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^{1}H NMR Assignment and Melting Temperature Study of Cis-Syn and Trans-Syn Thymine Dimer Containing Duplexes of d(CGTATTATGC)·d(GCATAATACG)[†]

John-Stephen Taylor,*[,‡,§] Daniel S. Garrett,[‡] Ian R. Brockie,[‡] Daniel L. Svoboda,[‡] and Joshua Telser[‖]

Department of Chemistry, Washington University, St. Louis, Missouri 63130, and Amoco Technology Company, P.O. Box 400, Naperville, Illinois 60566

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ABSTRACT: The preparation and spectroscopic characterization of duplex decamers containing site-specific cis-syn and trans-syn thymine dimers are described. Three duplex decamers, d(CGTATTATGC)·d(GCATAATACG), d(CGTAT[c,s]TATGC)·d(GCATAATACG), and d(CGTAT[t,s]TATGC)·d(GCATAATACG), were prepared by solid-phase phosphoramidite synthesis utilizing cis-syn and trans-syn cyclobutane thymine dimer building blocks (Taylor et al., 1987; Taylor & Brockie, 1988). NMR spectra (500 MHz 2D ^{1}H and 202 MHz 1D ^{31}P) were obtained in "100%" D_2O at 10 °C, and 1D exchangeable ^{1}H spectra were obtained in 10% D_2O at 10 °C. ^{1}H NMR assignments for H5, H6, H8, CH_3, H1′, H2′, and H2″ were made on the basis of standard sequential NOE assignment strategies and verified in part by DQF COSY data. Comparison of the chemical shift data suggests that the helix structure is perturbed more to the 3′-side of the cis-syn dimer and more to the 5′-side of the trans-syn dimer. Thermodynamic parameters for the helix $\rightleftarrows$ coil equilibrium were obtained by two-state, all or none, analysis of the melting behavior of the duplexes. Analysis of the temperature dependence of the T5CH_3 ^{1}H NMR signal gave $\Delta H = 44 \pm 4$ kcal and $\Delta S = 132 \pm 13$ eu for the trans-syn duplex. Analysis of the concentration and temperature dependence of UV spectra gave $\Delta H = 64 \pm 6$ kcal and $\Delta S = 178 \pm 18$ eu for the parent duplex and $\Delta H = 66 \pm 7$ kcal and $\Delta S = 189 \pm 19$ eu for cis-syn duplex. It was concluded that photodimerization of the dTpdT unit to give the cis-syn product causes little perturbation of the DNA whereas dimerization to give the trans-syn product causes much greater perturbation, possibly in the form of a kink or dislocation at the 5′-side of the dimer.

Cis-syn cyclobutane pyrimidine dimers are the major photoproducts produced upon exposure of DNA to sunlight [see Patrick and Rahn (1976) for a review] and have been correlated with mutation and skin cancer [see Brash (1988) for a recent discussion]. These photoproducts result from the photo [2 + 2] cycloaddition of the 5,6-double bond of two adjacent pyrimidine nucleotides in an anti glycosyl conformation. The cis-syn dimer of dTpdT[1] sequences is the major photoproduct of DNA, and as a consequence there is much interest in determining its structure–activity relationships. Cis-syn thymine dimers are the substrates for photolyases, pyrimidine dimer glycosylases, and the uvrABC excinuclease system (Friedberg, 1985). Cis-syn thymine dimers generally constitute blocks to replication, but recently it has been found that they can be bypassed by pol I of *Escherichia coli* in vitro in a nonmutagenic manner (Taylor & O'Day, 1990). The structural or physical properties of DNA containing the cis-syn dimer which contribute to its recognition by repair systems and its bypass by replication systems are unknown.

The cis-syn dimer of dTpdT itself has been extensively studied by NMR (Ogilvie, 1975; Liu & Yang, 1978; Rycyna & Alderfer, 1985, Kemmink et al., 1987c; Kan et al., 1988). The crystal structure of a phosphotriester derivative of the cis-syn dimer of dTpdT has been determined by X-ray crystallography and found to be somewhat different than what the solution-state NMR data indicate (Cadet et al., 1985; Hruska et al., 1986). NMR studies have also been reported on cis-syn dimer containing tri- and tetrathymidylates (Rycyna et al., 1988). Milligram quantities of d(GCGT[c,s]TGCG)·d(CGCAACGC) have been prepared by a direct photolysis route and the nonexchangeable ^{1}H NMR signals assigned (Kemmink et al., 1987a). In a separate study the exchangeable proton signals of the parent and cis-syn octamer duplexes were assigned and thermodynamic parameters for the helix $\rightleftarrows$ coil transition determined by analysis of the temperature dependence of one of the methyl proton signals (Kemmink et al., 1987b).

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[*] To whom correspondence should be addressed.

[‡] Washington University.

[§] Alfred P. Sloan Foundation Fellow, 1988–1990.

[‖] Amoco Technology Co. Present address: Squibb Institute for Medical Research, P.O. Box 191, New Brunswick, NJ 08903.

[1] Abbreviations: COSY, correlated spectroscopy; 2D, two dimensional; DQF, double quantum filtered; EDTA, ethylenediaminetetraacetic acid; NOE, nuclear Overhauser effect; NOESY, 2D NOE spectroscopy; TMP, trimethyl phosphate; dTpdT, thymidylyl-(3′→5′)-thymidine; T[c,s]T, cis-syn cyclobutane dimer of dTpdT; T[t,s]T, trans-syn cyclobutane dimer of dTpdT; TSP, 3-(trimethylsilyl)propionate.

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Evidence has accumulated to suggest that dimerization of a dTpdT unit causes changes in the structure and properties of duplex DNA. Studies in which plasmid DNA was titrated with UV light led to the conclusion that cis-syn thymine dimers unwind DNA by approximately 14° (Ciarrocchi & Pedrini, 1982). Analysis of the melting temperature behavior of d(TTTTT[c,s]TTTTT) with poly(dA) suggested that the cis-syn thymine dimer caused disruption of the hydrogen bonding of the two flanking base pairs (Hayes et al., 1971). In contrast, comparison of the imino proton spectra of the parent and cis-syn octamer duplexes indicated that there was little reduction in the hydrogen bonding of the adjacent base pairs and only weakening of those involving the dimer itself (Kemmink et al., 1987b). Two independent molecular mechanics studies of duplex decamers containing cis-syn thymine dimers led to differing conclusions regarding the effects of a dimer on DNA structure. One study predicted that a cis-syn thymine dimer causes little distortion of the B-DNA helix (Rao & Kollman, 1985) whereas another predicted that it induces a 27° kink or bend (Pearlman et al., 1986). NMR studies on the octamer duplex were in general accord with the idea that the dimer does not significantly perturb the B-DNA structure except in the immediate region flanking the dimer. A gel electrophoresis study of a polymerized cis-syn thymine dimer containing oligomer concluded that the dimer caused the DNA to bend by approximately 30° (Husain et al., 1988) as predicted Pearlman and co-workers.

In comparison to what is known about cis-syn thymine dimers, little is known about the structure and properties of trans-syn thymine dimers. These photoproducts result from photodimerization of a dTpdT unit in which the 5′-T is in a syn glycosyl conformation, thus precluding their formation in B-form DNA. Trans-syn thymine dimers can be produced in both native and denatured DNA, though more efficiently in the latter (Patrick & Rahn, 1976). This suggests that they are formed in single-stranded regions, as might be found in transcriptionally active genes, and may conceivably form in alternate conformations of duplex DNA, such as B-Z junctions (Taylor & Brockie, 1988). Most of the work on this class of photoproducts has focused on NMR studies of the trans-syn cyclobutane dimer of dTpdT (Liu & Yang, 1978; Kemmink et al., 1987c; Kan et al., 1988). Recently, we reported the synthesis and ^{1}H NMR characterization of a decamer containing a trans-syn thymine dimer, d(CGTAT[t,s]TATGC) (Taylor & Brockie, 1988).

In spite of all the work on pyrimidine dimers, the solution-state structure and properties of cyclobutane dimer containing DNA as a function of sequence are still not known. This has been due in large part to the lack of general methods for the preparation of site-specific pyrimidine dimer containing DNA in sufficient purity and quantity for detailed physical and biological studies. To this end, we have recently developed "building blocks" for the sequence-specific introduction of cis-syn (Taylor et al., 1987) and trans-syn (Taylor & Brockie, 1988) thymine dimers into oligonucleotides by solid-phase DNA synthesis technology (Figure 1). Herein we report the total synthesis and characterization of duplex decamers containing site-specific cis-syn and trans-syn thymine dimers.

MATERIALS AND METHODS

Sample Preparation. The synthesis and purification of d(CGTAT[c,s]TATGC), d(CGTAT[t,s]TATGC), and d(CGTATTATGC) have previously been described (Taylor & Brockie, 1988). The complementary strand to these three oligonucleotides, d(GCATAATACG), was prepared in a similar fashion. Predicted molar extinction coefficients at 260 nm (Fasman, 1975) were used to estimate the concentrations of single-strand oligonucleotides in solution. NMR samples were prepared by combining equimolar amounts of single-strand oligonucleotides and making up to a final volume of 0.7 mL of D_2O containing 100 mM NaCl, 10 mM sodium phosphate buffer, and 0.1% or 0.01% NaN_3. The pD of each sample was determined according to the equation: pD = pH meter reading + 0.4 (Glasoe & Long, 1960). Each sample was evaporated down twice under vacuum in a Savant Speedvac from "100%" D_2O (99.99% D from Merck Isotopes) before the final dilution and transfer to a Wilmad 538PP NMR tube. As an internal reference TSP was added to each sample for a final concentration one-tenth that of the duplex.

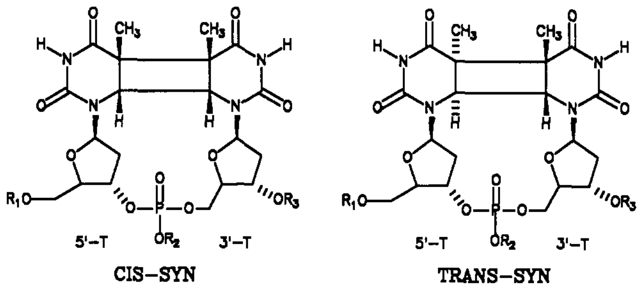

FIGURE 1: Cis-syn and trans-syn thymine dimers of dTpdT [$R_1 = R_3 = H$, $R_2 = (-)$] and the corresponding building blocks for their incorporation into DNA by solid-phase automated DNA synthesis [R_1 = DMT, $R_3 = P(OCH_3)(NC_4H_8O)$ or $P(OCH_3)(NC_6H_{14})$, $R_2 = CH_3$].

NMR Spectroscopy. All spectra were recorded on a Varian VXR-500 with a high-resolution proton probe and worked up with Varian VNMR version 6.1 software. Two-dimensional 500-MHz ^{1}H NOESY spectra were recorded in the phase-sensitive mode according to the hypercomplex method (Muller & Ernst, 1979; States et al., 1982; Keeler & Neuhaus, 1985) with the carrier frequency placed in the center of the spectrum. In order to optimize cross peak volumes, several NOESY experiments were acquired for each sample. Typically, experiments were performed nonspinning at a calibrated temperature near 10 °C, with a 0.2-s mixing time and 3.0-s relaxation delay time. Between 16 and 64 transients were collected for each 2048 points in t_2, and a total of 256 points were acquired in t_1. In order to ensure complete decay of the interferogram, Gaussian apodization factors of 0.032 and 0.127 were used in the t_1 and t_2 dimensions, respectively. In addition, a line broadening factor equivalent to 0.1 Hz was used in t_2. Exchangeable 1D proton spectra were collected at 10 °C in 90% H_2O using the $1-\bar{5}-10-\bar{1}0-5-\bar{1}$ binomial solvent suppression experiment (Hore, 1983) with a 10-kHz sweep width centered on the H_2O resonance. A 10-ms delay between the hard pulses gave an excitation maximum 5 kHz downfield of the H_2O resonance. Proton-decoupled 202.3-MHz ^{31}P spectra were recorded at 10 °C and referenced externally to 0.1 M TMP.

Temperature-Dependent NMR Spectroscopy. The 1D proton spectra of the decamers were taken at approximately 5 °C intervals from 5 to 80 °C for the parent and cis-syn duplexes and from 5 to 45 °C for the trans-syn duplex. Temperatures were calibrated against temperature-dependent shift differences in methanol and ethylene glycol (Van Geet, 1968; Kaplan et al., 1975). The shifts for the trans-syn decamer could be followed as a function of temperature (Figure 6) and were assigned on the basis of the low-temperature spectra and the spectra of isolated single strands at the higher temperatures. The changes in shifts were converted to fractional units, and K_{eq} was calculated for each temperature based on an all or none, two-state model for the helix $\rightleftarrows$ coil

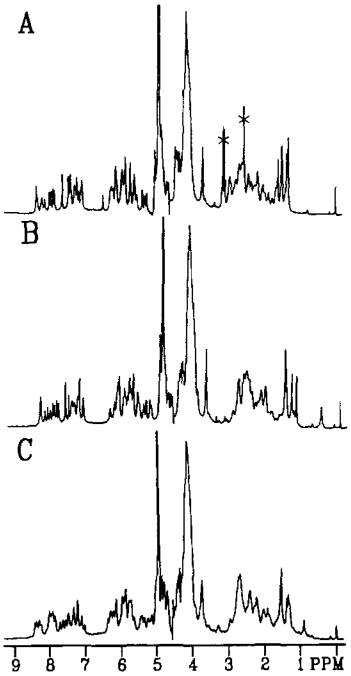

FIGURE 2: 500-MHz ^{1}H NMR spectra of the (A) parent, (B) cis-syn, and (C) trans-syn duplexes at 10 °C. Buffer conditions and chemical shift assignments are given in Table I. The peaks that are crossed out in (A) are due to residual EDTA. The large peak at 4.8 ppm in each spectrum is HOD.

equilibrium according to Marky and Breslauer (1987). Linear least-squares regression of ln K_{eq} vs $1/T$ gave ΔH and ΔS.

Temperature- and Concentration-Dependent UV Spectroscopy. The temperature dependence of the 260-nm absorbance of the parent and cis-syn duplexes was determined by one of us (J.T.) as previously described (Telser et al., 1989a,b). The concentrations used ranged from approximately 2×10^{-5} to 1.2×10^{-6} M in serial dilutions of a half. Thermodynamic parameters were extracted according to Marky and Breslauer (1987) by linear least-squares regression analysis of $1/T_M$ vs ln C_T, where C_T = total single strand concentration.

RESULTS

Selection and Synthesis of the Decamers. Decamers were chosen in order to form a complete turn of a B-DNA helix and to ensure that even a decamer containing a highly distorting lesion would form a duplex above 5 °C. A nonpalindromic sequence was chosen to ensure that only a duplex containing a single dimer could form, thereby avoiding complications associated with hairpin and homoduplex formation. Alternating purine–pyrimidine sequences were chosen to flank the dimer site so that an authentic sample of cis-syn decamer could be selectively produced by direct or sensitized photolysis of the parent duplex. In addition, alternating purine–pyrimidine sequences were expected to facilitate sequential ^{1}H NMR assignments (vide infra). High-melting, terminal d(GC)·d(GC) and d(GC)·d(CG) sequences were chosen in order to minimize fraying of the ends. These considerations ultimately led to the selection of d(CGTATTATGC)·d(GCATAATACG) as the sequence of the parent duplex.

The synthesis of the dimer-containing decamers and a ^{1}H NMR study on the single-strand form of the trans-syn decamer has been reported (Taylor & Brockie, 1988). The integrity of the cis-syn thymine dimer following the series of synthetic steps was established both enzymatically and spectroscopically. A 41-mer, constructed by ligating the cis-syn decamer to two other oligonucleotides, was found to be almost quantitatively cleaved at the site of the dimer by the cis-syn pyrimidine dimer specific T4 denV endonuclease V (Taylor & O'Day, 1990). In further support of its integrity, acetophenone-sensitized photolysis of the decamer d(CGTATTATGC) led to a product having the same characteristic ^{1}H NMR signals as found in the synthetic material.

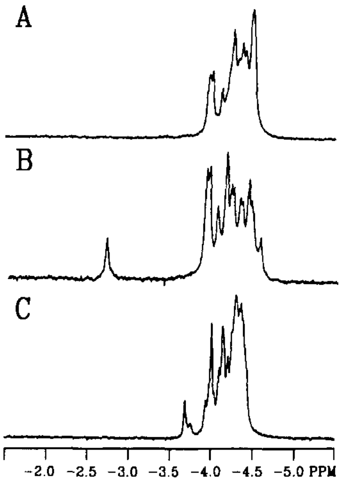

FIGURE 3: Proton-decoupled 202.3-MHz ^{31}P spectra of the (A) parent, (B) cis-syn, and (C) trans-syn duplexes at 10 °C in ppm relative to TMP. Buffer conditions are given in Table I.

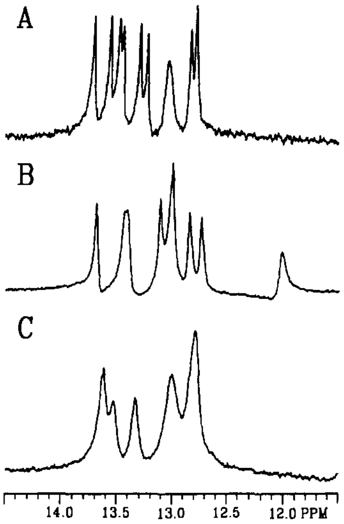

FIGURE 4: 500-MHz exchangeable ^{1}H spectra of the (A) parent, (B) cis-syn, and (C) trans-syn duplexes in 10% D_2O at 10 °C. Buffer conditions are given in Table I.

NMR Spectroscopy. Prior to acquiring the 2D NMR spectra, the optimal temperature for conducting the studies was determined by measuring the temperature dependence of the ^{31}P NMR spectra of the duplexes. A highly temperature-dependent ^{31}P NMR signal was detected at −2.7 ppm vs TMP in the spectrum of the cis-syn duplex, 1.2 ppm downfield from the remaining ^{31}P signals. At 10 °C this signal was sharp, becoming very broad at 20 °C and undetectable at higher temperatures. As a result, it was initially decided to conduct all 2D NMR experiments at 10 °C. Later temperature-dependent ^{1}H NMR studies indicated that the trans-syn duplex was completely duplex only up to 15 °C whereas the parent and cis-syn duplexes were completely duplex up to about 35 °C. The 1D ^{1}H and the proton-decoupled 1D ^{31}P NMR spectra of the three duplexes at 10 °C are shown in Figures 2 and 3. The *imino* proton spectra for the three duplexes (Figure 4) were obtained in 10% D_2O at 10 °C by use of a binomial solvent suppression pulse sequence. Homonuclear

Table I: ^{1}H NMR Assignments for the Parent,[a] Cis-Syn,[b] and Trans-Syn[c] Duplexes at 10 °C Relative to Internal TSP

		5′-C1	G2	T3	A4	T5	T6	A7	T8	G9	C10-3′
H6/H8	parent	7.65	8.01	7.31	8.38	7.23	7.42	8.34	7.10	7.88	7.43
	cis-syn	7.66	8.01	7.33	8.34	4.48	4.12	8.36	7.28	7.84	7.41
	trans-syn	7.61	7.99	7.20	8.24	3.77	3.90	8.42	7.20	7.85	7.35
H1′	parent	5.75	6.00	5.75	6.30	5.93	5.89	6.24	5.76	5.90	6.17
	cis-syn	5.76	6.03	5.82	6.41	5.59	5.48	6.22	5.84	5.92	6.17
	trans-syn	5.73	5.97	5.77	6.36	5.14	5.25	6.30	5.77	5.91	6.14
H5/CH3	parent	5.89		1.52		1.37	1.62		1.34		5.35
	cis-syn	5.87		1.52		0.51	1.48		1.21		5.27
	trans-syn	5.85		1.53		0.88	1.27		1.36		5.19
H2″	parent	2.44	2.82	2.55	2.98	2.58	2.61	2.97	2.38	2.69	2.19
	cis-syn	2.46	2.85	2.63	2.88	2.62	2.08	2.84	2.47	2.69	2.20
	trans-syn	2.42	2.76	2.37	2.69	2.48	1.93	2.96	2.40	2.68	2.20
H2′	parent	2.04	2.70	2.21	2.72	2.04	2.26	2.67	1.97	2.61	2.19
	cis-syn	2.08	2.69	2.27	2.60	1.97	2.08	2.52	2.09	2.56	2.20
	trans-syn	2.02	2.68	1.92	2.69	3.29	2.06	2.72	2.02	2.57	2.20
		3′-G20	C19	A18	T17	A16	A15	T14	A13	C12	G11-5′
H6/H8	parent	7.91	7.29	8.24	7.09	8.14	8.21	7.18	8.38	7.47	7.96
	cis-syn	7.90	7.31	8.23	7.16	8.08	8.15	7.17	8.35	7.49	7.99
	trans-syn	7.90	7.31	8.29	7.20	8.03	7.98	7.10	8.37	7.48	7.94
H1′	parent	6.15	5.63	6.17	5.66	6.13	5.98	5.57	6.29	5.64	5.98
	cis-syn	6.15	5.64	6.19	5.63	6.18	5.87	5.77	6.29	5.77	5.98
	trans-syn	6.14	5.63	6.21	5.45	5.88	5.88	5.71	6.27	5.70	5.95
H5/CH3	parent		5.28		1.33			1.49		5.41	
	cis-syn		5.30		1.35			1.54		5.41	
	trans-syn		5.34		1.31			1.51		5.41	
H2″	parent	2.35	2.28	2.85	2.42	2.92	2.94	2.43	2.97	2.46	2.79
	cis-syn	2.36	2.29	2.82	2.44	2.84	2.69	2.59	2.99	2.53	2.80
	trans-syn	2.38	2.28	2.84	2.25	2.56	2.94	2.21	2.96	2.48	2.79
H2′	parent	2.60	1.88	2.64	1.98	2.53	2.70	2.04	2.72	2.16	2.63
	cis-syn	2.59	1.89	2.62	2.07	2.52	2.56	2.12	2.71	2.23	2.65
	trans-syn	2.62	1.87	2.73	1.99	2.56	2.43	1.82	2.72	2.16	2.66

[a] 2.7 mM duplex, 100 mM NaCl, 10 mM sodium phosphate, pD 7.5, 0.1% NaN_3. [b] 1.46 mM duplex, 100 mM NaCl, 10 mM sodium phosphate, pD 8.5, 0.01% NaN_3. [c] 0.8 mM duplex, 100 mM NaCl, 10 mM sodium phosphate, pD 9.5, 0.1% NaN_3.

^{1}H NOESY spectra were acquired at 10 °C with a variety of mixing times, though 200–250 ms was found to lead to optimal intensities for both the intra- and internucleotide cross peaks in the H6/8 to H1′/H5/CH_3 portions of the phase-sensitive NOESY spectrum. Assignment of the proton NMR signals (Table I) was made according to general procedures [see Wuthrich (1986) for a general review and references]. Only partial assignment of the H3′, H4′, H5′, and H5″ protons has been possible and is not reported herein.

Assignment of CH5, H6; TCH$_3$, H6; and H2′, H2″ Pairs. Pairs of cytosine H5 and H6 signals were assigned by way of strong H5–H6 NOESY and COSY cross peaks and pairs of thymine H6 and CH_3 signals by way of strong NOESY and weak COSY cross peaks. Assignment of almost all of the H2′, H2″ pairs was made by way of strong H2′–H2″ COSY and NOESY cross peaks. In locating the H6 and CH_3 signals of the cis-syn and trans-syn thymine dimers, use was made of the fact that the H6 signals in the corresponding dinucleotide photoproducts appear in the 4–5 ppm range (Table II). The assignment of the methyl groups of the cis-syn dimer was confirmed by the presence of an NOE between them, consistent with their cis stereochemical relationship. A pair of H6 and CH_3 signals could be tentatively assigned to the 5′-T of the trans-syn dimer by the presence of a strong NOE between the H6 signal and an H1′ signal, as would be expected for a thymine in the syn, rather than anti, glycosyl bond conformation.

Assignment of H6/8, H5/CH$_3$, and H1′ of d(NPy) Subunits. The H6/8, H5/CH_3, and H1′ signals of d(NPy) sequences were readily assigned from the NOESY spectrum. In B-form DNA, where N refers to any nucleotide and Py refers to a pyrimidine nucleotide, the following four distances are within 5 Å: N(n)H6/8–Py(n + 1)H5/CH_3, Py(n + 1)-H5/CH_3–Py(n + 1)H6, Py(n + 1)H6–N(n)H1′, and N(n)-H1′–N(n)H6/8. Cross peaks in a NOESY spectrum corresponding to these distances can be connected to form boxes. Identification of these boxes allowed rapid assignment of these sets of proton signals. Nine such boxes were possible for the parent duplex, corresponding to unique d(GT), d(TT), and d(AC) sequences and two d(GC) and four d(AT) sequences. Many of the assignments of signals to d(NT) sequences could be confirmed by presence of NOEs between N(n)H1′ and T(n + 1)CH_3 which presumably arise indirectly via cross relaxation with N(n)H6/8 and/or T(n + 1)H6. In the cis-syn and trans-syn duplexes one of the d(AT) sequences is replaced by a d(AT[) sequence, where T[refers to a 5,6 cyclobutane adduct of the thymine. The unique d(TT) sequence in turn becomes a d(T[c,s]T) or d(T[t,s]T) sequence. Because of the altered structure of the duplex in the vicinity of the dimer not all of the cross peaks expected for a B-DNA conformation were observed.

The H1′–H6/8 Assignment Pathway. In idealized B-form DNA all of the intranucleotide H1′–H6/8 and internucleotide N(n)H1′–N(n + 1)H6/8 distances are within 5 Å, allowing one to "walk" along one strand of a duplex from H6/8 to H1′ of the same nucleotide and from there to H6/8 of the 3′-nucleotide and so on. Since the chemical shifts of AH8, CH6, GH8, and TH6 fall into fairly well-defined ranges, it was possible to first tentatively but rapidly assign the unique d(GT), d(AC), and d(TT) steps. Then, by making use of the known sequence of nucleotides and the assignments for the other d(NPy) steps, a unique and continuous path could be traced for the parent decamer and unique, but interrupted, paths for the cis-syn and trans-syn duplexes. This completed the assignment of almost all of the CH5/6, TCH$_3$, H6, and H8 signals of the three duplexes.

The H2′, H2″–H6/8 Assignment Pathway. The H2′ and H2″ protons were assigned in a manner similar to that used

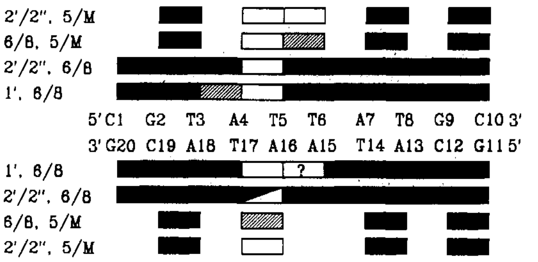

FIGURE 5: Summary of the internucleotide cross peak intensities observed in the 200-ms NOESY of the trans-syn duplex relative to those of the parent duplex. Cross peaks of readily observable intensity are indicated by solid bars, those of very weak intensity by hatched bars, and those which were unobservable by open bars. The question mark indicates that the intensity of the internucleotide cross peak could not be determined because of signal degeneracy. The half-filled bar indicates that cross peaks due to A16H2' and H2" cannot be distinguished because of signal degeneracy.

to assign the H1' protons. In B-form DNA intranucleotide H2'/2"-H6/8 distances and internucleotide N(*n*)H2'/2"-N(*n* + 1)H6/8 distances are within 5 Å of each other. Therefore, these signals could be assigned by tracing a path from H6/8 to either H2' or H2" of the same nucleotide and from these to H6/8 of the 3'-nucleotide and so on. Many of the H2'/H2" assignments for d(NPy) sequences were confirmed by way of NOEs between N(*n*)H2'/H2" and either C(*n* + 1)H5 or T(*n* + 1)CH_3. These NOEs presumably arise via intermediate cross relaxation with H6 of the C or T.

Interruptions in the Assignment Pathways. The sequential assignment pathway was interrupted in the cis-syn duplex by the lack of an observable A4H1'-T5H6 cross peak. Many more interruptions were found in the duplex containing the trans-syn dimer (Figure 5). The T3H1'-A4H8 cross peak was very weak, and all of the internucleotide NOEs expected for B-form DNA were entirely absent between A4 and T5. On the complementary strand, A16H1'-T17H6 and A16H2'/H2"-T17CH_3 were absent and A16H8-T17CH_3 was very weak.

AH2 Protons. Adenine H2 signals could be tentatively assigned for the parent duplex by consideration of the known interhelical and intrahelical cross relaxation pathways available for adenine H2s in B-DNA helices (Wuthrich, 1986). In B-form DNA the distance between H2s at d(AA)·d(TT) and d(TA)·d(TA) is sufficiently close to lead to an NOE. In d(CGTATTATGC)·d(GCATAATACG) two interstrand NOEs, A4H2-A18H2 and A7H2-A15H2, and one intrastrand NOE, A15H2-A16H2, are expected. These were readily detected in the NOESY spectra of the parent duplex, enabling the assignment of a signal at 6.4 ppm to A15H2. The H2s of the dimer duplexes could not be unambiguously assigned due to the disruption of the d(TAA)·d(TTA) section of the helix induced by the dimerization of the thymines. A unique NOESY cross peak was observed, however, in the spectrum of the trans-syn duplex. Examination of a trace along the T5CH_3 resonance led to the discovery of an NOE between T5CH_3 and a signal at 7.32 ppm. The only possible assignment for this signal was either A6H2, A15H2, or A16H2, as all the potential H1' or H6/8 assignments would have been for nucleotides far removed from the T5 site.

Melting Temperature Studies. Proton NMR spectra were obtained as a function of temperature for each of the duplexes in an effort to obtain thermodynamic parameters for their helix ⇄ coil equilibrium. Individual proton signals of the trans-syn duplex could be followed as a function of temperature (Figure 6), enabling calculation of ΔH and ΔS (Table III), assuming a two-state, all or none model. Unfortunately, individual proton signals of the parent and cis-syn duplex spectra could not be followed in the melting region due to extensive line broadening, making it impossible to obtain thermodynamic parameters by this method. The T_M was estimated to be 50 °C for the parent duplex at 2.7 mM, 46 °C for the cis-syn duplex at 1.5 mM, and only 26 °C for the trans-syn duplex at 0.8 mM. Thermodynamic parameters and more accurate T_M values could be obtained for the parent and cis-syn duplexes by analysis of UV melting temperature curves obtained for the duplexes in 1 M NaCl buffer (Table III). Thermodynamic parameters could not be accurately determined for the trans-syn duplex by this method because of its low melting temperature at the concentrations required for UV spectroscopic measurements.

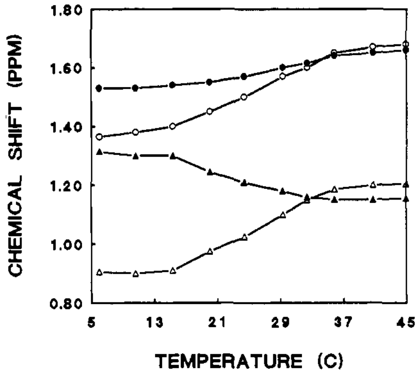

FIGURE 6: Temperature dependence of the T5 (△), T6 (▲), T8 (○), and T14 (●) methyl proton NMR signals of the trans-syn duplex. Buffer conditions are given in Table I.

DISCUSSION

Duplex Containing the Cis-Syn Thymine Dimer. The cis-syn decamer was synthesized by solid-phase phosphoramidite chemistry utilizing a cis-syn thymine dimer building block, prepared via sensitized photolysis of a derivative of dTpdT. It was conceivable that the cis-syn thymine dimer prepared in this manner is locked into a conformation that is different from that had it been formed in an oligonucleotide. In order to address this question, d(CGTATTATGC) was photolyzed with Pyrex-filtered light in the presence of the triplet sensitizer acetophenone. The ^1H NMR spectrum of the cis-syn decamer synthesized via the building block was virtually identical with that of the cis-syn decamer prepared by sensitized photolysis, indicating that the dimers were indeed in the same conformation.

In order to determine the optimal temperature for conducting the 2D NMR studies, ^1H and ^{31}P NMR spectra were acquired as a function of temperature. Although the characteristic upfield shift of the cyclobutyl methyl protons in the duplex appeared to remain constant at temperatures below 35 °C, a broad ^{31}P signal at −2.7 ppm relative to TMP began to appear at 20 °C which did not fully sharpen up until about 10 °C. Such behavior suggests that the phosphate giving rise to this signal may be involved in some sort of conformational equilibrium which is rapid at room temperature but slow at 10 °C. Downfield-shifted ^{31}P signals have been observed in DNA duplexes containing extrahelical bases (Patel et al., 1982) and abasic sites (Kalnik et al., 1989). In the former case the downfield-shifted signal was assigned to the phosphate on the 3'-side of the extrahelical base (Nikonowicz et al., 1989) and in the latter case to the two phosphates flanking the abasic site and the phosphate opposite the 5'-side of the abasic site. Unfortunately, it has not yet been possible to assign the downfield-shifted signal of the cis-syn duplex. Nonetheless,

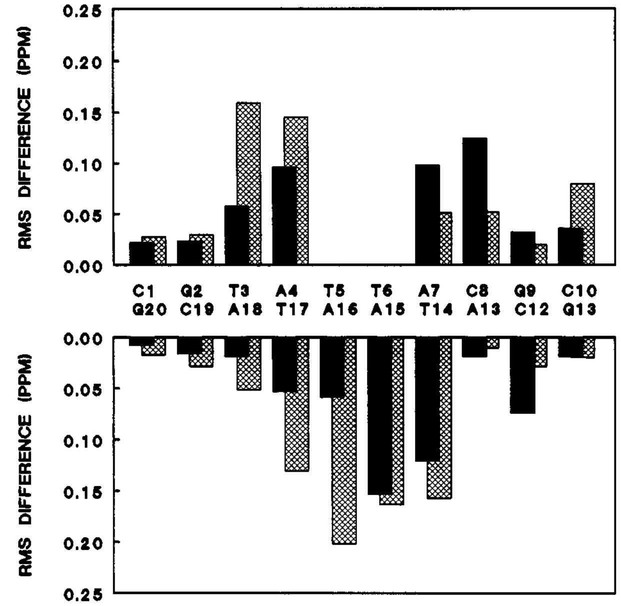

FIGURE 7: Root mean square chemical shift differences between the proton signals (H6/8, H5/CH$_3$, H1′, H2″, H2′) of a given nucleotide of the parent duplex and the cis-syn duplex (solid bars) or the trans-syn duplex (hatched bars). Shift differences for the T5 and T6 nucleotides have been omitted because they are principally due to changes in bonding and structure rather than conformation.

it is interesting to speculate that the conformational state giving rise to this signal may play an important role in the recognition of the cis-syn dimer by repair enzyme systems. As a result, it was decided to conduct initial solution-state structural studies at 10 °C.

The proton signals of the cis-syn duplex could be assigned in a straightforward way on the basis of standard NOE sequential assignment strategies for B-form DNA [see Wuthrich (1986) for a review]. The only interruption in the sequential assignment pathway was found at the d(A4T5) junction, where the A4H1′–T5H6 cross peak was not observed in the 250-ms NOESY spectrum. A similar interruption was also noted by Kemmink et al. (1987a) for the d(G3T4) junction in the cis-syn octamer duplex d(GCGT[c,s]TGCG)·d(CGCAACGC) and was attributed to an increase in the glycosyl angle of the 5′-T of the dTpdT site upon dimerization. The chemical shifts of the parent and cis-syn decamer duplexes are also quite similar to those of the octamer series. The large upfield shift of the CH$_3$ signal of the 5′-T of the dTpdT unit upon dimerization appears to be a characteristic feature of a dTpdT site flanked by purines. Whether such a large upfield shift occurs when the dTpdT site is flanked by pyrimidines remains to be determined. The nucleotide whose proton shifts were most affected by dimerization of the dTpdT site was A15, which is directly opposite the 3′-T of the dimer site. Sizable, but smaller, changes in chemical shifts occur adjacent to the dimer site and gradually reduce in magnitude as the distance from the site increases (Figure 7). Overall, the changes in shift induced by dimerization appear to be greater to the 3′-side of the dimer site, principally at T8, C12, and T14, than they do to the 5′-side and may indicate that the duplex is more distorted to the 3′-side of the dimer. This conclusion is similar to that reached by Pearlman et al. (1985) on the basis of modeling studies. They were only able to observe significant perturbations out one base pair from the 5′-side of the dimer and two base pairs from the 3′-side.

Though one cannot derive much in the way of precise structural information from 250-ms NOESY spectra, one hint as to the conformation of the cyclobutane ring of the cis-syn thymine dimer comes from analysis of the NOEs between its H6 and CH$_3$ protons. Whereas NOE cross peaks between T5CH$_3$ and T6CH$_3$ and between T5H6 and both T5CH$_3$ and T6CH$_3$ were present, that between T6H6 and T5CH$_3$ was not. The same was found to be true in a 70-ms NOESY expriment. These results require that the cyclobutane ring is in a conformation in which T5H6 and T6CH$_3$ are in axial positions and T5CH$_3$ and T6H6 are in equatorial ones (Figure 8A). In such a conformation, the two thymine rings of the dimer are twisted in a right-handed fashion, as are the bases in B-form DNA. This is the conformation used in the modeling studies of Pearlman et al. (1986) and is opposite to that found for the β-cyanoethyl phosphate derivative of the cis-syn dimer of dTpdT in the crystalline state (Cadet et al., 1985; Hruska et al., 1986). In the crystal structure, the two thymines are twisted in a left-handed fashion, placing T5CH$_3$ and T6H6 in axial positions (Figure 8B). These results suggest that the cis-syn dimer is conformationally flexible and that its conformation depends highly on its local environment.

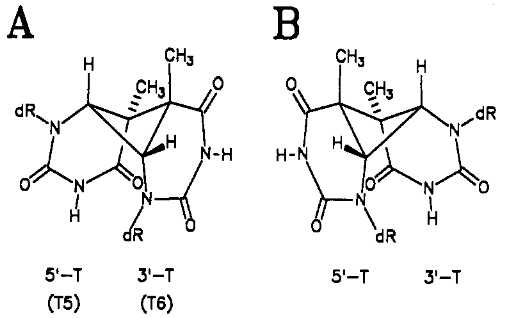

FIGURE 8: Puckered conformations of the cyclobutane ring of the cis-syn thymine dimer. (A) The conformation of the cyclobutane ring in the cis-syn decamer as suggested from the NOE data. (B) The conformation of the cyclobutane ring in the β-cyanoethyl phosphate derivative of the cis-syn dimer of dTpdT in the crystalline state (Cadet et al., 1985; Hruska et al., 1986).

In the imino region of the exchangeable proton spectrum of the cis-syn duplex one signal was found at 12.05 ppm, substantially upfield from the other signals and from the signals observed in the spectrum of the parent duplex (Figure 4). Two upfield imino signals were observed in the spectrum of d(GCGT[c,s]TGCG)·d(CGCAACGC), the most upfield of which appeared at 12.37 ppm, 1.62 ppm upfield from that in the parent, and was assigned to the 5′-T of the dimer (Kemmink et al., 1987b). The imino proton of the 3′-T of the dimer was also found to shift upfield, but only by 0.84 ppm, while the next greatest upfield-shifted signal (+0.32 ppm) was assigned to the imino proton of the A·T base pair on the 3′-side of the dimer. Though we have not yet been able to assign the upfield imino signal in the cis-syn decamer duplex, it is more than likely due to that of the 5′-T of the dimer and represents another characteristic of duplexes containing a cis-syn thymine dimer flanked by purines.

It was suggested that the large upfield shifts of the imino protons in the cis-syn octamer duplex indicated that hydrogen-bonding interactions between the dimer and the opposed adenines were significantly reduced, more so for the 5′-T (Kemmink et al., 1987b). It is important to consider the possibility, however, that the upfield shift of the imino proton of the 5′-T may be due in large part to ring current effects and/or to the change in the electronic structure of the thymine which occurs upon dimerization. In strong support of the former possibility is the fact that the 5′-TCH$_3$ signal of the dTpdT unit of the decamer duplex shifts upfield by 0.86 ppm upon dimerization, while the 3′-TCH$_3$ signal shifts upfield by 0.135 ppm. In the octamer duplex the corresponding upfield

Table II: Comparison of ^1H NMR Data of Cis-Syn and Trans-Syn Thymine Dimer Containing Oligonucleotides

	5′-T					3′-T				
	CH_3	H6	H1′	H2″	H2′	CH_3	H6	H1′	H2″	H2′
cis-syn										
dTpdT[a]	1.50	4.27	5.67	2.40	2.63	1.54	4.35	6.00	2.12	2.33
duplex octamer[b]	0.71	4.48	5.60	2.61	2.00	1.53	4.10	5.39	2.00	2.00
duplex decamer[c]	0.51	4.48	5.59	2.62	1.97	1.48	4.12	5.48	2.08	2.08
trans-syn										
dTpdT[d]	1.45	4.27	5.28	2.62	3.34	1.51	4.27	5.71	2.03	2.22
SS decamer[e]	1.20	4.05	5.14	2.40	3.27	1.13	3.70	5.50	1.88	1.73
duplex decamer[c]	0.88	3.77	5.14	2.48	3.29	1.27	3.90	5.25	2.06	1.93

[a] Hruska et al. (1975). [b] Kemmink et al. (1987a). [c] This work. [d] Liu and Yang (1978). [e] SS = single strand; Taylor and Brockie (1988).

shifts are 0.82 and 0.27 ppm (Table II). Therefore, it may not be possible to conclude on the basis of imino proton chemical shifts alone the extent to which hydrogen bonding of the dimer to the opposed adenines is diminished.

Duplex Containing the Trans-Syn Thymine Dimer. This work represents the first study of duplex DNA containing a trans-syn thymine dimer. As was the case for the cis-syn duplex, all of the proton NMR signals could be assigned via a sequential NOE assignment strategy valid for B-form DNA in spite of a number of interruptions in the assignment pathway. That T5 of the trans-syn duplex is indeed in a syn glycosyl conformation is supported by the presence of a strong T5H6–H1′ cross peak and weak T5H6–H2′ and T5H6–H2″ cross peaks. In a syn glycosyl orientation, T5H6 faces away from the H2′ and H2″ protons and sits over the H1′ proton (Figure 9C). The short T5H6–H1′ distance would also explain the origin of a cross peak between T5H1′ and $T5CH_3$ observed in the 200-ms NOESY which must occur via intermediate cross relaxation with T5H6. In further support of the syn glycosyl stereochemistry of the 5′-T of the dimer, the T5H2′ signal is shifted downfield by 1.25 ppm relative to the parent. This has been attributed to the syn glycosyl conformation of the T6 base which places the deshielding cone of the C2 carbonyl group directly over the H2′ proton (Liu & Yang, 1978) (Figure 9C). As expected for a trans-fused cyclobutane dimer (Figure 1), only intranucleotide NOE cross peaks were observed between the methyl and H6 signals of T5 and T6; internucleotide cross peaks were not detected. The anti glycosyl conformation of T6 was confirmed by the presence of strong intranucleotide NOE cross peaks between H6 and H2′/2″ expected for such a conformation (Figure 9D).

Major interruptions in the sequential NOE assignment pathway along with large changes in chemical shifts occurred at d(A4T5)·d(A16T17) (Figure 5) and can be understood to be a consequence of the syn glycosyl conformation of the 5′-T of the trans-syn dimer. Preliminary modeling studies (Figure 10B) indicate that, because of the syn conformation, H6 and CH_3 of T5 are greater than 5 Å from the H8 and H2′/H2″ protons of A4, explaining the lack of any observable internucleotide NOE cross peaks. The syn conformation of T5 also causes the bulky and hydrophobic methyl group to be thrust into a region normally reserved for base-pairing interactions (Figure 9C). The interruption of several internucleotide NOEs between A16 and T17 along with changes in ^1H NMR shifts relative to the parent may be due to conformational changes induced by the T5 methyl group. That the DNA duplex to either side of d(A4T5)·d(A16T17) could be almost completely assigned via a sequential NOE strategy for B-form DNA suggests that there may be a kink or dislocation at this junction. The generally upfield-shifted proton NMR signals and a weak internucleotide NOE between T3H1′ and A4H8 relative to the parent duplex suggest that the disruption induced by the thymine dimer may well extend beyond the junction. The most dramatic change in shift was observed for the T3H2′ signal, which shifted upfield by almost 0.3 ppm. Large chemical shift differences were also seen for T14 and A15. The large changes in shift may be the result of changes in the π-stacking interactions between the bases caused by the formation of the trans-syn dimer.

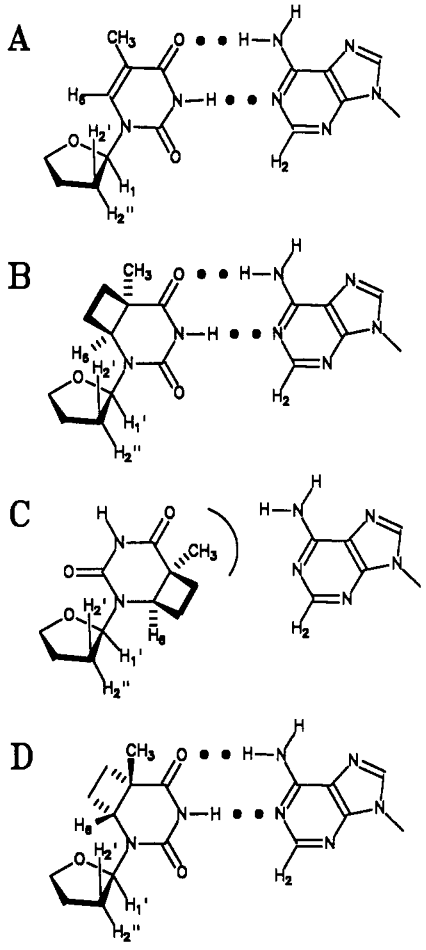

FIGURE 9: Schematic models for the d(T5T6)·d(A15A16) section of the parent, cis-syn, and trans-syn duplexes: (A) d(T5)·d(A16) and d(T6)·d(A15) of the parent duplex, (B) d(T5)·d(A16) of the cis-syn duplex, (C) d(T5)·d(A16) of the trans-syn duplex, and (D) d-(T6)·d(A15) of both the cis-syn and trans-syn duplexes. Dots indicate potential hydrogen-bonding interactions as deduced from preliminary modeling studies. The curve in (C) emphasizes the hydrophobic nature and steric bulk of the methyl group.

Insofar as chemical shift differences reflect differences in structure, the trans-syn dimer appears to induce greater distortion than the cis-syn dimer (Figure 7). Most of the distortion appears to lie to the 5′-side of the dimer, with the most significant changes observed at T3, A4, T14, A15, A16, and T17. As for the 3′-side of the dimer, the T6 methyl of the trans-syn duplex is significantly more shielded than the cor-

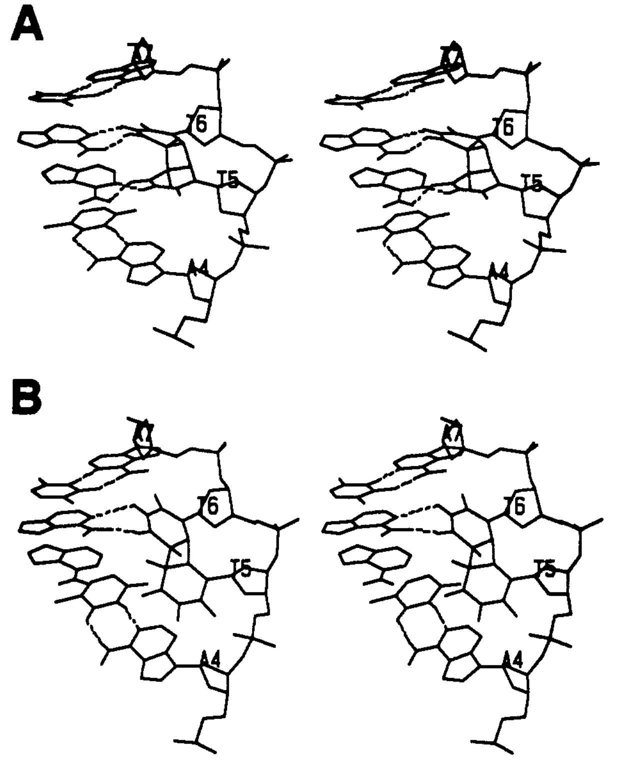

FIGURE 10: Models for the (A) cis-syn and (B) trans-syn duplexes which illustrate the general disposition of the dimers within a DNA duplex. The models were obtained by molecular mechanics minimization of a B-DNA duplex representing the central hexamer of the parent duplex in which the thymines were made to dimerize. Minimization was conducted with the SYBYL program and the Kollman force field including the electrostatic term and a distance-dependent dielectric constant. More accurate models await analysis of NOE buildup and coupling constant data. Only part of the hexamer and only the hydrogens involved in hydrogen bonding are shown to aid in visualization. Hydrogen bonds are indicated by dashed lines.

responding methyl in the cis-syn duplex. This suggests that the two d(T6A7) junctions have different conformations despite the similar anti glycosyl conformation of both T6s (Figure 9D). In spite of the seemingly greater distortion induced by the trans-syn dimer than the cis-syn dimer, no unusually shifted signals were observed in either the ^{31}P or the imino region of the exchangeable ^1H NMR spectra.

The lack of an unusually upfield-shifted imino proton signal in the NMR spectrum of the trans-syn duplex may be due to a number of possibilities. The imino proton most likely to be upfield shifted would be that of the 5′-T of the dimer as it is unlikely to be involved in any sort of hydrogen bonding. This is because the 5′-T is in a syn glycosyl conformation which positions the imino proton on the surface of the duplex, in what would be the major groove, away from the interior of the helix where the Watson–Crick base-pairing interactions take place (Figure 9C). This imino proton, though highly upfield shifted, would be unobservable due to rapid exchange with solvent. The other candidate for an upfield-shifted signal would be the imino proton of the 3′-T of the dimer. Unlike the 5′-T, the 3′-T is in an anti glycosyl conformation and does not preclude hydrogen bonding to the opposed adenine (Figure 9D). Lack of an observable upfield-shifted NMR signal for the 3′-T imino proton could therefore mean one of three things: (1) it is exchanging rapidly due to the distortion of the helix in this region, (2) it is fully hydrogen bonded, or (3) it is not fully hyrogen bonded but deshielding by the flanking A·T base pair offsets the upfield shift due to diminished hydrogen bonding. There is always the further possibility that the proton signals are much further upfield and are being obscured by the water suppression sequence used. Clearly, more experiments will be required to resolve this issue.

Table III: Thermodynamic Parameters for the Helix ⇌ Coil Equilibrium for Duplexes Containing Cyclobutane Dimers

		ΔH (kcal)	ΔS (eu)	T_M (°C)[a]	NaCl (M)
octamer	parent	67[b]	163[b]	110	1.0
	parent	49 ± 3[c]	131 ± 7[c]	70	0.2
	cis-syn	51 ± 7[c]	143 ± 20[c]	93	0.2
decamer	parent	74[b]	190[b]	93	1.0
	parent	64 ± 6[d]	178 ± 18[d]	64	1.0
	cis-syn	66 ± 7[d]	189 ± 19[d]	55	1.0
	trans-syn	44 ± 4[e]	132 ± 13[e]	33	0.1

[a] Calculated from the thermodynamic parameters for a single strand concentration of 10 mM. [b] Predicted values (Breslauer et al., 1986). [c] Derived from the temperature dependence of the T5CH$_3$ ^1H NMR signal in 200 mM NaCl and 50 mM sodium phosphate buffer at a pH of approximately 6 (Kemmink et al., 1987b). [d] Derived from temperature- and concentration-dependent UV measurements in 1 M NaCl, 10 mM sodium phosphate buffer, pH 7, and 0.1 mM EDTA; this work. [e] Derived from the temperature dependence of the T5CH$_3$ ^1H NMR signal shown in Figure 6.

Melting Temperature Studies. Another indication of the structural perturbation induced by the dimers comes from the thermodynamics of the helix ⇌ coil equilibrium. Thermodynamic parameters obtained from melting temperature studies on the cis-syn decamer duplexes are tabulated in Table III and are similar to those reported for the octamer duplexes (Kemmink et al., 1987b). The 9 °C drop in melting temperature upon formation of cis-syn dimer in the decamer calculated from the thermodynamic data corresponds quite well to that of 8 °C previously estimated from melting temperature studies of dimer-containing DNA (Rahn & Patrick, 1976).

The thermodynamic parameters suggest that dimerization of the dTpdT site to give the cis-syn product does not greatly affect the ability to form a duplex. What little destabilization is induced by the cis-syn dimer appears to result from an entropy effect, a conclusion contrary to what would be expected solely on the basis of configurational entropy and hydrogen-bonding and base-stacking interactions. Dimerization of a dTpdT unit would be expected to reduce the number of conformations of the coil state without changing that of the duplex state and hence lower ΔS rather than raise it. Likewise, disruption of hydrogen-bonding and π-stacking interactions upon dimerization would be expected to lower ΔH rather than raise it. A crude estimate of the reduction in configurational entropy can be made by assuming that nine rotatable bonds or pseudorotatable groups become frozen upon dimerization, the two glycosyl bonds, the two sugar rings, and the five internucleotide bonds. If one assumes that each rotatable bond or group had two accessible conformations prior to dimerization, then ΔS is given by $-9R \ln 2$ or -12.5 eu (Bloomfield et al., 1974). Of course, the above analysis is simplistic and does not take into account solvent and counterion effects or chain dynamics. It is clear that more work will be required in order to obtain more precise thermodyamic parameters and other studies to determine their origin.

The change in the thermodynamic parameters for helix ⇌ coil transition upon formation of the trans-syn duplex are in the direction expected for a loss of configurational entropy in the coil form and a loss of hydrogen-bonding and π-stacking interations in the duplex. The large difference in the melting behavior between the cis-syn and trans-syn duplexes can be readily ascribed to the difference between the conformation of the glycosyl bond of the 5′-T of the dimers (Figure 9B,C). Both dimers have the 3′-T in the anti conformation. In the trans-syn dimer the glycosyl bond of the 5′-T is in the syn conformation, which places the methyl group in the center of the helix, where it would be expected to interfere with the hydrogen-bonding and π-stacking interactions and lead to a decrease in ΔH for duplex formation. The cis-syn dimer on the other hand has the 5′-T in the anti orientation and is therefore similar in conformation and hydrogen-bonding properties to a B-form thymine.

Biological Implications. The large destabilizing effect of the trans-syn dimer on the duplex form of DNA and the minimal effect of the cis-syn dimer helps explain the action of polymerase I of *E. coli* on DNA templates containing these photoproducts. We recently found that a cis-syn thymine dimer contained within the same sequence as used in this study can be bypassed in vitro by polymerase I in a nonmutagenic manner (Taylor & O'Day, 1990). Under the same conditions the trans-syn dimer cannot be bypassed (unpublished results). The ability of the cis-syn thymine dimer to be bypassed in a nonmutagenic manner is in accord with the overall conclusion that it does not significantly disrupt the originally encoded Watson–Crick hydrogen-bonding interactions or perturb the structure of the DNA. The inability of the trans-syn dimer to be bypassed is in accord with the overall conclusion that the methyl of the 5′-T of the dimer causes signifiant disruption of the Watson–Crick hydrogen-bonding interactions and possibly introduces a kink or dislocation into the DNA.

An intriguing consequence of the differences between cis-syn and trans-syn thymine dimer containing DNA, yet to be tested, is that the trans-syn dimer, because it is more distorting and destabilizing, may be more easily detected and repaired. Even if it remains undetected by repair systems when in the duplex form, it could elicit their action by impeding the progress of a polymerase, or failing this, lead to cell death. The cis-syn dimer, on the other hand, because it is less distorting and destabilizing may be harder to detect and repair, making it more likely to be encountered and bypassed by a polymerase. The precise relationship, however, between the structure and properties of DNA containing cis-syn and trans-syn dimers and their biological activity remains to be elucidated and is consequently under study.

SUPPLEMENTARY MATERIAL AVAILABLE

NOESY spectra and the sequential pathways used to assign H6/8, H1′, and H5/CH_3 of the parent, cis-syn, and trans-syn duplexes (7 pages). Ordering information is given on any current masthead page.

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