Solution Structures of Psoralen Monoadducted and Cross-Linked DNA Oligomers by NMR Spectroscopy and Restrained Molecular Dynamics$^{1,2}$

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ABSTRACT: We have used two-dimensional $^1$H NMR spectroscopy to determine the solution structures of the 4'-[(hydroxymethyl)-4,5',8-trimethylpsoralen (HMT) furanside monoadduct (MAf) and the photo-isomeric HMT interstrand cross-linked (XL) DNA oligonucleotide d(5'-GGCTAGCG-3')$_2$. The determination of the structure was based on total relaxation matrix analysis of the NOESY cross-peak intensities using the program MARDIGRAS. Improved procedures to consider the experimental “noise” in NOESY spectra during these calculations have been employed. The NOE-derived distance restraints were applied in restrained molecular dynamics calculations. Twenty final structures each were generated for both the MAf and XL from both A-form and B-form dsDNA starting structures. The root-mean-square (rms) deviations of the coordinates for the 40 structures for the MAf and XL were 1.12 and 1.10 Å, respectively. The rmsd of the MAf with respect to the XL is 2.20 Å. The local DNA structure is distorted in both adducts, with the helix unwound by 34° and 25° for the MAf and XL, respectively, and an overall helical repeat of 11 base pairs, caused by intercalation of the HMT. The MAf is a photochemical intermediate on the path to interstrand XL. Considerable local structural distortion is induced by both adducts, but the DNA returns to B-form structure within three base pairs of the damage site. There is no significant bend in the helix axis of either the MAf or the XL. We have evaluated the accuracy of the two major methods of converting NOESY data into interproton distances, the isolated spin-pair approximation (ISPA) and the complete relaxation rate matrix analysis (RMA). Both methods were evaluated by comparing the resulting calculated interproton distances generated to known covalently fixed distances in the HMT. The overall structures were evaluated by checking their agreement with biophysical evidence from non-NMR techniques. Only the modified RMA method gave correct interproton distances.

Psoralens are a class of photomutagenic (Cassier et al., 1984; Fujita, 1984; Misra & Vos, 1993; Sage et al., 1993; Yang et al., 1994) and photochemotherapeutic agents (Corash et al., 1981; Morel et al., 1992; Lin et al., 1993) that produce monoadducts and interstrand cross-links in DNA and RNA capable of blocking replication (Shi et al., 1988a,b) and transcription (Sastry et al., 1993; Huang et al., 1994). Psoralens have been used for the treatment of vitiligo, psoriasis, and other cutaneous diseases (Fitzpatrick et al., 1982; Pathak & Fitzpatrick, 1992). Thymidine is the primary nucleoside in DNA at which photoreaction occurs. The reaction takes place between the 3,4 (pyrone) or 4',3' (furan) double bonds of the psoralen and the 5,6 double bond in pyrimidines (Staub et al., 1981; Kanne et al., 1982a,b; Shi et al., 1990). The effects of psoralen adducts on the structure and function of DNA have been extensively studied (del Olmo et al., 1993; Sastry et al., 1993; Huang & Sancar, 1994; Islas et al., 1994; Ramaswamy & Yeung, 1994). Previous studies have shed light on the mechanism of psoralen adduct formation and their biological consequences (Van Houten et al., 1986a, 1988; Sancar & Sancar, 1988; Smith, 1988; Cheng et al., 1988; Sladek et al., 1989; Smith et al., 1989).

4'-(Hydroxymethyl)-4,5',8-trimethylpsoralen (HMT,$^1$ 1), is a synthetic psoralen derived from the natural product 4,5',8-trimethylpsoralen (TMP). HMT has greatly enhanced water solubility relative to TMP and forms high yields of adducts with DNA. The structures of the three main adducts between an isolated thymidine nucleoside and HMT (Cimino

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$^2$ Coordinates for the HMT furanside monoadducted (MAF) and the HMT interstrand cross-linked (XL) DNA oligonucleotide d(5'-GGCTAGCG-3')$_2$ have been deposited in the Brookhaven Data Bank under Identifier codes 203D and 204D.

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1 Abbreviations: AMBER, assisted model building with energy refinement; dsDNA, double-stranded DNA; EDTA, ethylenediaminetetraacetic acid; MARDIGRAS, matrix analysis of relaxation for discerning the geometry of an aqueous structure; MOPAC, molecular orbital package; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; RANDMARDI, randomized MARDIGRAS; RMD, restrained molecular dynamics; TPPI, time-proportional phase incrementation; HMT, 4'-(hydroxymethyl)-4,5',8-trimethylpsoralen; AMT, 4'-(aminomethyl)-4,5',8-trimethylpsoralen; TMP, 4',5',8-trimethylpsoralen; ISPA, isolated spin-pair approximation; COSY, correlation spectroscopy; UM, unmodified DNA oligomer 5'-GGCTAGCG-3'; MAf, psoralen furanside monoadduct; XL, psoralen interstrand cross-link; DQF-COSY, double-quantum-filtered correlation spectroscopy; RMA, relaxation rate matrix analysis.
et al., 1985) are shown in Figure 1. The photoadducts have a cis-syn stereochemistry about the cyclobutane rings determined by the noncovalent intercalation complex. The reaction of psoralen with DNA has been proposed to take place via the intercalation of the planar psoralen into double-stranded nucleic acid, followed by absorption of a long-wavelength UV photon (320–410 nm) causing the psoralen to react with pyrimidine bases to form either a furanside or pyroneside monoadduct. The yield of furanside monoadduct relative to pyroneside adduct in DNA depends upon the presence or absence of the psoralen 4-methyl group (Kanne et al., 1984). The predominant monoadduct formed by psoralens with a 4-methyl group is the furanside monoadduct. An interstrand cross-link can be formed by absorption of a second photon by the furanside monoadduct in a site with an adjacent pyrimidine base on the opposite strand of double-stranded DNA (or RNA). Pyroneside monoadducts do not absorb light at wavelengths above 320 nm and cannot be driven to interstrand cross-links with long-wavelength UV light. All of the psoralen–DNA adducts can be photoreversed by exposure to 254 nm UV light (Cimino et al., 1986) or, in the case of either furanside monoadduct and cross-link, by treatment with base (Shi et al., 1988a; Yeung et al., 1988). The presence of a psoralen monoadduct or an interstrand cross-link stabilizes the helix to thermal denaturation relative to unmodified DNA (Shi & Hearst, 1986).

There are conflicting reports in the literature regarding the structural consequences of psoralen-induced damage to dsDNA. Flow linear dichroism provided evidence for pseudointercalative adduct geometry of the furanside monoadduct (Vigny et al., 1987). Models based on the structure of the isolated furanside psoralen–thymine monoadduct led to the proposal that a psoralen cross-link induces a sharp kink in and a large unwinding of the DNA duplex (Pearlman et al., 1985). A model derived from NMR data for the octamer d(5'-GGGTACCC-3')2 cross-linked with the psoralen AMT [4'-aminomethyl]-4,5',8-trimethylpsoralen] exhibited a kink at the site of photoalkylation (Tomic et al., 1987). Such results contradict studies (Sinden & Hagerman, 1984; Haran & Crothers, 1988) in which psoralen cross-links did not alter the mobility of DNA fragments in a gel shift assay, indicating that the cross-links do not induce a bend into the DNA helix axis.

Due in part to the controversy surrounding the solution structure of the XL and the lack of structural information about the MAf, we have determined the structures of the HMT furanside monoadduct of d(GCGTACGC)2 (MAf) and the HMT interstrand cross-link d(GCGTACGC)2 (XL) using 1H NMR. We recently reported a preliminary account of the structural and functional characterization of these molecules (Spielmann et al., 1995a). NOESY spectra were used to obtain interproton distance estimates using the RANDMARDI procedure (H. Liu, H. P. Spielmann, N. B. Ulyanov, D. E. Wemmer, and T. L. James, unpublished results), a variation of the MARDIGRAS complete relaxation matrix approach (Borgias & James, 1988; Borgias et al., 1990; Schmitz et al., 1992). Distance estimates derived using this approach were then used as restraints in molecular dynamics simulations (RMD). Since many NOE contacts were observed between the HMT adducts and the surrounding DNA, the resulting structures have fairly high resolution and allowed determination of local conformational features in the DNA structure after HMT photobinding. We discuss the conformational and dynamic changes that the DNA undergoes upon adduct formation, and we also discuss the conformation of the MAf with respect to interstrand cross-link formation.

We have evaluated the accuracy of the two major methods of converting NOESY data into interproton distances, the isolated spin-pair approximation (ISPA) and complete relaxation matrix analysis. The HMT-modified DNAs are unusual in that there are a significant number of covalently fixed proton pairs spanning the range of distances observable by the NOE. These provide a very important test for the evaluation of the accuracy of the determined distances.

**EXPERIMENTAL PROCEDURES**

**Materials.** The DNA oligomers were synthesized on a 10-mol scale using the phosphite triester method on an Applied Biosystems Inc. (Foster City, CA) automated DNA synthesizer. The DNA oligomer samples were cleaved from the solid support and deprotected using standard methods.

We use the following numbering scheme to describe the UM, MAf, and XL:

5' - G1 C2 G3 T4 A5 C6 G7 C8 -3'  
3' - C16 G15 C14 A13 T12 G11 C10 G9 -5'

**Synthesis of Psoralen-Modified DNAs.** The MAf and XL were synthesized and purified as described (Spielmann et al., 1992). Single-stranded MAf was formed by irradiation of an HMT + d(5'-GGGTACGC-3')2 solution with 406.7 nm light from a krypton ion laser. The resulting MAf did not absorb light at the irradiation wavelength, allowing it to accumulate in the reaction without formation of a cross link. A single isomer of the MAf was produced in the synthesis. The furanside monoadducted DNA was separated from the unmodified DNA using a multistep HPLC purification procedure. The XL was formed by irradiation of an HMT + d(5'-GGGTACGC-3')2 solution with 365 nm UV light from an argon ion laser. The major product was separated from the reaction mixture using a multistep HPLC purification procedure.

**NMR Sample Preparation.** (1) Unmodified DNA (UM). The purified octamer duplex was dissolved in 500 μL of 10 mM phosphate buffer (pH 7) containing 200 mM NaCl.
lyophilized several times from D$_2$O, and finally dissolved in 500 $\mu$L of 99.996% D atom D$_2$O. The resulting NMR sample was 6 mM in UM.

(2) MAf. In order to prepare a homogeneous NMR sample of the double-stranded MAf heteroduplex, the HMT monoadduced DNA strand must be hybridized with its unmodified complement. This is possible because the MAf heteroduplex is approximately 1.3 kcal/mol more stable than the unmodified DNA duplex and 3.1 kcal/mol more stable than the doubly modified DNA homoduplex (Shi & Hearst, 1986). Formation of the MAf heteroduplex required annealing one monoadduced octamer strand and one unmodified d(5'-GGCTACGCG-3') (UM) strand. The purified, single-stranded monoadduced oligomer was taken up in 80 $\mu$L of 1 M NaCl. The UM duplex was dissolved in 200 mM NaCl/10 mM Tris-HCl-d$_{11}$ buffer (pH 7.0), lyophilized from D$_2$O repeatedly, and finally taken up in 200 $\mu$L of 20 mM Tris-HCl-d$_{11}$ buffer (pH 7.0)/400 mM NaCl and then lyophilized. Each sample was lyophilized several times from D$_2$O, and the octamers MAf and UM were dissolved in 400 and 70 $\mu$L of 99.996% D atom D$_2$O, respectively. The single-stranded octamer MAf was then titrated with the more concentrated UM to give duplex DNA. The titration was monitored by observing the appearance of two resonances assigned to the C8 protons of the adenines in the $^1$H NMR spectrum in D$_2$O.

Additions of the UM duplex were made until the resonances corresponding to single-stranded MAf disappeared. The resulting sample was 8 mM in MAf heteroduplex, 200 mM in NaCl, and 10 mM in Tris-HCl-d$_{11}$ buffer (pH 7.0).

(3) XL. The purified XL was dissolved in 500 $\mu$L of 10 mM phosphate buffer (pH 7) containing 200 mM NaCl, lyophilized several times from D$_2$O, and finally dissolved in 500 $\mu$L of 99.996% D atom D$_2$O. The resulting NMR sample was 6 mM in XL.

Proton NMR Studies. Phase-sensitive two-dimensional proton NOESY spectra in D$_2$O were recorded on a Bruker AMX 600 MHz NMR spectrometer. Proton chemical shifts were indirectly referenced using the residual HOD resonance. The two-dimensional NOESY spectra in D$_2$O were recorded on a Bruker AMX 600 MHz NMR spectrometer. A spectral width of 4000 Hz in both t$_2$ and t$_1$ was used, with a repetition delay of 2.5 s; 64 scans were averaged per t$_1$ value: 512 FIDs were recorded in t$_1$ and 2048 points in t$_2$. Soft-COSY experiments were run as described (Emsley et al., 1993).

Calculation Methods. (A) Distance Constraints. Cross-peaks in the NOESY spectra arise from dipole-dipole relaxation between nuclei that are within ca. 5 Å. The intensities of these NOE cross-peaks provide information about the internuclear distance based on the initial NOE buildup relationship:

$$\text{NOE} = f(t_c) \frac{1}{r^6}$$

where $f(t_c)$ is a function of the correlation time $t_c$ and represents the influence of molecular motion on the observed NOE. Given a known distance $r_{ij}$, an unknown distance $r_{kl}$ can be directly calculated from the relationship:

$$r_{ij} = r_{kl} \left( \frac{\text{NOE}_{kl}}{\text{NOE}_{ij}} \right)^{1/6}$$

where NOE$_{kl}$ and NOE$_{ij}$ are the NOEs between atoms $k$ and $l$ and atoms $i$ and $j$, respectively. The validity of these equations is limited. Equation 1 is valid only for two-spin relaxation. In practice, NOE cross-peaks result from multispin relaxation. Relaxation matrix methods have been developed to account for multispin effects. The $f(t_c)$ term in eq 1 may be canceled in eq 2 only under the assumption that the molecule tumbles as a rigid isotropic unit where all interproton vectors have the same effective correlation time. In most cases, macromolecules are not rigid bodies, and internal motions in the molecule cause different effective correlation times for different interproton vectors. This assumption leads to an estimated error of 10% in the calculated distances (Borgias & James, 1988).

The integrated volumes of symmetry-related cross-peaks above and below the diagonal were treated as independent measurements to more accurately reflect the scatter in the data. For the MAf, this gave 10 sets of integrated cross-peak intensities, two for each of the five mixing times, and for the XL, this gave 8 sets of integrated crosspeak intensities, two for each of the four mixing times. Not all cross-peaks were present at all mixing times. The spin-lattice relaxation rates for A5 H2 and A13 H2 and other representative protons were measured by the inversion recovery method. The measured intensities of the NOESY cross-peaks were scaled for the effects of partial saturation according to $A = 1/(1 - \exp(-T/T_1))$, where $A$ is the correction factor, $T$ is the recycle delay for the experiment, and $T_1$ is the longitudinal relaxation rate for the proton in question from which the magnetization originated.

(B) Data Analysis. The data were converted into interproton distances by three different methods, the two-spin approximation, the MARDIGRAS iterative relaxation matrix method, and the RANDMARDI modification of MARDIGRAS.
(1) Isolated Spin-Pair Approximation (ISPA). The volume for a given cross-peak as a function of the mixing time was fitted to a second-order polynomial function that was forced through zero time and zero intensity. The slope of the curve at zero mixing time was taken to be the buildup rate for that NOE. This was repeated for each measured cross-peak. The interproton distances were calculated using eq 2, where \( r_{ij} \) was a known distance, for example, the sugar \( H2' - H2'' \) (1.8 Å) or cytidine \( H5 - H6 \) (2.52 Å).

(2) Relaxation Matrix Approach (MARDIGRAS). The MARDIGRAS method was used to interpret the NOE data (Borgias & James, 1988; Borgias et al., 1990). The distances returned by MARDIGRAS for the data sets were averaged, and standard deviations for the measurements were calculated using the program CALSD (Schmitz et al., 1992). A total of 342 experimentally measured NOE intensities were included in the MARDIGRAS calculation of the MAf data, and 327 were used in the XL. MARDIGRAS uses internal calibrations when it iteratively fits the data to a model of the molecule. In particular, it resets the cytosine \( H5 \) to \( H6 \), the thymidine 5-methyl to \( H6 \), and deoxyribose \( H2' \) to \( H2'' \) interproton distances to the values assumed from the covalent geometries. NOE intensities for these known distances are used to normalize the NOE intensities in the model. The program does not reset fixed interproton distances within the HMT adducts because these distances were intentionally left out of the reference file. The magnetization transfer for these protons is calculated and provides a check of how well the MARDIGRAS procedure is working. Agreement between the actual and the experimentally determined distances indicates that the MARDIGRAS procedure yields more accurate interproton distances than the ISPA procedure (see discussion below).

(3) RANDMARDI Procedure. The RANDMARDI procedure (Liu et al., unpublished results) of the complete relaxation matrix analysis method MARDIGRAS was used to calculate interproton distance RMD bounds from the resulting integrated peak intensities. The RANDMARDI procedure was used to include the effects of experimental noise in the relaxation matrix calculations. RANDMARDI adds a random number from within a specified range to each input intensity used in a MARDIGRAS calculation. For the calculations performed on the HMT adducts a noise level of 1–5 times the integrated intensity of the smallest cross-peak was used as the “noise” range. The dynamic range of observed cross-peak intensities was 1000. In the RANDMARDI procedure, 30 different intensity sets from each experimental data set were generated, and MARDIGRAS calculations were performed on all of them. Resulting distances were then averaged together to form one bounds file for each data set. The bounds files from all of the mixing times were then combined into a single bounds file from which the RMD restraint file is generated. Upper and lower bounds were the average interproton distances \( \pm 1 \) SD calculated from all of the MARDIGRAS runs. A complete description of the RANDMARDI theory and procedure will be given elsewhere (Liu et al., unpublished results). Good agreement between the actual and the experimentally determined distances indicates that the RANDMARDI procedure yielded the most accurate interproton distances of the three methods tested (see Tables 1 and 2 and discussion below).

(C) Starting Structures. The starting models for the refinement were generated using standard Arnott parameters for A-form and B-form DNA in the Biopolymer module of InsightII (version 2.3.0) (Biosym Technologies, San Diego) and assigned potentials using the AMBER force field. The HMT moiety was created using the Builder module of InsightII, and partial charges were calculated using semiempirical AM1 parameters as implemented by the MOPAC module of InsightII. The furanside monoadduct was assembled by covalently bonding the HMT with thymidine T4 in the oligomer and then editing the atom types and connectivities to form the cyclobutane ring. For the crosslink, a second covalent modification was made between the pyrone ring of the HMT and thymidine T12. Starting models were generated by energy minimizing the MAf and XL models using only the AMBER force-field function of the Discover module (version 2.9.5) of InsightII with 100 steps of steepest descents minimization, followed by conjugate gradient refinement to an rmsd of 0.01 Å. The distance bounds generated using the RANDMARDI procedure were then applied to the models, which were minimized again to arrive at the starting structures. The five methyl groups of the psoralen and thymidines were represented as pseudoatoms at the geometric center of mass of the three protons.

(D) Restrained Molecular Dynamics. The distance restraints obtained from the RANDMARDI calculations were incorporated into an RMD procedure. The RMD and energy minimization calculations were performed using Discover. The models were displayed using InsightII. The NOE-derived distance restraints were applied to the models.
followed by 20 ps of restrained molecular dynamics at 350 K for 4 ps, followed by warming to 400 K for 4 ps and then cooling to 200 K in 50 K steps of 3 ps each. The final structure was then energy minimized to a maximum derivative of 0.01 Å. The pseudoenergy term used to enforce the distance constraints was

$$E_{\text{constr}} = \begin{cases} k_1(r_2 - r_1)^2 & \text{when } r_2 \geq r_1 \\ 0 & \text{when } r_1 \geq r \geq r_2 \\ k_2(r_3 - r_2)^2 & \text{when } r_3 \geq r_1 \\ 
$$

where \( r_2 \) and \( r_1 \) define the upper and lower bounds of the flat region of the potential, corresponding to the distance bounds derived from the cross-peak volumes, and \( k_1 \) and \( k_2 \) are force constants that could be independently assigned to each constraint. All of the NOE-derived distance constraints were assigned an upper and lower bound force constant of 50 kcal/(mol·Å²). The width of the flat region of the potential well reflects the accuracy of a given constraint (see below). An additional 18 distance constraints were included to enforce Watson-Crick hydrogen bonding throughout the calculations. Three hydrogen bonds were included for each of the six G-C base pairs where the upper and lower margins were set to 1.7–2.1 Å. No hydrogen bond restraints were used for the A-T base pairs. Water and counterions were ignored in all calculations. Helix parameters were calculated with the program CURVES 3.1 (Lavery & Sklenar, 1988, 1989).

RESULTS

Spectral Analysis and Assignment of Exchangeable DNA Protons. Melting studies were performed to determine the stability of the helix in the adducted DNAs. One-dimensional NMR spectra in H2O were acquired at various temperatures while observing the imino proton resonances (Figure 2). Sharp imino resonances imply protection from exchange due to hydrogen bonds. The imino proton resonances were assigned by 1D NOE experiments and confirmed by NOESY experiments performed in H2O.

(1) MAf. A single species was observed when the adducted strand of the MAf was titrated with UM in the NMR to form heteroduplex DNA. This confirms the observation by Shi and Hearst (1986) that the presence of a psoralen monoadduct stabilizes a duplex. It is commonly expected that intercalation induces a large shift (≈0.5–1 ppm) (Assa-Munt et al., 1985a,b) on imino proton resonances adjacent to the binding site. The T4 imino proton resonance is sharp and shifted upfield approximately 2 ppm relative to the T4 imino of the free DNA. The larger than expected upfield shift is a consequence of the formation of the cyclobutane linkage between the psoralen and T4. A similar large upfield shift was observed for the imino proton resonances of a thymine dimer in a duplex DNA (Taylor et al., 1990). In the crystal structure of the isolated MAf nucleoside, the imino proton is directly over the center of the psoralen benzene ring (Peckler et al., 1985). The upfield shift is likely due in part to a ring current effect. The T12 imino is slightly broader and resonates at 13.2 ppm. The G1 and G9 imino resonances broaden with increasing temperature and become unobservable at 35 °C. The remaining six imino resonances all broaden with increasing temperature in a homogeneous fashion. The T4 imino resonance remains the sharpest signal in the spectrum even at 50 °C. These data indicate that normal hydrogen bonds are formed in the MAf. The decrease in the signal-to-noise ratio of the higher temperature spectra in Figure 2A results from broadening due to melting of the DNA duplex.

(2) XL. The imino spectrum of the XL shows more symmetry than the imino spectrum of the MAf (Figure 2B). The imino resonances of both T4 and T12 in the XL are shifted upfield by 2 ppm relative to free DNA as a consequence of forming the cyclobutane rings of the adduct. There is a marked asymmetry in the stability of the helix on the two sides of the adduct in the HMT cross-linked (GCGTACGC)3. The two base pairs on the pyrimidine side of the adduct are destabilized relative to both normal DNA and the furan side base pairs. The T12 imino begins to broaden at 20 °C and becomes unobservable at 40 °C. The adjacent G11 imino resonance begins to broaden at 40 °C and is unobservable at 50 °C. The T4 imino proton resonance remains sharp even at 50 °C. The G3 imino resonance is sharp and only shows broadening above 50 °C. Chemical shifts indicate that the DNA returns to a normal state three base pairs from the site of adduction on both sides of the psoralen since at that point they are equivalent to UM. We interpret these data to mean that a hydrogen bond with enhanced stability is formed between T4 and A13, while a weak or nonexistent hydrogen bond is formed between T12 and A5. The hydrogen bonds for G3 are stabilized relative to those for G7 and G15. We ascribe a normal hydrogen-bonding pattern to the G3-C14 , G11-C6 , G7-C10 and the G15-C2 base pairs. Similar results were observed for the AMT crosslinked oligomer (GGGTACCC)2 (Tomic et al., 1987). Hence, the localized destabilization of hydrogen
bonding on the pyrone face of the cross-link appears to be a general result for psoralen adducts and not a function of the particular psoralen used.

Assignment of Nonexchangeable DNA Protons. The NOESY spectra for the MAf in Figure 3A and for the XL in Figure 3B exhibit the characteristic features of dsDNA sequential connectivities from aromatic H6/H8 protons to both intra- and interresidue H1' and H2'/'"'. However, the sequential connectivities are interrupted in both molecules at the 5'-TpA-3' base pair step. This is clear evidence of intercalation by the HMT adduct in both the MAf and XL. Figure 3 also includes a few of the observed cross-peaks between the HMT adducts and the oligonucleotide. Aromatic (H6, H5, H8, and H2) and most of the deoxyribose proton (H1', H2', H2", H3', H4', and H5'/H5") resonances of dsDNA in the MAf and XL were assigned in the conventional way (Hare et al., 1983; Wuthrich, 1986). In normal B-form DNA, the H5' proton of the deoxyribose has a close contact with the H1' of the base on its 5' face. It is possible to distinguish some of these cross-peaks from other cross-peaks in the same region of the spectrum in the short mixing time NOESY spectrum. The proton resonance assignments for the MAf and XL are given in Table 3.

Intercalation Site of HMT. The d(GCGTACGC)Z duplex (UM) has only eight spectroscopically distinct residues due to its self-complementary nature. The 2-fold symmetry of the UM duplex is broken by HMT adduct formation in the MAf and XL. In the MAf, the HMT is covalently attached to aromatic part of the NOESY spectrum (250 ms mixing time) of the (A) MAf and (B) XL. The sequential H1'—H6/H8—H1' connectivity pathway for the furanside strand is indicated by a solid line, and the connectivity pathway for the pyroneside strand is indicated by a dotted line. The interrupted connectivities at the 5'-TpA-3' base pair step due to intercalation are indicated.

Table 3: Chemical Shift Values of the HMT MAf and XL of d(GCGTACGC)Z at 25 °C

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*The values are given relative to the HOD signal at 4.78 ppm.*

Assignment of Nonexchangeable DNA Protons. The NOESY spectra for the MAf in Figure 3A and for the XL in Figure 3B exhibit the characteristic features of dsDNA sequential connectivities from aromatic H6/H8 protons to both intra- and interresidue H1' and H2'/'"'. However, the sequential connectivities are interrupted in both molecules at the 5'-TpA-3' base pair step. This is clear evidence of intercalation by the HMT adduct in both the MAf and XL. Figure 3 also includes a few of the observed cross-peaks between the HMT adducts and the oligonucleotide. Aromatic (H6, H5, H8, and H2) and most of the deoxyribose proton (H1', H2', H2", H3', H4', and H5'/H5") resonances of dsDNA in the MAf and XL were assigned in the conventional way (Hare et al., 1983; Wuthrich, 1986). In normal B-form DNA, the H5' proton of the deoxyribose has a close contact with the H1' of the base on its 5' face. It is possible to distinguish some of these cross-peaks from other cross-peaks in the same region of the spectrum in the short mixing time NOESY spectrum. The proton resonance assignments for the MAf and XL are given in Table 3.

Intercalation Site of HMT. The d(GCGTACGC)Z duplex (UM) has only eight spectroscopically distinct residues due to its self-complementary nature. The 2-fold symmetry of the UM duplex is broken by HMT adduct formation in the MAf and XL. In the MAf, the HMT is covalently attached...
The HMT in the XL is attached to the DNA on the furanside by a cyclobutane ring to T4 and on the pyroneside by a cyclobutane ring to T12. The strand-specific assignments of the unsymmetrical modified DNAs were resolved by observation of NOE connectivities between the psoralen methyl protons and the adenosine H8 protons. Although the regular sequential path in NOESY spectra is interrupted at the intercalation site, there is a sequential path across the two strands through the psoralen. There is only a single possible path of each type, conclusively defining the intercalation site. These results indicate that the HMT is intercalated in the central 5'-TpA-3' site in both adducted molecules.

Formation of the MAf causes the H6 proton of the T4 thymidine to undergo a dramatic upfield shift from 7.3 to 3.9 ppm. The H6 protons of T4 and T12 in the XL undergo upfield shifts from 7.3 to 3.9 and 4.4 ppm, respectively.

In both molecules, the corresponding resonances for the terminal pseudo-symmetry-related G1-C16 and C8-G9 base pairs were degenerate, and most of the corresponding pseudo-symmetry-related resonances for the C2-G15 and G7-C10 base pairs were also degenerate.

Assignment of the HMT Protons. NOEs from the HMT H5 proton to the 4-methyl group and to the HMT H3 protons of the psoralen assigned the pyrone ring protons. In the NOESY spectra of both the MAf and XL there are cross-peaks from HMT H5 to the diastereotopic 4'-hydroxymethyl protons on the furan ring, the T4-methyl and spin diffusion mediated cross-peaks to both the HMT 5'-methyl protons and the HMT 8-methyl protons.

Intermolecular HMT-DNA Contacts. It was possible to assign many NOEs between the HMT and DNA protons in the adducts. There were 47 NOEs from the adduct to the surrounding bases and sugar residues in the MAf and 49 in the XL. Cross-strand NOEs are uncommon in regular DNA, arising only from AH2 to AH2 and H1' cross-peaks in suitable sequences. There are 15 cross-strand NOEs from the psoralen to the opposite strand bases and sugar residues in the MAf. The presence of the cross-strand NOEs confirms the intercalated nature of the MAf and tightly constrains the conformation of the damaged region of the DNA. The HMT is localized relative to the unmodified strand by NOEs from the 4-methyl group to T12 H6, to A13 H8, and to the T12 methyl group. The HMT 8-methyl shows prominent NOEs to both the A5 H2 and A13 H2. NOEs between the 4'-hydroxymethyl protons and T4 H6 confirm the presence of the cyclobutane ring and its cis-syn geometry. In the XL, the covalent geometry of the adduct imposes a fixed pattern of NOEs that arise from the substituents on the pyroneside cyclobutane ring in addition to the furanside resonance pattern seen in the MAf. The covalent structure of the cross-link fixes the orientation and position of T4 relative to T12 and bridges the helix. The protons of the T4-HMT-T12 adduct give rise to many NOEs to the surrounding bases, and the orientation of the interstrand adduct in the helix is tightly constrained relative to the other bases. The pattern of NOEs found in the XL is similar to that for the MAf. However, the intensities of corresponding cross-peaks are different in the two molecules. This reflects the different interproton distances and geometries in the MAf and XL.

Structure Calculation. More than 400 NOE cross-peaks were observed in the NOESY spectrum for both the MAf and XL obtained with a mixing time of 250 ms. Some of them resulted predominantly from spin diffusion and were consequently not observed in NOESY spectra with shorter mixing times. The accuracy of the integration of some cross-peaks was hampered by spectral overlap. A summary of observed NOEs used in the structure determination are included in Tables 4 and 5.

Distance Restraints. In both the MAf and XL there are well-resolved direct and spin diffusion mediated NOEs among the psoralen protons. There is no ambiguity in the distances separating these protons due to the rigid covalent geometry of the psoralen adducts. The NOE intensities of cross-peaks arising from protons that are known to be a fixed distance apart were used to check the accuracy of the calculated distances. The HMT MAf and XL systems
contains covalently determined distances that span the range of observable NOE contacts from 2.35 to 6.94 Å. The HMT-thymidine adduct has a variety of single protons and methyl groups that cross-relax each other. These psoralen protons are also cross-relaxed by the nearby protons in the DNA that are not rigidly connected to the adduct. For the MAF, there were 67 NOEs involving the HMT-T4 base unit protons, 52 NOEs from the HMT-T4 base unit to the surrounding DNA, and 15 inter-HMT-T4 NOEs. For the XL, a total of 90 NOEs involved the T12-HMT-T4 adduct, 61 NOEs from the T12-HMT-T4 unit to the surrounding DNA, and 29 intra-HMT-T4 NOEs. For the MAF, 61 NOEs from the T12-HMT-T4 unit to the surrounding DNA, and 15 intra-HMT-T4 NOEs. For the MAf, there were 67 NOEs involving the HMT-T4 base unit protons, 52 NOEs from the HMT-T4 base unit to the surrounding DNA, and 15 inter-HMT-T4 NOEs. For the XL, a total of 90 NOEs involved the T12-HMT-T4 adduct, 61 NOEs from the T12-HMT-T4 unit to the surrounding DNA, and 29 intra-HMT-T4 NOEs.

(1) **ISPA.** This method failed to produce a model for the MAF or the XL that was able to fit the observed NOE intensities with the covalently fixed distances in the HMT because it does not account for spin diffusion. Some of the distances calculated were up to 1.3 Å too short. A number of cross-peaks in the MAF and XL NOESY spectra become visible only at the longer (175 or 250 ms) mixing times resulting from magnetization transfer due to spin diffusion. The assumption that NOEs result only from direct magnetization transfer is inappropriate only for NOEs recorded with very short mixing times where signal to noise is poor. The use of the two-spin approximation is inappropriate for commonly used mixing times in cases where there is spin diffusion. Failing to compensate for spin diffusion in a molecule can lead to artificially short distances derived from the intensities measured from cross-peaks in the spectra.

(2) **MARDIGRAS.** The data were reinterpreted using MARDIGRAS, developed by James and co-workers to give interproton distances that account for spin diffusion. The distance bounds returned by the procedure were better than ISPA but also did not correctly fit all the observed NOE intensities to the psoralen covalent geometry. The actual distances for the MAF fell within the range of the MARDIGRAS and were consequently not observed in NOESY spectra with shorter mixing times. The absence of a measured cross-peak at some of the mixing times was taken into account during the statistical analysis of the results generated by the RANDMARDI procedure (see above). Cross-peak integrals that corresponded to fixed distances in the DNA were not converted into distance restraints for use in the RMD simulations. MAF. The integrated intensities from 327 NOESY cross-peaks for the MAF were used in the total relaxation matrix analysis. Ten sets of measured NOE intensities were converted to distance restraints using the RANDMARDI procedure. The calculations returned 328 interproton distances that are not covalently fixed in either the HMT or DNA in the MAF (Table 4). XL. The integrated intensities from 327 NOESY cross-peaks for the XL were used in the total relaxation matrix analysis. Eight sets of measured NOE intensities were converted to distance restraints using the RANDMARDI procedure. The calculations returned 295 interproton distances that are not covalently fixed in either the HMT or DNA in the XL (Table 5).

The reliability of these distance restraints was examined by comparing the covalently fixed distances in HMT-thymine adducts to the distances obtained from the RANDMARDI calculations (Tables 1 and 2). The almost complete agreement between the actual and the experimentally determined distances indicates that the RANDMARDI procedure yields accurate interproton distances. The MARDIGRAS/RANDMARDI calculations were performed with various values of the correlation time for the complex in the range 2-5 ns. The results were fairly insensitive to the actual value, and a correlation time of 3.75 ns was chosen for the final calculations.

### Table 5: Distribution of NMR-Derived Distance Restraints Used in RMD Calculations of the XL

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| total 98    | 15 113         | 34 8     |

| *The restraints were derived from RANDMARDI calculations. The 327 NOE connectivities gave a total of 295 usable distance restraints. Eighteen distance restraints ensuring Watson−Crick base pairing were also included in the calculations. *Restrains to the HMT 4-hydroxymethyl protons.
NOEs to the diastereotopic HMT 4'-hydroxymethyl group protons were included in the RMA calculation. The distances from these protons to others in the MAf and XL are highly dependent upon the rotational conformation of the group. Because it was not possible to stereospecifically assign them, they were not included in the comparison of the actual and experimentally derived distances. Uncertainties in the distance constraints arise from internal motion in the molecule that averages the intensity of the NOE, integration errors, and the incomplete experimental description of the relaxation matrix.

Generation of Interproton Restraints. The bounds for use in RMD simulations were determined by combining the results from all of the individual MARDIGRAS calculations performed during the RANDMARDI procedure into one set. The distance bounds were calculated individually for each proton pair corresponding to a NOESY cross-peak included in the RANDMARDI calculations. Each individual restraint was generated from the average distance calculated for that proton pair from the MARDIGRAS calculations, ±1 SD calculated for that proton pair. Tables 4 and 5 give a summary of the distance restraints used in the RMD calculations. For the MAf the range of differences between the upper and lower bounds for the 328 NOE-derived restraint bounds used (cf. Table 4) was between 0.12 and 2.96 Å with an average flat well potential width of 1.18 Å. For the XL, the range of differences between the upper and lower bounds for the 295 NOE-derived restraint bounds used (cf. Table 5) was between 0.16 and 3.67 Å with an average flat well potential width of 1.53 Å. This range between the upper and lower bounds represents the accuracy of the restraints calculated from the NOESY data. Weak NOESY cross-peaks that arose primarily from spin diffusion generally had the greatest difference between their upper and lower bounds.

Deoxyribose Ring Conformations. The DQF-COSY spectra were acquired in an attempt to get J-coupling information to determine the pseudorotation angle of the deoxyribose residues (Ernst et al., 1987; Gochin et al., 1990). In B-form DNA the deoxyribose adopts a predominantly C2-endo conformation where the H2' and H2'' protons may be distinguished from one another by their coupling patterns with H1' in a DQF-COSY spectrum. The deoxyribose H1' to H2' cross-peak is characterized by a strong active coupling and a weak passive coupling, while the H1' to H2'' crosspeak is characterized by a more complex pattern. The assignment of H2' and H2'' protons from DQF-COSY coupling patterns must be made with caution because the patterns are a function of the sugar pucker. The H1' proton is closer to the H2'' proton than to the H2' proton in all conformations accessible to the sugar. A comparison of the relative intensities of the H1' to H2' and the H1' to H2'' NOESY cross-peaks allowed unambiguous assignment of all H2' and H2'' protons. Interpretation of the coupling patterns observed for the H1' to H2' and H2'' cross-peaks in the MAf and XL was difficult due to broad lines in the DQF-COSY spectra which led to cancellation of the antiphase multiplets and chemical shift degeneracy for many of the deoxyribose ring protons that further obscured the coupling patterns. High-resolution selective-COSY methods were employed in an attempt to extract backbone angle constraints for use in the modeling. It was shown that the deoxyribose sugars of the residues in and around the HMT binding site in both the MAf and the XL are in a complex mixture of rapidly equilibrating conformations (Emsley et al., 1993; Spielmann et al., 1995a). The biological implications of these complex conformational states are described elsewhere (Spielmann et al., 1995a,b).

Structure Convergence. A total of 346 restraints were used for the MAf, and a total of 313 restraints were used for the XL including the hydrogen-bonding restraints. Twenty final structures each were generated for both the MAf and XL starting with dsDNA in B-form and A-form. All the structures converged to the same conformation. The root-mean-square (rms) deviations of the coordinates of the 40 structures for the MAf and XL were 1.12 and 1.10 Å, respectively. The rmsd of the MAf with respect to the XL is 2.20 Å. The resulting total violations of the MAf summed to 11.3 Å for the A-form starting structure and to 12.6 Å for the B-form starting structure and for the XL summed to 8.4 Å for the A-form starting structure and to 9.3 Å for the B-form starting structure. Seven violations greater than 0.25 Å but less than 0.5 Å were found in the structures of the MAf derived from A-form coordinates, six for the MAf derived from B-form coordinates, five for the XL derived from A-form coordinates, and eight for the XL derived from B-form coordinates. The majority of these residual errors are localized near the HMT 4'-hydroxymethyl group and arise from the influence of the apparent conformational heterogeneity of that group and to the conformational heterogeneity of the sugars on the RMA. Different views of the calculated structures are given in Figures 4-6.

DISCUSSION

Description of the Structures. Qualitative analysis of the pattern of NOEs between the psoralen and the surrounding bases and sugars indicates that the HMT is intercalated into the helix in both the MAf and XL. In both the MAf and the XL the 8-position of the psoralen is in the minor groove, and the 4', 5', 3', 4-, and 5-positions of the psoralen are in the major groove. Furthermore, the adducted base(s) still adopt(s) a Watson-Crick type of base-pairing geometry. The enhanced stability of the MAf relative to the UM is due to strong stacking interactions between the psoralen and the surrounding bases, in addition to the continued presence of normal Watson-Crick hydrogen bonds. As a consequence of the covalent addition of the psoralen in the MAf, the long axis of the HMT is nearly parallel to the T4-A13 axis. In the MAf there are four nonadducted bases in the immediate vicinity of the HMT adduct (G3, A5, T12, and A13) in addition to the photoadducted thymidine.

NMR-derived restraints are short range in nature, and some helical parameters are better defined by the experimental data than others. In addition, the anisotropic distribution of observable interproton contacts by NMR in free dsDNA (largely confined to the ribbon of the sugar—phosphate backbone) defines some helical parameters poorly. In the case of the MAf and XL, there are a large number of NMR-derived restraints in the center of the helix due to the presence of the HMT. These HMT—DNA contacts are distributed fairly isotropically and therefore lock down the conformation for the central four base pairs of both molecules.

(1) MAf. The adenine bases are stacked coplanar with the psoralen in the MAf (Figure 4A). The six-membered ring of A5 is stacked onto the HMT 8-methyl group, and the adenine 6- amino group is stacked directly on top of the central benzene ring of HMT. The pyrimidine ring of T12 in the MAf is centered over the H3 of the HMT, and there
FIGURE 4: Stereoview of the stick plot of the structure of the (A, left) MAf and (B, right) XL. Deoxyribose protons have been omitted for clarity. (Top) One of the calculated structures showing the calculated global helix axis. (Bottom) Superposition of 10 of the 40 structures obtained by RMD calculations.
is good van der Waals contact between the 4-methyl group of the HMT with the 5-methyl group of the thymidine.

(2) XL. As a consequence of XL formation, A5 is displaced relative to its position in the MAf (Figure 4B). In the XL, the six-membered ring of A5 still makes contact with the 8-methyl group, but the 6-amino group is now positioned over the furan ring of the HMT. There is nearly complete overlap of the six-membered ring of A13 and the pyrone ring of the HMT in the MAf. This stacking arrangement is retained upon conversion of the MAf to the XL. T12 is in contact with the 4-methyl and positioned over the H3 substituent of the HMT pyrone ring in the MAf. T12 is located directly over the pyrone ring when the MAf is converted to XL.

The stacking between T4 and G3 is perturbed in the MAf and XL by the upward protrusion of the adducted base into G3. In addition, the interaction between G11 and T12 in the XL is distorted. Cyclobutane ring formation changes the hybridization of the thymidine 5 and 6 carbons from sp² to sp³, forcing the 5-methyl group (5-Me) and 6-H of the adducted thymidine away from the HMT with respect to the pyrimidine ring. The adjacent guanine stacks onto the surface formed by the adducted thymidine 5-Me, 6-H, and pyrimidine ring that is much less tilted than the pyrimidine ring alone.

There is a 1 μs relaxation delay between the absorption of the photon that induces monoadduct formation and the ability of the MAf to then undergo further chemistry to form the cross-link (Johnston et al., 1977). Absorption of a photon by the MAf chromophore is not prohibited; rather, it is necessary for the system to complete a conformational change caused by the MAf cyclobutane ring formation before the reaction can occur. A significant local reorganization in the conformation of the DNA is required to accommodate the conversion of the MAf to the XL (Spielmann et al., 1995a). The MAf structure presented here is presumed to be the post-1 μs relaxed structure that is competent to absorb a photon and form a cross-link. Indeed, irradiation of the MAf heteroduplex with 366 nm light results in quantitative transformation to the cross-link (Spielmann et al., 1992). The 3.47 Å separation of T12 C-5 and MAf C-4 and the 3.35 Å separation of T12 C-6 and MAf C-3 change to 1.54 Å each upon XL formation. The MAf-T4 adduct moves 1.3 Å into the major groove relative to the helix axis. T12 rolls down toward the HMT when the pyroneside cyclobutane ring is formed, but it is not displaced relative to the helix axis. T12 shifts into the major groove and across the helix axis toward T12 upon cross-link formation. A5 moves 1.0 Å toward the major groove upon cross-link formation and rolls slightly into the minor groove because of a steric clash between the exocyclic amino group and the 4'-hydroxymethyl group of the HMT. A13 moves slightly into the minor groove when the 4-methyl group of the HMT clashes with the five-membered ring of A13 upon cross-link formation.

Surface Accessibility of the Adducts. A space-filling model of the structure of the MAf is shown in Figures 5A and 6A. The view of the major groove (Figure 5A) shows the solvent-accessible edge of the HMT between the T-A base pairs. The 5'-methyl group is unobscured by any of the functional groups on the DNA. The 4'-hydroxymethyl group of HMT is clearly in a sterically unhindered position, whereas the 5-position is sterically crowded by the psoralen 4'-hydroxymethyl group and the 4-methyl group, but access is not obscured by the DNA. The 5-position of HMT is directly over the T4-O4 to A13-N6 hydrogen bond. The pyrone ring 4-methyl group shows van der Waals contact to the T12 methyl group. The pyrone H3 of the psoralen is buried in a pocket bounded by the five-membered ring of A13, the T12 base, and the sugars from T12 and A13. Pyridopсоралens have a pyridine ring fused to the 3,4 double bond of the pyrone ring, and these compounds form monoadducts with DNA exclusively (Moysan et al., 1993). The steric requirements for a furanoid monoadduct of this type are greater than those of HMT. Based on our model, there would
have to be a significant reorganization of the MAf structure for the HMT adduct to accommodate such a large substituent at the 3-position. DNA is flexible and has increased dynamics at the site of the photoadduct formation.

The view of the minor groove (Figure 6A) shows the edge of the HMT between the T-A base pairs. The 8-methyl group makes a close van der Waals contact with the O4' of A5. The ester oxygens of the pyrone ring are also solvent accessible. The view of the minor groove shows how the 8-position of the HMT, the pyrone ring carbonyl, and O1 make up part of the floor of the minor groove. It also shows that access to the furan ring in the minor groove is obscured by the T4 and A5 sugar residues. The buckle introduced into the T4-A13 base pair by the photoaddition of T4 to the HMT is quite clear. Unwinding of the DNA helix at the TpA step and an elongation of the distance between the adenines from 3.3 to 6 Å to accommodate the intercalated adduct are apparent.

Figures 5B and 6B show similar space-filling models of the structure of the XL. The solvent-accessible functional groups in both the major and minor groove are qualitatively the same as those in the MAf. In the major groove view of the XL (Figure 5B) the overwinding of the helix in the vicinity of the HMT is evident. The T4 5-methyl and T12 5-methyl groups are conspicuously closer together in the XL than they are in the MAf. It is also evident that the psoralen has been pulled across the helix toward T12 in the XL. The view of the minor groove (Figure 6B) shows the edge of the HMT between the TA base pairs. The 8-methyl group is no longer in van der Waals contact with the O4' of A5 because the psoralen has been shifted toward T12 and displaced toward the major groove.

A large number of synthetic psoralens have been produced that bear substituents on the 4'-, 5-, and 8-positions and that retain the ability to form both monoadducts and crosslinks (Cimino et al., 1985; Bisagni, 1992). The structures in Figures 5 and 6 show that functionalization of the 4'- and 5-positions does not introduce unfavorable steric interactions between the psoralen and the surrounding DNA in the monoaadduct. Based on the MAf and XL structures, both the 5' and the 4-positions of the psoralen could be substituted with a group larger than methyl without adverse steric interaction with the surrounding nucleic acid. The 8-methyl group is in the minor groove in a somewhat more sterically congested site.

**Helix Parameters.** There are three major categories of helical parameters: axis—base pair, intra-base pair, and inter-base pair parameters (Dickerson et al., 1989). These parameters are discussed for the MAf and XL with respect to the calculated global helix axis below. However, the parameters reported for the terminal base pairs of the MAf and XL are probably less reliable because of dynamic processes and a lower density of restraints and are included only for completeness. The DNA returns to B-form geometry three base pairs away from the site of damage. Adjustments in the helix geometry of the three base pairs on either side of HMT compensate for the distortions introduced by the psoralen damage in the MAf and XL. These perturbations away from canonical B-form DNA are described by the helix parameters shown in Figure 7.

**Global Helix Axis.** The global helix axis is straight in the XL, and the MAf shows only a small 8° bend (Figure 4). The bend in the helix axis was calculated by taking the inner product of the vectors describing the axis segments between the C2-G15-G3-C14 and the C6-G11-G7-C10 base pair steps. The final conformation of the terminal base pairs was considered to be suspect due to their unresolved dynamic motions. The MAf helix axis can be divided into three segments, the first running through the first four base pairs, the second traversing the T4-A13-A5-T12 base pair step, and the third running through the last four base pairs of the octamer. The first and third segments are almost parallel with each other, while the second describes a slip dislocation of the two helical segments caused by the HMT photoadduct. The dislocation in the helix axis in the MAf is probably due to the favorable stacking of the A5 onto the 8-methyl group of the HMT and the steric interaction of the 4'-hydroxymethyl group with A5.
The MAf helix axis is not kinked by the adduct because A5 and A13 remain coplanar with the HMT. G3 of the MAf is rolled slightly into the major groove contributing to the small bend into the major groove. We believe that bends of this magnitude cannot be accurately determined with current NMR methods and are difficult to measure with any technique. The presence or absence of a small bend induced by the MAf might be more reliably measured by other techniques, such as gel mobility assays, that are beyond the scope of the present work. The formation of the XL introduces a large buckle into the T4-A13 and T12-A5 base pairs. Cross-link formation can be accommodated without kinking because A5 and A13 remain coplanar with the HMT. G3 stacks onto the 5-Me and modified ring of T4, and G11 stacks onto the 5-Me and modified ring of T12 coplanar with the HMT in the XL (Figure 4). The unbent axis of the XL is in agreement with gel mobility shift data (Sinden & Hagerman, 1984; Haran & Crothers, 1988).

Axis—Base Pair Parameters. The calculated parameters for shear, stretch, stagger, and opening are influenced but not completely determined by the restraints used to enforce base pair hydrogen bonding. For the base pairs in and around the photodamage site, these parameters are determined by the HMT-DNA base NOEs because no hydrogen-bonding restraints were included for the thymidine-adenosine base pairs in the RMD procedure.

Y-Axis Displacement. The Y-axis displacement in the cross-link is that found in the normal B- and A-form DNA. There are significant negative Y-axis displacements in the MAf. The global axis is displaced toward the adducted strand and becomes increasingly more negative 3' to the adduct. The HMT−T monoadduct does not introduce the same amount of A-form-like character as does the T−HMT−T cross-link into the helix. The A-form-like character is asymmetrically distributed between the strands in the MAf, with the HMT damaged strand being more A-form like and the unmodified strand more B-form like.

Intra-Base Pair Parameters. The calculated parameters for shear, stretch, stagger, and opening are influenced but not completely determined by the restraints used to enforce base pair hydrogen bonding. For the base pairs in and around the photodamage site, these parameters are determined by the HMT-DNA base NOEs because no hydrogen-bonding restraints were included for the thymidine-adenosine base pairs in the RMD procedure.

Shear. For the MAf, the shear changes from positive to negative across the TpA step, compensating for the intercalation of the HMT and returning to essentially B-form values at the ends of the helix. There appears to be no net shear introduced into the MAf-adducted helix. This was also observed in the structure of the bisintercalator TOTO−DNA complex (Spielmann et al., 1995). The covalent geometry imposed on the cross-linking site in the XL does not show this behavior. The values start out negative and only show a small negative shear immediately 5' to the intercalation site. The hydrogen-bonding edges of the two adducted thymidines are rotated relative to each other into the major
Buckling has been observed in the DNA bases of perpen-
the HMT. The buckling is a mechanism by which the
the perpendicular intercalators where the DNA buckles to
mophore. The buckling is primarily a consequence of the
covalent nature of the thymidine adduct geometry. Because
the adjacent guanine bases stack on the "platform" of the
adnene base paired with the thymidine. This offset is
manifested by a compensatory change in stagger when going
from the damaged base to the base 5' to the adduct.

Buckle. The large deviations in the buckle from the
normal A- and B-DNA value of 0° are a direct consequence
of the intercalation and photoadduction of the HMT. Unlike
the perpendicular intercalators where the DNA buckles to
maximize van der Waals contacts with the chromophores (Williams et al., 1992), the A5 and A13 in the adduct site
stack coplanar with the HMT and are normal to the helix
axis, maximizing van der Waals contacts with the chromo-
phore. The buckling is primarily a consequence of the
covalent nature of the thymidine adduct geometry. Because
the adjacent guanine bases stack on the "platform" of the
adducted thymines, there is no propensity for the adduct
to introduce a bend into the helix or add additional buckle
to the binding site (Spielmann et al., 1995a). The binding
pocket for the XL adduct is defined by the surfaces of G3,
A5, G11, and A13, with the two thymines being an integral
part of the intercalating agent. The symmetry of the
distortions introduced in the DNA by MAf and XL formation
is evident in the relative sign and distribution of the buckle
across the HMT photobinding site. It is also evident that
the effect of the adduct formation on the buckle rapidly
diminishes a few base pairs distal to the site of damage in
both the MAf and XL. The buckle in T12 of the MAf
appears to be a direct effect of stacking of that base with
the HMT. The buckling is a mechanism by which the
unadducted thymidine increases its van der Waals interac-
tions with the intercalated monoadduct which does not reach
all of the way across the helix (Williams & Gao, 1992).
Buckling has been observed in the DNA bases of perpen-
dicular intercalator—DNA complexes by both X-ray crystal
structures and NMR solution structures (Wang et al., 1984;
Addess et al., 1993; Spielmann et al., 1995c). The HMT
monoadduct is similar to a parallel intercalator such as
ethidium, but the MAf does not reach all the way across the
helix. The T12-A5 base pair in the MAf buckles to create a
cavity that optimizes the interactions between the psoralen
and the surrounding DNA. Although the plane of the
pyrimidine ring of T4 is at an angle of between 44° and 53°
with respect to the helix axis, the base 5 and 6 carbons have
converted from planar sp² hybrids to strained and tetrahedral
sp³ centers in the cyclobutane ring. This forces the T4
5-methyl group and the T4 H6 up into G3. To maintain
stacking, G3 H8 makes a contact with the "folded thymidine" T4
at the 5-methyl, O2, and N3H. In doing so, the effect of the
dramatic thymidine buckle on the helix axis is greatly dimin-
ished.

Propeller Twist. A large propeller twist is introduced into
the T4-A13 base pair in the MAf because the cyclobutane
ring formation twists T4 relative to the helix axis and A5
and A13 remain essentially coplanar with the HMT tricyclic
nucleus. This effect is duplicated for the T4-A13 and A5-
T12 base pairs of the XL. The effect of the distorted
thymines on the propeller twist is quite localized, with the
remaining base pairs in the helixes having essentially normal
values.

Inter-Base Pair Parameters. The roll, slide, and shift in
regular A-DNA and B-DNA conformations are essentially
zero. These parameters show large and correlated changes
from the equilibrium values in the MAf and XL structures.

Slide. The slide of the T4-A5 base pair step changes sign
in response to the formation of the interstrand cross-link,
reflecting the contraction of the two strands toward each
other. This change in the sign and magnitude of the slide
induced by the conversion of MAf to XL seems to be
propagated to each base pair step of the two molecules.

Rise. The rise values for corresponding base pair steps
in the MAf and XL almost exactly mirror each other. The
rise for all of the base pair steps except for the 5'-TpA-3'
is close to that of A-form DNA. The T4-A5 base pair
has a rise of 7.6 Å in the MAf and 8.4 Å in the XL, reflecting
the intercalation and structural deformation introduced by
the HMT photoadduct. These values are somewhat larger
than that induced by simple intercalation. This is due to
the cyclobutane ring formation which pushes the added
thymines away from coplanarity with the rest of the bases
in the helix.

Twist. The helix is unwound by 34° in the MAf and by
25° in the XL. This agrees well with the unwinding angle
for the XL of 28 ± 4° determined by a DNA supercoiling
assay (Wiesehahn & Hearst, 1978). The helix has an overall
helical repeat of 11 base pairs in both the MAf and the XL,
consistent with intercalation of the HMT. The distribution
of the unwinding along the molecule is different in the MAf
and the XL. The MAf helix is underwound at the T4-A5
base step as a result of the insertion of the psoralen.
The underwinding in the MAf is distributed over the T4-A5
and A5-C6 steps. The TA base step in the XL is overwound to
39° due to the covalent geometry imposed on the DNA by
the HMT cross-link. The overwinding at the XL T4-A5 step
is compensated for by the underwinding of the C2-G3, G3-
T4 and A5-C6 steps. The destabilization of the helix on
the pyroneside of the adduct is due, in part, to the change in
helix twist at the TpA step brought about by the covalent
connection of the two DNA strands.

Roll. The roll values for corresponding base pair steps
in the MAf and XL almost exactly mirror each other. Positive
roll opens the angle between base pairs toward the minor
groove, resulting in a bend into the major groove, while a
negative roll results in a bend into the minor groove. Excep-
t for the G3-T4 base pair step, there are only small deviations
in the roll for both molecules away from canonical A- and
B-form DNA values. The large -37° roll found for the G3-
T4 base pair step in both the MAf and the XL is a result of
Table 6: Backbone Angles (deg) for the HMT MAf and XL of d(GCGTACGC)Z

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propeller twisted and buckled six-membered ring of the

Table 6: Backbone Angles (deg) for the HMT MAf and XL of d(GCGTACGC)Z Psoralen Monoadduct and Cross-Link Structure

residues in and around the adduct site. The calculated values

pyroneside adduct of the XL because the hydrogen-bonding

The adjustments that the helix makes to accommodate the

is also evidence of conformational heterogeneity in the sugar

represent averages of different conformations and are

there are at least two technical factors which contributed to

the problems with the AMT XL structure that are unrelated to

the differences in the psoralen and DNA sequence. First, the

contribution of spin diffusion to the NOE intensities was not

accounted for in the AMT XL system. In the present work we have

used the RANDMARDI modification of the relaxation matrix

analysis program MARDIGRAS to obtain accurate interproton

distances from the NOE data. The current calculations show that

there are important spin diffusion pathways in both the MAf and

the XL molecules. Significant bending of the helix and highly

strained, unrealistic distortions of the psoralen and thymidine

bases were observed in initial models of the HMT XL and HMT

MAf generated from experimental NOE data using distance

restraints derived by the isolated spin-pair approximation

(ISPA). Indirect magnetization transfer makes significant

contributions to the peak intensities in these psoralen–DNA

complexes. Second, modern simulated annealing (SA) and

restrained molecular dynamics (RMD) protocols were unavailable

when the AMT XL structure was reported. The previously reported

structure was refined from a bent starting model, introducing a

conformational bias. Attempts to refine the AMT XL structure from a

straight B-form DNA starting model without SA or RMD protocols

led to strained conformations with high energies and significant
distance restraint violations. The modern algorithms used in the

present work are much more efficient at overcoming local

energy barriers encountered during the structure refinement.

Bent DNA models derived from NMR data can be artifacts due to

the higher density of NOEs in the major groove and the anisotropic

character of DNA. Incorrect NOE-derived distance restraints can be partially satisfied by bending the DNA.

Implications for DNA Repair. The MAf and XL are recognized by the (A)BC excinuclease and serve as excellent models to study the structural and dynamic motifs that cellular repair enzyme systems may recognize (Sancar & Sancar, 1988; Cheng et al., 1988b, 1991). The structural differences between the MAf and the XL allow examination of common structural or dynamic features that may be recognized by the enzyme system. Understanding how the (A)BC excinuclease recognizes psoralen damage to DNA
will assist in the elucidation of the mechanism of lesion recognition. Excision repair systems efficiently recognize and remove many structurally diverse DNA damaging agents while the wide range of normal DNA microstructures triggers a response that is 3–5 orders of magnitude lower in rate (Sancar & Sancar, 1988; Lin & Sancar, 1992; Huang et al., 1994). Recognition of damaged DNA by the (A)BC excinuclease repair system is initiated by the binding of the UvrA2B1 complex to the damage site. The UvrA2B1 protein complex, utilizing energy provided by its ATPase activity, sharply bends the DNA into the major groove at the 11th phosphodiester bond 5' to the lesion, leaving the UvrB component tightly bound to the DNA (Shi et al., 1992). The “molecular matchmaker” UvrA then dissociates from the complex (Sancar & Hearst, 1993). The matchmaking mechanism uncouples the damage recognition event by UvrA from the subsequent excision steps by UvrB and UvrC. In the absence of UvrA, UvrB appears to have no affinity for DNA. Recognition of DNA damage by these systems has been proposed to take place by the detection of structural changes and/or the alteration of the dynamic behavior of the DNA (Sancar & Sancar, 1988; Lin & Sancar, 1989; Pu et al., 1989). It has been proposed that damage causes local instability in the helix and that the (A)BC excision repair system recognizes this excessive or unusual deformability (Williams & Gao, 1992). The conformations of the UM sugars have been compared with those of MAF and XL, and the HMT damage destabilizes the preference for C2-endo sugar conformation of the four nucleotides T4, A5, T12, and A13. The MAF or XL permits them to adopt a larger range of rapidly interconverting conformers with a significantly altered population distribution with respect to unmodified DNA (Emsley et al., 1993; Spielmann et al., 1995a). These changes in the equilibrium distribution of the deoxyribose conformations and the implied coupled “crankshaft motions” of the phosphate backbone detected for the MAF and XL may be recognized as the signal that positions the DNA repair system at the location of the DNA damage.

The DNA repair system in this model recognizes changes in the equilibrium behavior of the DNA surrounding the adduct, not specific structural features induced by the damage (Lin & Sancar, 1989; Pu et al., 1989). Other DNA oligomers covalently modified with drugs that are recognized by cellular repair systems and have radically different structures from the MAF and the XL show evidence of altered helix dynamics by NMR (O’Handley et al., 1993). We hypothesize that the repair enzymes target lesions by detecting the conformational flexibility of the sugar–phosphate backbone induced by DNA damaging agents. There are numerous amino acid contacts to the backbone phosphates observed in high-resolution X-ray crystal structures of protein–DNA complexes (Beamer & Fabo, 1992). The flexibility of the phosphate esters in the recognition and formation of protein–DNA contacts has been proposed as a mechanism for discrimination of different operator sites by the lac repressor (Karslake et al., 1992; Botuyan et al., 1993). Flexibility of the backbone is a more general case of localized sequence-specific conformational variations in DNA that are potentially recognized by proteins. We propose that the backbone of undamaged DNA has a certain stiffness (firmness) that is ignored by the UvrA2B1 system and that introduction of a region of greater conformational freedom (softness) into the DNA backbone at the adduct is recognized as damage by the UvrA2B1 complex. Determination of the precise backbone dynamic modes that are changed by lesion formation will require other experiments such as the measurement of order parameters and further analysis of homonuclear and heteronuclear coupling constants for the sugar residues by NMR.

CONCLUSION

We have presented clear evidence of a single HMT adduct intercalated in the central 5'-TpA-3' site in the MAF and XL. We have shown that neither the MAF nor the XL show unusual bending as a result of adduct formation. The RANDMARDI procedure yields structures of molecules in solution that are more consistent with magnetization transfer theory and agree better with results from other physical techniques. A better understanding of the fundamental processes that allow photobinding of organic molecules to DNA will aid in the design of new and improved nucleic acid specific agents that interfere with DNA metabolism. High-resolution structures and dynamics information of the various reaction products between psoralen and nucleic acids are required to understand the detailed mechanism of the photoreactions and the biological function of the adducts. The ability of psoralen adducts to stop replication, transcription, and other DNA metabolic activities such as the activity of restriction endonucleases is the same activity that is exploited by a number of chemotherapeutic agents.

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REFERENCES
