDO₂. They also revealed the presence of significant levels of deletion mutants, consistent with the presence of DNA cross-links (37,102,103).

Structural Analysis of (S,S)-BD-(61-2,3) Cross-link

The major groove orientation of the (S,S)-BD-(61-2,3) cross-link was consistent with the observation of few NOEs between the butadiene cross-link and the DNA, placing the butadiene protons far from major groove non-exchangeable DNA protons. Nevertheless, the NOEs between the butadiene H α and H α' protons and major groove C⁵ H5 proton of the 5' neighboring

base served to locate the cross-link in the major groove. The NOEs observed between the butadiene $H_{\alpha,\alpha'}$, H_β and the exchangeable proton T^{17} N3H further oriented the cross-link in the major groove. The *anti* conformation of the two hydroxyl groups at C_β and C_γ with respect to the C_β - C_γ bond resulted in a decrease of the twist angle between base pairs $X^6 \cdot T^{17}$ and $Y^7 \cdot T^{16}$, and an approximate 10° bending of the cross-linked DNA duplex. This was accompanied by disruption of Watson-Crick hydrogen bonding at base pair $X^6 \cdot T^{17}$. The observation of a break in sequential NOE connectivity between T^{16} H6 and T^{17} H1' (Figure 1) and the 0.46 and 0.29 ppm upfield shifts of the T^{17} N3H and T^{17} H6 resonances, respectively (Figure 2), supported the conclusion that Watson-Crick base-pairing was disrupted at base pair $X^6 \cdot T^{17}$. This probably also explained the reduction in the T_m of the (*S,S*)-BD-(61-2,3) cross-linked duplex as compared to that of the unmodified *ras61* duplex. The potential formation of a hydrogen bond between the C_β -OH group of the cross-link and either T^{16} O^4 or T^{17} O^4 , predicted by the rMD calculations, probably stabilized the conformation of the cross-link.

Comparison with the (*R,R*)-BD-(61-2,3) Cross-linked Adduct

Like the (*R,R*)-BD-(61-2,3) cross-link, the (*S,S*)-BD-(61-2,3) cross-link was oriented in the major groove of the DNA. For both cross-links, Watson-Crick base pairing was disrupted at base pair $X^6 \cdot T^{17}$, whereas base pair $Y^7 \cdot T^{16}$, base pairing was less perturbed. The major conformational difference between the (*R,R*)-BD-(61-2,3) and (*S,S*)-BD-(61-2,3) cross-links was in the conformation of the four carbon butadiene chain. For the (*R,R*)-BD-(61-2,3) cross-link, the butadiene linkage existed in an extended chain conformation, placing the two hydroxyl groups at C_β and C_γ in the *syn* conformation with respect to the C_β - C_γ bond. This allowed the (*R,R*)-BD-(61-2,3) cross-link to be accommodated within the major groove of the DNA with minimal structural perturbation of the duplex. In contrast, the *anti* conformation of the two hydroxyl groups at C_β and C_γ with respect to the C_β - C_γ bond in the (*S,S*)-BD-(61-2,3) cross-link required greater structural perturbation to the DNA duplex, resulting in a decreased twist between base pairs $X^6 \cdot T^{17}$ and $Y^7 \cdot T^{16}$, and an approximate 10° bending of the cross-linked duplex.

Structure-Activity Relationships

The present results confirm a role for stereochemistry in modulating the conformations of the diastereomeric (*R,R*)- and (*S,S*)-BD-(61-2,3) butadiene-derived cross-links. While the (*R,R*)-BD-(61-2,3) cross-link existed in the extended chain conformation with minimal perturbation of the DNA duplex, the (*S,S*)-BD-(61-2,3) cross-link created a greater structural perturbation at base pairs $X^6 \cdot T^{17}$ and $Y^7 \cdot T^{16}$. These differential structural perturbations could influence the recognition and biological processing of these cross-links. Although both (*R,R*)- and (*S,S*)-BD-(61-2,3) cross-links were highly mutagenic in both *Escherichia coli* and COS-7 cells, the (*S,S*)-BD-(61-2,3) cross-link exhibited a somewhat lower overall mutagenic frequency (20%) than that of (*R,R*)-BD-(61-2,3) cross-linked adduct (54%) (58).

Park et al. (56) proposed a model in which the differences between the ability of different BDO₂ stereoisomers to induce N7,N7-dG cross-links might be caused by different orientations of functional groups in stereoisomeric N7-(2'-hydroxy-3',4'-epoxybut-1'-yl)-guanine intermediates. Thus, for *S,S*- and *R,R*-N7-(2'-hydroxy-3',4'-epoxybut-1'-yl)-guanine, the oxirane and the 2'-hydroxy group reside on one side of the plane formed by the carbon chain, while, in the cross-link arising from the *meso* BDO₂, the oxirane and the 2'-OH are on different sides of the plane, potentially influencing the site of the second alkylation. Molecular modeling suggested that formation of a hydrogen bond between the 2'-hydroxy group in *S,S*- and *R,R*-N7-(2'-hydroxy-3',4'-epoxybut-1'-yl)-guanine and N3 of the 3'-neighbor dG would position the oxirane favorably for alkylation at N7-dG in the opposite strand. In contrast, formation of a hydrogen bond between the 2'-hydroxyl and 3'-neighbor dG in *meso*-DEB-induced *S,R* and *R,S* epoxy alcohol intermediates would stabilize the conformation in which the oxirane faced

N7-dG in the opposite strand. As a result, the regiochemistry of cross-linking would differ. Likewise, for the *S,S*-*N*⁶-(2',3',4'-trihydroxybut-1'-yl)-deoxyadenosyl adduct hydrogen bond formation was observed between the -OH of the side chain and the *O*⁴ of thymine in the opposite strand, while, for the *R,R* adduct, hydrogen bonding was formed between the -OH and the thymine *O*⁴ in the opposite DNA strand (104).

Biological Significance

Mammalian mutagenesis studies performed on B6c3F1 *lacI* transgenic mice showed point mutations at both dG and dA; the primary adenine-specific point mutations were A→T transversions (105–109). The site specific mutagenesis experiments on both stereoisomers of *N*⁶,*N*⁶-dA intrastrand cross-linked adducts yielded primarily A→G mutations (58), indicating that the BDO₂-induced cross-linked adducts did not represent the source of the predominant A→T mutations induced by butadiene in bacterial or in mammalian cells. Nonetheless, butadiene was reported to induce A→G transitions in the *H-ras* locus (110), to which might be attributed to BDO₂-induced cross-links. The N1-deoxyinosine adducts represent another potential source of A→G transitions. Site-specific mutagenesis studies of the N1-deoxyinosine adducts arising from BDO in the COS-7 cells revealed that they were strongly mutagenic with respect to A→G mutations. Consequently, the specific DNA adducts arising from exposure to BD and responsible for inducing A→T mutations remain to be determined. Mutagenesis studies of the (*S,S*)-BD-(61-2,3) cross-linked adduct suggested that A→G transitions at the 3' adenine were the primary point mutations in both *E. coli* and COS-7 cells, the same results were observed for the (*R,R*)-BD-(61-2,3) cross-linked adduct. This observation suggested that both stereoisomeric cross-linked adducts facilitate mis-incorporation of dCTP opposite cross-linked nucleotide Y⁷. Examination of the structure of a mismatched dC opposite the *N*⁶, *N*⁶-dA cross-linked adduct will be of interest. The predominant adducts formed upon exposure of DNA to butadiene epoxides are N7-dG adducts (111). The N7-N7 guanine interstrand cross-linked adduct has been determined to be the major DNA-DNA cross-link induced by BDO₂ (17). It will also be of considerable interest to examine the structures of N7-guanine adducts.

Summary

The (*S,S*)-BD-(61-2,3) cross-linked adduct at *N*⁶ in the ras61 coding sequence oriented in the major groove, resulting in the opening of base pair X⁶.T¹⁷. Two potential conformations obtained from NMR data refinement exhibited similar structures with different hydrogen bonding interactions between the C_β-OH group of the cross-link and T¹⁶ *O*⁴ or T¹⁷ *O*⁴, respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Expanded plots from a NOESY spectrum showing sequential NOE connectivities from aromatic to anomeric protons. **A.** Nucleotides C¹→G¹¹ of the modified strand of the (*S,S*)-BD-(61-2,3) cross-linked adduct. **B.** Nucleotides C¹²→G²² of the complementary strand of the (*S,S*)-BD-(61-2,3) cross-linked adduct. The experiment was carried out at a mixing time of 250 ms and 800 MHz.

Figure 2.

A. NOE connectivity for the imino protons for the base pairs from G²·C²¹ to A¹⁰·T¹³. Note the breaks in connectivity between the imino resonances for base pairs and between base pairs C⁵·G¹⁸ and X⁶·T¹⁷ and between X⁶·T¹⁷ and Y⁷·T¹⁶. The experiment was carried out at a mixing time of 250 ms and 800 MHz. **B.** NOE cross-peaks between exchangeable protons and non-exchangeable protons: a1, G¹⁸ N1H→G¹⁸ N²H1; a2, G¹⁸ N1H→G¹⁸ N²H2; a3, G¹⁸ N1H→A⁴ H2; a4, G¹⁸ N1H→X⁶ H2; b1, G⁸ N1H→G⁸ N²H1; b2, G⁸ N1H→G⁸ N²H2; b3, G⁸ N1H→A⁹ H2; b4, G⁸ N1H→Y⁷ H2; c1, G³ N1H→G³ N²H1; c2, G³ N1H→G³ N²H2; c3, G³ N1H→A⁴ H2; c4, G³ N1H→G² N²H1; d1, G² N1H→G² N²H1; d2, G² N1H→G² N²H2; d3, G² N1H→G³ N²H1; d4, G² N1H→G³ N²H2; e1, T¹⁷ N3H→X⁶ H2; e2, T¹⁷ N3H→Y⁷ H2; f1, T¹⁹ N3H→A⁴ H2; f2, T¹⁹ N3H→G³ N²H1; f3, T¹⁹ N3H→G¹⁸ N²H1; f4, T¹⁹ N3H→G¹⁸ N²H2; g1, T¹⁴ N3H→A⁹ H2; g2, T¹⁴ N3H→A¹⁰ H2; g3, T¹⁴ N3H→G⁸ N²H1; g4, T¹⁴ N3H→G⁸ N²H2; h1, T¹⁶ N3H→Y⁷ H2; i1, T¹³ N3H→A¹⁰ H2. The experiment was carried out at 10 °C.

Figure 3.

Expanded NOESY spectrum showing the assignments for the butadiene protons in the (*S,S*)-BD-(61-2,3) duplex. The experiment was carried out at 250 ms mixing time and 800 MHz. The temperature was 25 °C.

Figure 4.

Tile plot showing NOE cross-peaks between BD and DNA of the (*S,S*)-BD-(61-2,3) cross-linked duplex. Cross-peaks a, C⁵ H5 → BD H_α; b, C⁵ H5 → BD H_{α'}; c, T¹⁷ N3H → BD H_α; d, T¹⁷ N3H → BD H_{α'}; e, T¹⁷ N3H → BD H_β. The experiment was at 250 ms mixing time and 800 MHz.

A

B

Figure 5.

Chemical shift differences of protons of the (*S,S*)-BD-(61-2,3) cross-linked adduct duplex relative to the unmodified *ras61* oligodeoxynucleotide. **A.** Nucleotides C¹ → G¹¹ of the (*S,S*)-BD-(61-2,3) cross-linked duplex. **B.** Nucleotides C¹² → G²² of the (*S,S*)-BD-(61-2,3) cross-linked duplex. Black bars represents the deoxyribose H1' protons; gray bars represent the purine H8 or pyrimidine H6 protons, respectively; white bars represent the cytosine H5 protons.

Figure 6.

A. Stereoview of superimposed conformation I structures of the (*S,S*)-BD-(61-2,3) cross-linked duplex emergent from the simulated annealing rMD protocol. **B.** Stereoview of superimposed conformation II structures of the (*S,S*)-BD-(61-2,3) cross-linked duplex emergent from the simulated annealing rMD protocol. The structures resulted from randomly seeded calculations.

Figure 7.

Sixth root residuals between calculated NOE intensities and experimental NOE intensities (R_1^x values) as a function of position in the (*S,S*)-BD-(61-2,3) adduct. **A.** Nucleotides C¹ → G¹¹ of conformation I with hydrogen bonding between β-OH and T¹⁶ O⁴. **B.** Nucleotides C¹² → G²² of conformation I. **C.** Nucleotides C¹ → G¹¹ of conformation II with hydrogen bonding between β-OH and T¹⁷ O⁴. **D.** Nucleotides C¹² → G²² of conformation II.

Figure 8. Stick models of the (*S,S*)-BD-(61-2,3) cross-linked duplex predicted by rMD calculations. **A.** Conformation I with hydrogen bonding between β -OH and T¹⁷ O⁴. **B.** Conformation II with hydrogen bonding between β -OH and T¹⁶ O⁴. The butadiene moiety is in red.

Figure 9.

Views from the major groove of the duplex DNA of the (S,S)-BD-(61-2,3) and the (R,R)-BD-(61-2,3) cross-linked adduct duplexes at the lesion site predicted by rMD calculations, as compared to the unmodified *ras61* oligodeoxynucleotide duplex. **A.** Conformation I of the (S,S)-BD-(61-2,3) cross-linked duplex detailing base pairs X⁶.T¹⁷ (green) and Y⁷.T¹⁶ (purple). **B.** Conformation II of the (S,S)-BD-(61-2,3) cross-linked duplex detailing base pairs X⁶.T¹⁷ (green) and Y⁷.T¹⁶ (purple). **C.** The (R,R)-BD-(61-2,3) cross-linked duplex detailing base pairs X⁶.T¹⁷ (green) and Y⁷.T¹⁶ (purple). **D.** The unmodified *ras61* duplex detailing base pairs A⁶.T¹⁷ (green) and A⁷.T¹⁶ (purple). The butadiene moiety is in red.

Figure 10.

Base stacking of the (*S,S*)-BD-(61-2,3) and the (*R,R*)-BD-(61-2,3) cross-linked duplexes at the lesion site predicted by rMD calculations, as compared to the unmodified *ras61* duplex. **A.** Conformation I of the (*S,S*)-BD-(61-2,3) cross-linked duplex detailing stacking of the base pairs X⁶.T¹⁷ (green) and Y⁷.T¹⁶ (purple). **B.** Conformation II of the (*S,S*)-BD-(61-2,3) cross-linked duplex detailing stacking of the base pairs X⁶.T¹⁷ (green) and Y⁷.T¹⁶ (purple). **C.** The (*R,R*)-BD-(61-2,3) cross-linked duplex detailing stacking of the base pairs X⁶.T¹⁷ (green) and Y⁷.T¹⁶ (purple). **D.** The unmodified *ras61* duplex detailing stacking of the base pairs A⁶.T¹⁷ (green) and A⁷.T¹⁶ (purple). The butadiene moiety is in red.

Scheme 1.
Reactive Metabolites of Butadiene.

A.

B.

Scheme 2.

The *ras61* oligodeoxynucleotide (A), and the chemical structure of (B) the (2*S*,3*S*)-N⁶-(2,3-dihydroxybutyl)-2-deoxyadenosyl cross-linked adduct and nomenclature.

Table 1

Analysis of the rMD-Generated Structures of the ras61 (*S,S*) *N*⁶,*N*⁶-dA Cross-Linked Adduct.

NMR restraints	
total no. of distance restraints	413
interresidue distance restraints	69
intraresidue distance restraints	339
DNA-cross-link distance restraints	5
empirical restraints	
H-bonding restraints	39
sugar pucker restraints	90
backbone torsion angle restraints	104
Structural statistics	
NMR R-factor (R_1^x) ^{a,b,c}	
<rMDIi>	0.0903 ± 0.0140
<rMDIIj>	0.0848 ± 0.0142
rmsdI of NOE violations (Å)	0.0200
rmsdII of NOE violations (Å)	0.0195
pairwise rmsd (Å) over all atoms	
<rMDIi> vs <rMDIav>	0.553 ± 0.13
<rMDIIj> vs <rMDIIav>	0.559 ± 0.17

^aThe mixing time was 250 ms.

^b $R_1^x = \sum | (a_O)_i^{1/6} - (a_C)_i^{1/6} | / \sum | (a_O)_i^{1/6} |$, where a_O and a_C are the intensities of observed (non-zero) and calculated NOE cross-peaks.

^c<rMDIi>, 5 converged structures of conformation I starting from randomly seeded calculations; <rMDIIj>, 7 converged structures of conformation II starting from randomly seeded calculations; <rMDIav>, average of 5 converged structures of conformation I; <rMDIIav>, average of 7 converged structures of conformation II; rmsdI, rmsd of conformation I; rmsdII, rmsd of conformation II.