Nuclear Magnetic Resonance Studies of Cis-Syn, Trans-Syn, and 6-4 Photodimers of Thymidylyl(3′-5′)thymidine Monophosphate and Cis-Syn Photodimers of Thymidylyl(3′-5′)thymidine Cyanoethyl Phosphotriester†

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ABSTRACT: Three out of four possible photodimers of thymidylyl(3′-5′)thymidine monophosphates (i.e., cis-syn, 6-4, and one of the trans-syn) and two structural isomers (i.e., *R* and *S* forms) of *cis-syn*-thymidylyl(3′-5′)thymidine cyanoethyl phosphotriester have been isolated and purified from the reaction mixtures after UV irradiation and studied by multinuclear magnetic resonance spectroscopy. All five inter thymine base linked photodimers have grossly similar structures which are quite different from those of the parent thymidylyl(3′-5′)thymidine. The base of Tp- is in the syn conformation, and that of -pT is in the anti conformation. The sugar puckering of Tp- is dominated by the ^2E conformer, but in -pT it is in ^4E; except for the conformer around the $C_{5'}$-$O_{5'}$ bond, the 6-4 isomer is very similar to those of cis-syn and trans-syn conformation. As expected, there are sugar-phosphate backbone distortions in the phosphotriesters, due to the neutralization of the negative charge of the phosphate. In general the structures of all five photodimers are very close to those of the cis-syn photodimer of thymidylyl(3′-5′)thymidine monophosphate cyanoethyl ester as studied by X-ray diffraction [Cadet, J., Voituriez, L., Hruska, F. E., & Grand, A. (1985) *Biopolymers 24*, 897-903; Hruska, F. E., Voituriez, L., Grand, A., & Cadet, J. (1986) *Biopolymers 25*, 1401-1417]. While the trans-syn photodimer has two structural isomers, only one [C_6(of Tp-)-*R*] was produced by the UV irradiation and studied.

Photodimerization of pyrimidine nucleobases is a well-established fact in isolated DNA (Fisher & Johns, 1976) as well as in cellular DNA (Patrick & Rahn, 1976). These photoreactions may be the results of direct absorption of far- and near-ultraviolet (UV)[1] (Wang, 1960; Paterson et al., 1981) or of energy transfer from photosensitizng agents in their triplet excited state (Elad et al., 1967; Khattak & Wang, 1972). UV photolysis of pyrimidine bases (Fisher & Johns, 1976) or of their corresponding nucleosides (Ben-Hur et al., 1967; Cadet et al., 1985b) may led to the formation of the cis-syn, trans-syn, cis-anti, and trans-anti stereoisomers. However, the formation of the anti-type photodimers is unlikely in oligonucleotides for steric reasons. Another class of DNA photoproduct which deserves increasing attention due to its possible high mutagenic potential (Brash & Haseltine, 1982; Wood et al., 1984) is the pyrimidine-pyrimidone (6-4) photoadduct (Varghese & Wang, 1967; Franklin et al., 1985; Franklin & Haseltine, 1986). Early studies have shown that far-UV photolysis of oxygen-free aqueous solutions of thymidylyl(3′-5′)thymidine (d-TpT) generated two isomers of internal cyclobutane-type dimers (Johns et al., 1964; Peason, 1965). The conformational features of the major photoproduct, which was characterized as a cis-syn diastereoisomer, were investigated by 220-MHz ^{1}H nuclear magnetic resonance (NMR) analysis (Hruska et al., 1975). It was reported that acetophenone-mediated photosensitization of d-TpT gave rise to cis-syn and trans-syn photodimers which were partly characterized by ^{1}H NMR spectroscopy (Liu & Yang, 1978). More recently, it was suggested that near-UV irradiation of d-TpT in the presence of acetone, which serves as a photosensitizer, was able to generate the pyrimidine-pyrimidone (6-4) photoproduct (d-T6p4T) (Umlas et al., 1985). However, no chemical or spectroscopic evidence was provided to substantiate this hypothesis.

Increasing attention is being devoted to the determination of the conformational properties of oligodeoxyribonucleotides containing cyclobutapyrimidines (pyr[]pyr). It appears that the conformational changes caused by a pyrimidine dimer in a DNA chain are probably involved in the recognition of the photolesion by repair enzymes (Pearlman et al., 1985; Rao et al., 1984). Two recent theoretical calculation studies of the conformational changes associated with the presence of a cis-syn cyclobutathymidine in a short oligodeoxyribonucleotide have led to different conclusions regarding either the formation of a local distortion (Rao et al., 1984) or a more severe kink

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[1] Abbreviations: 1D (2D) NMR, one- (two-) dimensional NMR; COSY, chemical correlated spectroscopy; CPK model, Corey-Pauling-Koltun model; DNA, deoxyribonucleic acid; ^2E (^3E or ^4E), $C_{2'(3'or4')}$ endo conformer; EDTA (ethylenedinitrilo)tetraacetic acid; FAB-MS, fast atom bombardment mass spectrometry; *gg* (*gt*, *g'g'*), gauche-gauche (gauche-trans, gauche'-gauche'); HPLC, high-performance liquid chromatography; *J*, coupling constant in hertz; Hz (MHz), cycles (megacycles) per second; NMR, nuclear magnetic resonance spectroscopy; NOE (NOESY), nuclear Overhauser enhancement (spectroscopy); pyr[]pyr, dipyrimidine photodimer; TMS, tetramethylsilane; d-TpT, thymidylyl(3′-5′)thymidine monophosphate; UV, ultraviolet; *cs* (*ts*, *CEcs*), cis-syn, (trans-syn) photodimer of d-TpT (β-cyanoethyl phosphotriester); 6-4, pyrimidine-pyrimidone (6-4) photoadduct.

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Chart I: Stereoisomers of Photodimerization of Deoxythymidylyl(3′–5′)deoxythymidine Phosphate

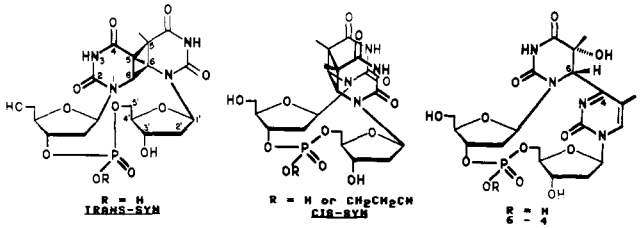
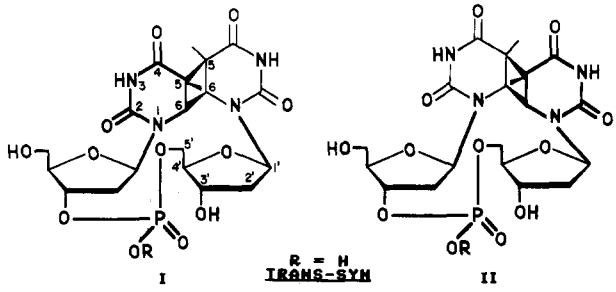


Chart II: Two Structural Isomers of *ts*



(Pearlman et al., 1985). However, a preliminary ^1H NMR study (Kemmink et al., 1987) of a short duplex containing a T[]T photodimer (GCGT[]TGCG·CGCAACGC) found that the DNA duplex formed a small distortion of the B-form. The major conformational change in the hexamer duplex occurred at the site of the thymine dimer. X-ray structure analysis of the cis-syn photodimer of the d-TpT with the *S*-cyanoethyl phosphotriester (Cadet et al., 1985a; Hruska et al., 1986) has revealed that the two pyrimidine bases are rotated by −29° from the position of direct overlap of their corresponding atoms. This represents a major distortion of DNA, since in DNA adjacent thymines are rotated by +36°. The present work constitutes an extension of this study of the conformational properties of these photodimers in the solid state to aqueous solution by using ^1H, ^{31}P, and ^{13}C NMR analyses. The comparison of structures has been extended also to the corresponding deprotected *cis-syn*-d-TpT photodimer in order to assess the possible role of the cyanoethyl group in the conformational changes. Other d-TpT photoproducts that have been included in this study are the trans-syn photodimer and the pyrimidine–pyrimidone (6–4) photoadducts (Rycyna & Alderfer, 1985) which are also biologically relevant photolesions (Charts I and II).

Experimental Procedures

Preparation of d-TpT Cyanoethyl (CE) Phosphotriester. The cyanoethyl phosphotriester of d-TpT has been prepared according to Letsinger and Ogilvie's procedure (Letsinger & Ogilvie, 1969) by using unprotected thymidine for the condensation with 5′-*O*-tritylthymidine 3′-*β*-cyanoethyl phosphate (pyridinium salt). Both *R* and *S* isomers of 5′-d-TrTp(CE)T and d-Tp(CE)T have been successively purified by preparative HPLC on a PAK 500 silica gel cartridge using a LC 500 chromatograph (Waters Associates, Milford, MA) equipped with a Model 401 refractometer index detector. The eluent consisted of a mixture by volumes of ethyl acetate/2-propanol/water (75:16:9). Deprotection of the cyanoethyl phosphotriester derivative of the d-TpT was achieved by treatment with ammonia-saturated methanol for 15 h at room temperature.

Far-UV Irradiation. Steady-state photolysis experiments were carried out with a Rayonet photochemical reactor (Southern New England Ultraviolet Co., Hamden, CT) using

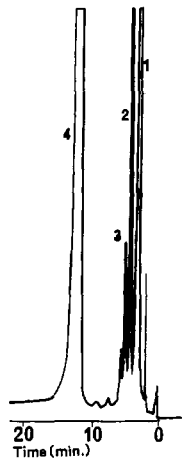


FIGURE 1: HPLC profile of d-TpT after UV irradiation. The eluent solution is 0.1 M ammonium acetate + 5% CH_3CN. The flow rate is 2 mL/min, and the column is a Whatman analytical octadecylsilyl silica gel ODS-3. Peaks: 1, *cs*; 2, 6–4; 3, *ts*; 4, unreacted d-TpT.

16 RPR-2537 A lamps. The aqueous solution of 1 mM d-TpT was irradiated in RQV-218 quartz reaction vessels (F.O.B., Middletown, CT) at a temperature that was maintained below 40 °C with a fan.

Isolation of the d-TpT Photoproducts. The mixture of the d-TpT photoproducts has been separated by ion-suppression HPLC on an analytical octadecylsilyl silica gel ODS-3 column (Whatman, Clifton, NJ; 25 × 0.46 cm i.d.) (Zon et al., 1985). The eluent was 0.1 M aqueous ammonium acetate plus 5% acetonitrile (Figure 1) (Franklin et al., 1982).

FAB-MS Analysis. Fast atom bombardment (FAB) mass spectrometry (MS) was carried out in a Model MS 50 spectrometer equipped with a commercially available FAB gun. Desorption of the molecules was obtained by exposure to a beam of 8-keV xenon atoms of the sample in a glycerol mull.

NMR: Sample Preparation. All five samples were dissolved in 0.4 mL of 99.8% D_2O solution with 0.01 mM EDTA and 10 mM phosphate buffer solution (pH 7.0). In order to eliminate possible intermolecular interaction, the concentrations of all samples were 1 mM.

^{13}C and ^{31}P NMR. ^{13}C and ^{31}P NMR spectra were obtained by a Bruker WM-300 NMR spectrometer with a 5-mm $^{13}C/^1H$ dual probe (operated at 75.47 MHz for ^{13}C) and a 10-mm broad-band probe (operated at 121.49 MHz for ^{31}P), respectively, in the quadrature detecting Fourier transform mode. The spectrometer was also upgraded with a ASPECT 3000 microcomputer with 256K word memory and a Winchester fixed disk drive with 160-Mbyte storage capability. A gated pulse sequence with nuclear Overhauser enhancement (NOE) was applied to avoid heating the samples due to the continuing decoupler power applied to the sample. In order to keep long-term stability of the magnetic field, an internal lock channel monitors the ^2H signal from the solvent (^2H_2O). A BVT-1000 temperature regulator was used to keep the temperature constant. The temperature in this experiment was 28 °C unless otherwise indicated. The digital resolutions were 0.925 and 0.365 Hz/point for ^{13}C and ^{31}P, respectively.

Due to the low sensitivity of naturally abundant ^{13}C in very low concentration, only the carbons with relatively short spin–lattice relaxation time were recorded (see Results).

^1H NMR. Several NMR spectrometers were used for this project. A 600-MHz spectrometer (operated, however, at 611.1 MHz for ^1H) located in Carnegie-Mellon University, Pittsburgh, PA, and a Bruker WM-500 (operated 500.13 MHz for ^1H) located at Yale University, New Haven, CT, were used mainly for the conventional one-dimensional (1D) NMR

Table I: Chemical Shifts in ppm of ^{13}C Atoms in Five d-TpT Photodimers

		$C_{1'}$	$C_{2'}$	$C_{3'}$	$C_{4'}$	$C_{5'}$	C_2	C_4	C_5	C_6	Me	others	
CEcs-R	Tp	84.97	32.86	69.85	81.92	62.32	*a*	*a*	44.99	54.62	17.84	CH_2CN	19.96
	pT	89.22	36.81	69.85	82.98	64.24	*a*	*a*	41.98	52.72	17.84	OCH_2	66.32
CEcs-S	Tp	85.69	33.36	70.12	82.53	62.37	*a*	*a*	44.13	55.38	17.73	CH_2CN	20.12
	pT	90.30	36.64	69.86	83.17	63.24	*a*	*a*	37.01	53.39	18.13	OCH_2	63.59
cs	Tp	86.12	34.33	76.45	83.96	62.03	*a*	*a*	51.82	60.25	17.78		
	pT	88.22	37.01	69.71	84.08	66.36	*a*	*a*	47.33	56.23	17.17		
ts	Tp	86.13	34.12	78.72	83.54	64.31	*a*	*a*	47.83	62.67	20.76		
	pT	92.48	36.88	68.83	85.30	66.43	*a*	*a*	46.52	59.70	22.00		
6–4	Tp	83.1	35.9	70.7	83.1	59.7	*a*	*a*	73.2	58.7	26.0		
	pT	88.9	35.9	70.7	86.7	65.6	*a*	*a*	*b*	*b*	14.5		
d-TpT[c]	Tp	85.96	38.32	75.73	86.43	61.83	152.32	167.21	112.25	138.23	12.35		
	pT	85.62	39.45	71.20	85.80	65.65	152.48	167.05	112.28	138.12	12.38		

[a] Not observed. [b] Out of spectral window; includes carbon in the group CN. [c] From Rycyna and Alderfer (1985).

spectra. A Bruker AM-500 located at Bruker Instrument, Inc., was used for the phase-sensitive 2D NOESY (Aue et al., 1976; Frechet et al., 1983). Finally, a Bruker WM-300 which was described in the previous section equipped with a 10-mm ^1H probe (operated at 300.13 MHz for ^1H) was used for both 1D and 2D COSY and also 2D NOESY (Aue et al., 1976; Nagayama et al., 1980; Ernst et al., 1987).

The digital resolutions of 1D spectra were 0.305 and 0.183 Hz/point for 500 or 600 and 300 MHz, respectively.

2D COSY acquisition was obtained by a $90°-t_1-45°-\text{FID}(t_2)$ pulse sequence. The second 45° pulse is to reduce the peak intensity on the diagonal line (Aue et al., 1976). Data sets consisted of 512 and 256 data points for t_2 and t_1 domains, respectively. This gives a digital resolution of 7 Hz/point with spectral window of around 2000 Hz. A total of 128 t_1 values were taken during the sampling and then zero-filled to 256. Both time domains were multiplied by a sine bell function during the Fourier transform. A total of 64–128 scans were recorded for each t_1 value.

Two-dimensional NOESY acquisition was obtained by a pulse sequence of $90°-t_1-90°-\tau_m-90°-\text{FID}(t_2)$. Here the τ_m is a mixing time. A randomized τ_m (between 0.5 and 0.8 s) was used to eliminate the zero-quantum transition (Bodenhausen et al., 1984). In order to remove the quadrature image, an incremental phase change after first pulse and a 32-step phase cycling were used (Marion & Wüthrich, 1983; Bodenhausen et al., 1984). The t_2 domain has 1024 data points, and 128 t_1 values were taken and then zero-filled to 512 or 1024. A similar window function as in 2D COSY was applied in Fourier transform. A total of 128–256 scans were recorded for each t_1 value.

NMR: Spectral Simulation. The chemical shift and coupling constant values were determined accurately by spectral simulation of the 1D ultra-high-resolution spectra (611.1 MHz). This task was done on an ASPECT 2000 microcomputer with Bruker software PANIC.

Results

Isolation and Characterization of Photoproducts Arising from 254-nm Irradiation of d-TpT. Far-UV irradiation of 1 mM d-TpT in aqueous solution gave rise to three main photoproducts which have been separated by ion-suppression HPLC on an analytical octadecylsilyl silica gel ODS-3 column or a semipreparative C-18 reversed-phase column. Three compounds have lost the characteristic absorption of 2,4-dioxopyrimidines at 254 nm. Two of the photoproducts were found to undergo photoreversal to the parent d-TpT when exposed to far-UV light (250 nm) in aqueous solution. This is indicative of a cyclobutane-type structure. Further confirmation was provided by FAB-MS (presence of a characteristic peak at m/z 444 corresponding to the loss of a sugar fragment from the 5′-nucleotidyl unit) and ^1H NMR (upfield shift for the methyl and C_6-H proton resonances and the change of the patterns of C_6-H doublets). It should also be mentioned that the stereochemistry of the main photodimer which shows the fastest eluting HPLC mobility has been determined by X-ray diffraction analysis using the cyanoethyl ester derivative (Cadet et al., 1985a). The last photoproduct which exhibits fluorescence properties has been characterized as the 6–4 pyrimidine–pyrimidone adduct on the basis of FAB-MS (Rycyna & Alderfer, 1985) and ^1H NMR analysis.

^{31}P NMR. The proton-decoupled ^{31}P NMR spectra of these five photodimers (for short they will be called *cs*, *ts*, *CEcs-R*, *CEcs-S*, and 6–4.) showed only a singlet as expected, except the *CEcs-R* has an extra minor peak which may indicate a possible impurity. Fortunately, the impurity does not interfere with the analysis as described in the following sections. The ^{31}P chemical shift values of *cs*, *ts*, 6–4, *CEcs-R*, and *CEcs-S* are −0.64, −0.10, 0.41, −3.64, and −4.35 ppm with respect to 85% H_3PO_4, respectively ("−" means upfield). It is clear that the neutralization of phosphate charge shifts the ^{31}P signal to upfield, as expected (Pramanik & Kan, 1987). It is worthwhile to note that the ^{31}P chemical shift of 6–4 is slightly different than those reported by Rycyna and Alderfer (1985) which may be due to slight temperature or concentration difference or the quality of 85% H_3PO_4 used.

^{13}C NMR. The resonances of carbon atoms C_2 and C_4 have not been seen after long accumulation times (24 h) due to their long spin–lattice relaxation times or the dilute concentration of sample or both. Thus, the downfield region was not recorded. As a consequence, the signals of C_5 and C_6 of -pT in 6–4 and the CN of the β-cyanoethyl group were also omitted. However, this does not influence the conclusions. The assignment was done by comparison with the previous study (Rycyna & Alderfer, 1985) and by $^{13}C/^1H$ chemical shift correlated spectroscopy (data not shown). Thus, the ^{13}C signals of *cs*, *ts*, and 6–4 can be assigned, and their chemical shift values are reported with respect to TMS (Table I).

In turn, the ^{13}C spectral patterns of *CEcs-R* and *CEcs-S* are quite similar to those of *cs*, except for two new carbon signals, one that resonates at 20 ppm and was assigned to the methylene group next to CN and another that resonates at 63 ppm and was assigned to the methylene carbon next to oxygen atom in the β-cyanoethanol group. (The CN was not recorded as explained in previous paragraph.) The assignment was done by a comparison of the ^{13}C chemical shift of 3-ethoxypropionitrile (Johnson & Jankowski, 1972) which contains an $-OCH_2CH_2CN$ group.

The assigned ^{13}C NMR spectra of all five photodimers are listed in Table I.

^1H NMR: Assignment. The assignments of the ^1H resonances of all five photodimers were done by analysis of 1D

Table II: Chemical Shift in ppm (from DSS) of All Five Photodimers as Well as d-TpT at 28 °C in Aqueous Solution

		1′	2′	2″	3′	4′	5′	5″	H_6	CH_2CN	CH_2O
ts	Tp	5.25	3.80	2.58	4.73	4.12	3.70	3.67	4.21		
	pT	5.69	2.20	1.99	4.25	3.87	4.16	4.20	4.20		
cs	Tp	5.67	2.61	2.39	4.66	4.14	3.72	3.68	4.36		
	pT	5.98	2.30	2.10	4.33	3.93	3.99	4.11	4.34		
CEcs-R	Tp	5.71	2.69	2.51	4.08	4.20	3.71	3.69	4.11	2.96	4.39
	pT	6.21	2.38	2.26	4.22	3.96	4.27	4.45	4.23		
CEcs-S	Tp	5.38	2.83	2.25	5.06	4.30	3.72	3.70	4.08	2.98	4.38
	pT	6.00	2.38	2.18	4.21	4.02	4.02	4.35	4.34		
6–4	Tp	6.20	2.15	1.44	3.85	3.99	3.70	3.68			
	pT	6.50	2.58	3.02	4.83	4.13	3.96	3.80			
TpT	Tp	6.21	2.35	2.57	4.79	4.19	3.84	3.79			
	pT	6.32	2.39	2.37	4.60	4.12	4.16	4.09			

Table III: ^1H–^1H and ^1H–^{31}P Coupling Constants (in Hz) of All Five Photodimers and d-TpT at 30 °C in Aqueous Solution

		1′–2′	1′–2″	2′–2″	2′–3′	2″–3′	3′–4′	4′–5′	4′–5′	5′–5″	2′–p	3′–p	4′–p	5′–p	5″–p	6–6
ts	Tp	10.4	5.1	−14.1	5.2	0.8	0.5	5.5	5.7	−12	0.7	3.7				6.0
	pT	11.0	4.7	−13.5	8.4	1.2	4.6	4.0	2.0	−12.3			2.2	4.0	4.1	
cs	Tp	8.7	5.4	−13.6	5.4	3.2	3.1	3.8	3.8	−12.3	1.0	3.2				
	pT	8.8	5.8	−13.6	7.9	3.4	4.8	5.9	1.6	−11.8			1.0	6.2	4.3	
CEcs-R	Tp	10.35	4.75	−14.0	4.4	1.2	0.5	4.2	5.2	−12.0	0.7	3.7				
	pT	6.95	6.15	−14.0	8.5	7.0	~0	8.5	1.8	−12.2			6.8	5.7	9.3	
CEcs-S	Tp	10.1	4.7	−13.5	5.0	1.5	0.5	3.8	4.8	−12	0.8	4.7				
	pT	8.85	5.9	−13.4	8.4	3.6	4.0	9.5	1.5	−12			6.8	6.0	3.9	
6–4	Tp	8.6	0	−14.0	11.5	7.6	9.0	4.2	2.0	−13	~0	3.0				
	pT	7.6	3.0	−14.9	7.2	6.9	4.6	4.1	2.0	−13						
TpT	Tp	4.49	6.14	−14.08	6.49	3.43	3.42	3.62	4.5	−12.34		6.54				
	pT	6.83	6.86	−14.14	7.00	3.83	3.83	3.42	3.3	−11.85			2.4	4.3	4.5	

spectra (i.e., double resonance spectroscopy, observation of peak area integration, peak patterns, and relaxation times) and by 2D COSY spectra. The coupling connectivity of the sugar protons can be extracted from these 2D spectra (Figures 2 and 3). For the sake of clarity, these connectivities (i.e., assignments) were marked by solid lines for -pT portion (also corresponds to "|" marks for 1D, Figures 2 and 3) and by broken lines for Tp- portion (also corresponds to "o" marks). Although a few off-diagonal signals were missing (for example, between $H_{1'}$ and $H_{2''}$ of *ts* in Figure 2b) due to the small magnitude of the coupling constant, there is no major hindrance to the assignment. The coupling between the protons of two methylenes in the OCH_2CH_2CN functional group can also be recognized as marked by θ and connected by dotted lines (Figure 3). The $-OCH_2-$ can be assigned to the peak at 4.35 ppm due to its coupling to the phosphorus atom. Thus, the other one at 2.98 ppm is assigned to $-CH_2CN$. The C_6-H's can be easily recognized as two doublets with an AB pattern (Becker, 1980), with the exception of 6–4. In 6–4, the two C_6-H's are singlets because they do not exhibit any scalar coupling to each other like *cs*, etc. The assignments of the methyl groups were done by NOE (both 1D and 2D, see following section). Thus, all proton resonances of all five photodimers can be assigned, and their chemical shift values as well as those of d-TpT are listed in Table II.

^1H NMR: Coupling Constants. Proton–proton and phosphorus–proton coupling constant values (J) were obtained by analyzing the 1D spectra at 611.1 MHz (Figure 4). Due to this extremely high field, most of the splitting patterns are first order. However, the double resonance technique was also applied as needed. The accurate J values were obtained by computer simulation as also shown in Figure 4. The entire list is tabulated in Table III except for those in the β-cyanoethyl group; i.e., all $^3J_{HH}$ = 5.9 Hz, $^3J_{PH}$ = 7.6 and 7.9 Hz, and $^4J_{PH}$ = 1.0 and 1.2 Hz for *CEcs-R* and *CEcs-S*, respectively. The difference between the three-bond and four-bond P–H couplings makes the assignment of these two CH_2 groups easy, as mentioned in the previous section.

Table IV: A Summary of Key NOE's in All Five Photodimers[a]

	ts		*cs*		6–4		*CEcs-R*		*CEcs-S*	
	Tp-	-pT	Tp-	-pT	Tp-	-pT	Tp-	-pT	Tp-	-pT
C6–H–C1′–H	p	a	p	a	p	a	p	a	p	a
C6–H–C2′–H	a	p	a	p	a	p	a	p	a	p
C6–H–C3′–H	a	a	a	a	a	p	a	a	a	a
C1′–H–C4′–H	p	p	p	p	p	p	p	p	p	p
C1′–H–C2′–H	a	a	a	a	a	a	a	a	a	a
C1′–H–C2″–H	p	p	p	p	p	p	p	p	p	p

[a] p, present; a, absent.

^1H NMR: NOE. The NOE's will reveal the spatial relationship of protons. Thus, 2D NOESY becomes a popular tool for structure determination. The 2D NOESY spectra of these five compounds are very similar. Thus, Figure 5 shows a 2D NOESY of cs as an example. There are NOE's between C_6-H and $C_{1'}$-H of Tp-; C_6-H and $C_{2'}$-H of -pT; C_6-H and the methyl group; $C_{1'}$-H and $C_{4'}$-H; and $C_{1'}$-H and $C_{2'}$-H″ of both Tp- and -pT.

In 6–4, there is a slight difference due to the different nature of the photodimer. There is an NOE between C_6-H and $C_{3'}$-H of -pT.

A summary of those NOE relationships in all five photodimers is found in Table IV.

Discussion

At the beginning, it should be stated that the absolute configurations of *CEcs-R* and *CEcs-S*, although they can be determined by NMR (Pramanik & Kan, 1987), were determined by earlier X-ray diffraction studies (Cadet et al., 1985a; Hruska et al., 1986).

After the assignment of all ^1H, ^{13}C, and ^{31}P resonances of these five photodimers is done, the three-dimensional structure of these compounds can be determined from their coupling constant, chemical shift, and NOE data.

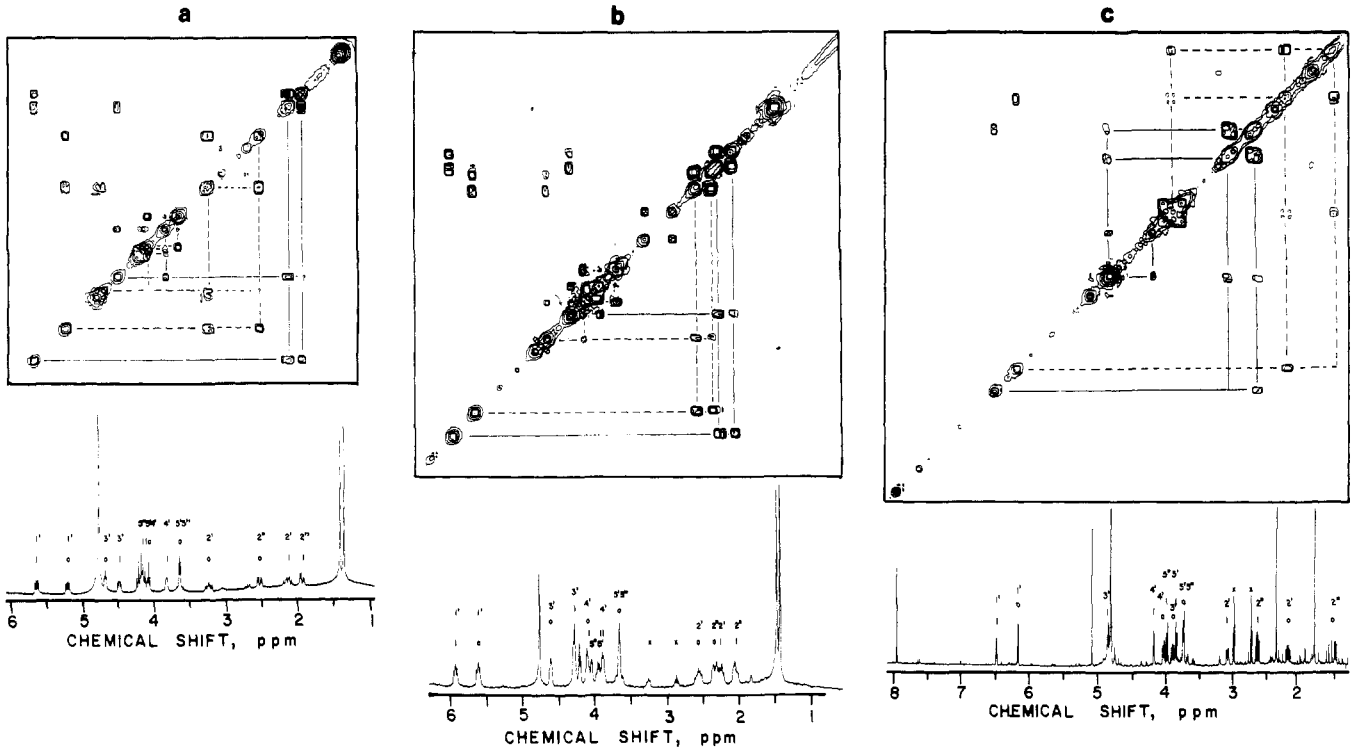


FIGURE 2: One-dimensional (bottom) and two-dimensional COSY (top) ^{1}H NMR spectra of photodimers of deoxythymidylyl(3′–5′)thymidine monophosphate. "|" is for the -pT portion, connected by solid lines, and "o" is for the Tp- portion, connected by broken lines, of the dimer. All assignments are given on the top of the 1D spectrum. "x" represents impurities. "?" in 2D spectra marks the missing off-diagonal peaks due to the small coupling constant. (a) *ts*; (b) *cs*; (c) 6–4.

The Glycosyl Bond. The NOE results (Figure 5 and Table IV) provide an unambiguous picture of the conformation about the glycosyl bond. The Tp- and -pT are syn and anti, respectively, for all five photodimers. This result concurs with the X-ray diffraction study of *CEcs-S* (Cadet et al., 1985a; Hruska et al., 1986).

As shown in Chart II, there are two possible structural isomers for *ts*; i.e., the pyrimidine base ring of Tp- can be in front (toward the reader) of the -pT [a C_6(Tp-)-*R* form], or the other way around [a C_6(-pT)-*R* form] (II of Chart II). These two isomers can be built by using a CPK model. According to the NOE results, *ts*-I (Chart II) can be ruled out, because in this form the C_6-H of Tp- points toward the reader and is close to both $C_{1'}$-H of Tp- and $C_{2'}$-H of -pT. The C_6-H of -pT, on the contrary, points to the back (away from the reader) of the paper and is close to its own $C_{2'}$-H and $C_{1'}$-H of Tp-. However, only NOE's from C_6-H to $C_{1'}$-H of Tp- and C_6-H to $C_{2'}$-H of -pT were observed. These results fit *ts*-II (Chart II). From the model, the C_6-H of Tp- points back and close to $C_{1'}$-H only; the C_6-H of -pT points to $C_{2'}$-H only.

Besides $C_{2'}$-H, the $C_{3'}$-H of the -pT portion has also received an NOE from its C_6-H in 6–4. Thus the base ring of -pT is still anti but may be projected toward the middle of the $C_{2'}$–$C_{3'}$ bond of the sugar. Thus, the C_6-H is close to both to $C_{2'}$-H and $C_{3'}$-H. This result agrees with the 5*R*,6*S* configuration noted in the previous study (Rycyna & Alderfer, 1985).

Sugar Pucker. In this section, the coupling constants (Table III) will play an important role. The sum of $J_{1'2'}$ and $J_{3'4'}$ of *cs*, *CEcs-S*; and the Tp- in *ts* and *CEcs-R* are close to the typical value (10.8 ± 0.4 Hz) of deoxydinucleotide monophosphates (Cheng & Sarma, 1977). Thus, the ^2E percentage of these furanose conformers can be calculated as indicated in Table V (Cheng & Sarma, 1977). It is worthwhile to note that the high percentage of ^2E conformer in Tp- in 6–4 may also be ^2E dominated due to large $J_{1'2'}$. Thus, the furanose of Tp- in all five photodimers is in the ^2E conformation. This result agrees with the X-ray diffraction study of *CEcs-S* (Cadet et al., 1985a). For the sugar pucker of -pT, the large $J_{2'-3'}$ and $J_{3'4'}$, and small $J_{1'2'}$ of *ts*, *CEcs-R*, and 6–4 (Table III) indicate the sugar conformation favors the ^3E conformer (Cheng & Sarma, 1977). In addition, there is an NOE observed between $C_{1'}$-H and $C_{4'}$-H of -pT (Figure 5). Thus, the sugar pucker of -pT of these five photodimers may be an ^4E, a conformation between ^{2}E and ^{3}E (Altona & Sundaralingam, 1972). It is interesting to note that a weaker NOE can also be observed between $C_{1'}$-H and $C_{4'}$-H of the Tp- portion (Figure 5). This may well be evidence that the sugar is not fixed in one conformation in solution. Thus, the sugar conformations of the -pT portion of all five compounds either are in ^4E (from NOE data) or slightly favor the ^{3}E conformer (from coupling constant data). This is in rough agreement with X-ray diffraction data. However, the X-ray diffraction study will have difficulty in demonstrating the equilibrium of two forms.

Table V: Conformation and Population Distribution of Conformers of the Sugar Backbone of the Five Photodimers

		^2E	*gg*	*g′g′*	ϕ (deg)
ts	Tp	9.50	25.7		
	pT	*a*	78.3	78.3	187/293
cs	Tp	73.8	62.8		
	pT	65.7	63.9	69.7	185/295
CEcs-R	Tp	95.4	44.2		
	pT	*a*	35.7	48.1	187/293
CEcs-S	Tp	95.0	52.5		
	pT	69.0	27.6		191/289
6–4	Tp	*a*	77.3		
	pT	*a*	77.3	100	184/294
TpT	Tp	68.8	80		
	pT	65.0	60	79	199/281

[a] $J_{1'2'} + J_{3'4'} > 10.8$ Hz.

The Exocyclic Bonds. The sugar–phosphate rotamers of $C_{4'}$–$C_{5'}$ (γ) and $C_{5'}$–$O_{5'}$ (β) can be determined by the ^{1}H–^{1}H

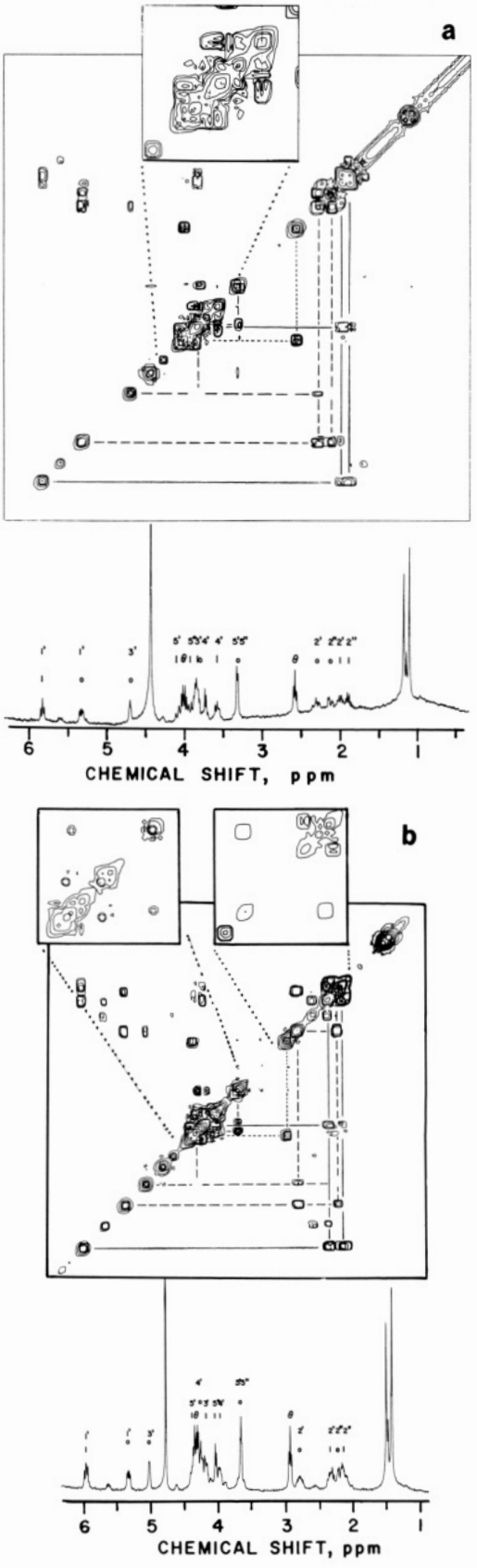


FIGURE 3: One-dimensional (bottom) and two-dimensional COSY (top) ^{1}H NMR spectra of cis-syn photodimers of deoxythymidylyl-(3′–5′)thymidine cyanoethyl phosphotriesters. "θ" marks the methylene groups of the cyanoethyl group. The rest of the symbols are the same as in the previous figure, except the coupling between the two methylene groups of the cyanoethyl portion is connected by dotted lines. The inserted box(es) on top of the 2D spectra is (are) the expanded plot of the areas defined by the dotted lines. (a) *R* form; (b) *S* form.

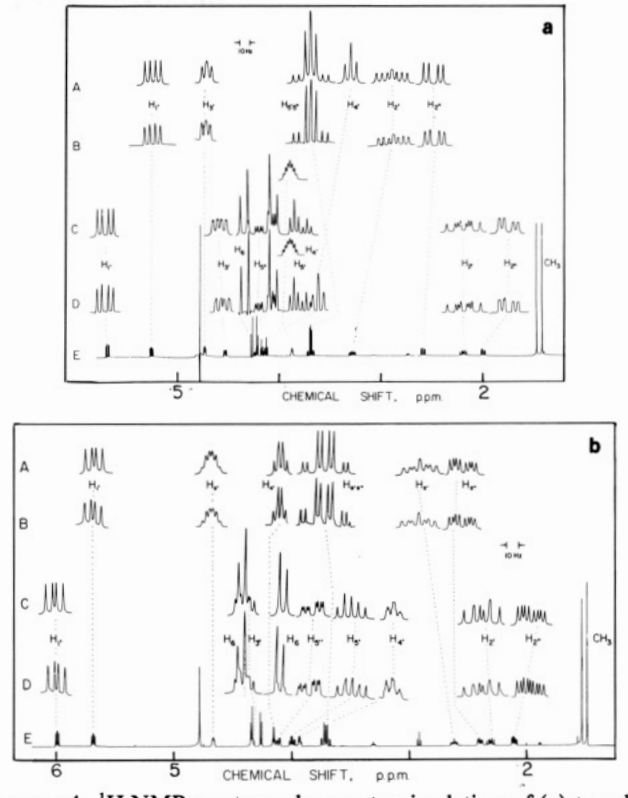


FIGURE 4: ^{1}H NMR spectra and computer simulations of (a) *ts* and (b) *cs* at 611.1 MHz. The entire spectrum is on row E. The expanded and simulated patterns are in rows B, D and A, C, respectively.

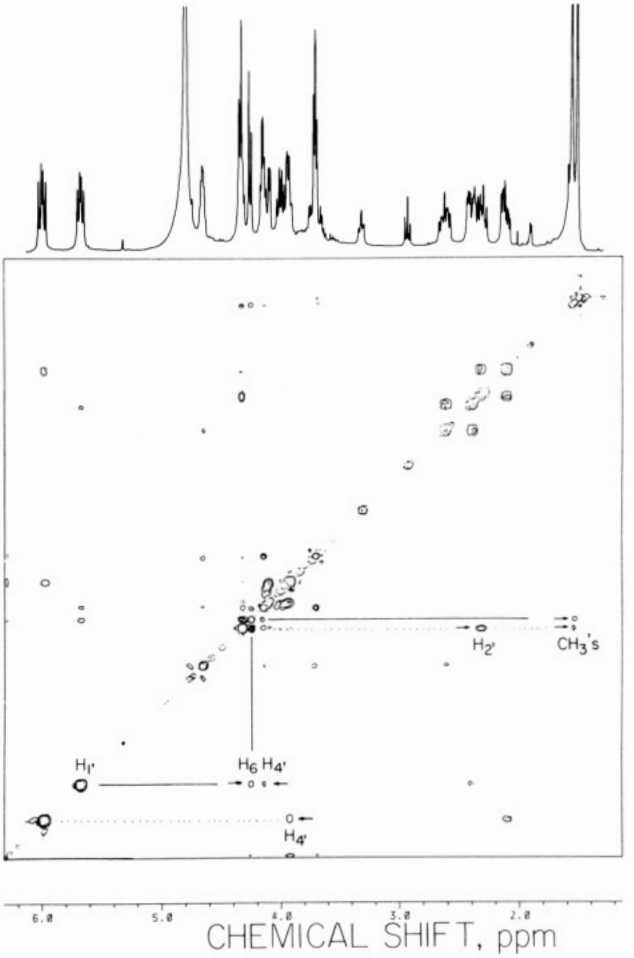


FIGURE 5: One-dimensional (top) and two-dimensional (bottom) proton NOESY spectra of cis-syn. The detailed peak assignment is the same as in Figure 2.

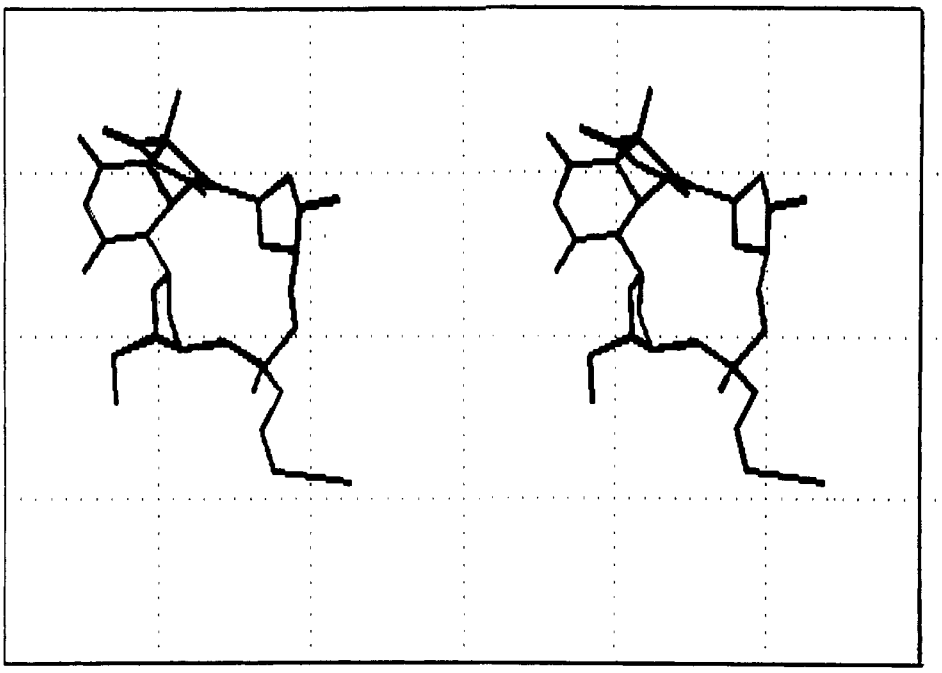


FIGURE 6: Stereo drawing of *CEcs-S* based on the coordinates published by Cadet et al. (1985a).

and ^1H–^{31}P constants in Table III (Cheng & Sarma, 1977; Cheng et al., 1984), and the results are listed in Table V.

The ϕ value of $C_{3'}$–$O_{3'}$ can also be estimated by $J_{P3'}$ values (Cheng & Sarma, 1977). This is almost the only value that is equal to that of their parent dimer d-TpT (Table V). Other values are quite different from those in d-TpT. For instance, the conformers around γ in *CEcs-R* and *CEcs-S* do not favor *gg* at all. This is because $J_{4'5'}$'s of all five dimers are larger and $J_{4'5''}$'s are smaller than those in d-TpT (Table III). Model building indicates that this bond tends to be *gt*. This result is exactly the same as X-ray diffraction studies (Cadet et al., 1985a; Hruska et al., 1986). However, the conformation around the same (γ) bond in *ts* and *cs* is dominated *gg*. The difference may be caused by the bulky β-cyanoethyl group and the elimination of the negative charge of the phosphate group (Pramanik & Kan, 1987). The sugar–phosphate backbone has been twisted. Examining the *g'g'* conformers will confirm the above observation. Again the *g'g'* in *CEcs-R* is low but is high in *CEcs-S*. This is a similar trend to other phosphotriester diastereoisomers (Miller et al., 1982).

It is interesting to note that the *g'g'* conformer of 6–4 is 100%. This may be the result of the C_6 (Tp-) to C_4 (-pT) linkage of the dimer.

CONCLUSION

All five photodimers are under structural stress due to the interthymine base linkage. They are quite different from their parent dimer, d-TpT. The base of Tp- is in the syn conformation and -pT in anti conformation. The sugar pucker of Tp- is ^2E dominated, but in -pT it is in ^4E. Although two possible *ts* isomers can be formed, only one (i.e., *ts*-II in Chart II) is produced by UV irradiation. The reason maybe is that *ts*-II is less tightly packed. Both *cs* and *ts* are very similar in structure. The distorted sugar–phosphate backbone in *CEcs-R* and *-S* may be due to the bulky β-cyanoethyl group and the elimination of the negative charge of phosphate group. Surprisingly, the structure of 6–4 is very similar to those of both *cs* and *ts* except for the conformation around bond $C_{5'}$–$O_{5'}$ of -pT. All results are in agreement with those obtained in X-ray diffraction studies (Cadet et al., 1985a; Hruska et al., 1986) (Figure 6).

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