Eliciting DNA Photoproduct-Specific Antibodies with a Dinucleotide Photoproduct Antigen

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Received April 29, 1994®

Abstract: DNA photoproduct-specific antibodies have proven to be very useful for the quantification of DNA photodamage induction and repair in cells by radio immunoassays (RIAs) and enzyme-linked immunoassays (ELISAs). RIAs and ELISAs for quantifying cis–syn, (6-4), and Dewar photoproducts of dipyrimidine sites have been reported that utilize both polyclonal and monoclonal antibodies, though nothing has yet been reported for quantifying trans–syn dimers. Photoproduct-specific monoclonal antibodies are attractive analytical agents because they have the potential advantage of being able to differentiate between photoproducts of the four possible dipyrimidine sites and their degradation products. With few exceptions, heterogeneous mixtures of photoproduct-containing DNA obtained by irradiation of DNA have been traditionally used as the antigens, and as a consequence the polyclonal and monoclonal antibodies elicited cannot discriminate well between photoproducts of different dipyrimidine sequences. A possible solution to this problem is to raise monoclonal antibodies against individual photoproducts of dinucleotide sites. To investigate this idea, a set of dinucleotide photoproduct antigens were prepared by covalently linking the four major photoproducts of thymidylyl-(3′→5′)-thymidine, the cis–syn, trans–syn-I, (6-4), and Dewar products, to bovine serum albumin through a flexible aliphatic chain. Mice were immunized against the (6-4) dinucleotide antigen and the serum assayed by ELISA against photoproducts of both dinucleotides and oligonucleotides. Photoproduct-containing DNA for the assays was prepared by ligating photoproduct-containing octamers to biotinylated oligonucleotides to form 49-mers that could be bound to microtiter plates via avidin. The serum exhibited very high titers (≈100 000) for the (6-4) product of both the dinucleotide and the oligonucleotide and was highly selective for the (6-4) product, demonstrating that the (6-4) photoproduct of the dinucleotide is an effective antigen for eliciting antibodies that can recognize the (6-4) product in DNA.

Introduction

The major UV-induced photoproducts of DNA are the cis–syn, trans–syn, (6-4), and Dewar photoproducts of dipyrimidine sites, and of these the cis–syn and (6-4) photoproducts are thought to be responsible for much of the lethal, mutagenic, and carcinogenic effects of sunlight (Figure 1).[1,2] The precise relationship between a DNA photoproduct and its biological activity is poorly understood, however, because of a lack of highly sensitive and specific methods for quantifying photoproduct formation and repair and methods for the preparation of site-specific photoproduct-containing DNA for biological studies. Monoclonal antibodies are an attractive candidate for both purposes as they often show high substrate binding affinity and specificity and can be used in radio immunoassays (RIAs) and enzyme-linked immunoassays (ELISAs) and as immunoaffinity reagents for the purification of photoproduct-containing DNA. By linking DNA cleaving agents to DNA photoproduct-specific antibodies, one could generate photoproduct-specific affinity reagents that could be used to map photoproduct sites at the DNA sequence level and possibly function as excision repair enzymes with potential therapeutic uses. The recent isolation of DNA cleaving antibodies[3] suggests that it may even be possible to directly elicit antibodies capable of cleaving DNA specifically at photoproduct sites. Likewise, the demonstration that antibodies can be elicited that can photocatalytically revert cyclobutane dimers of thymine (the base portion only)[4] suggests the possibility of eliciting antibodies that would be capable of photorepairing cyclobutane pyrimidine dimers in DNA.

Over the past 10 years, a number of polyclonal[5–8] and monoclonal[9,10] antibody assays have been developed against various classes of DNA photoproducts.[11] Almost invariably, UV-irradiated calf thymus DNA has been used as an antigen which is a heterogeneous mixture of photoproduct classes and sites and thus induces a broad spectrum of photoproduct-binding antibodies, thereby making it difficult to develop highly specific assays or monoclonal antibodies. In principle, higher specificity could be achieved by eliciting the antibodies with homogeneous antigens, but there have been very few reports to date of utilizing such an approach. In one study, a monoclonal antibody recognizing a cis–syn thymine dimer was obtained by using the purified cis–syn thymine dimer-containing tetranucleotide d(GTTG) as a hapten.[9] Early attempts to raise polyclonal antibodies against the base portion of a cis–syn thymine dimer resulted in serum that bound the cis–syn dimer of thymine strongly but only bound very weakly to cis–syn thymine dimers in DNA.[12] Conversely, it has been observed that antibodies raised against photolyzed DNA bound tightly to cis–syn thymine dimers in DNA but not to the cis–syn

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® Abstract published in *Advance ACS Abstracts,* September 1, 1994.

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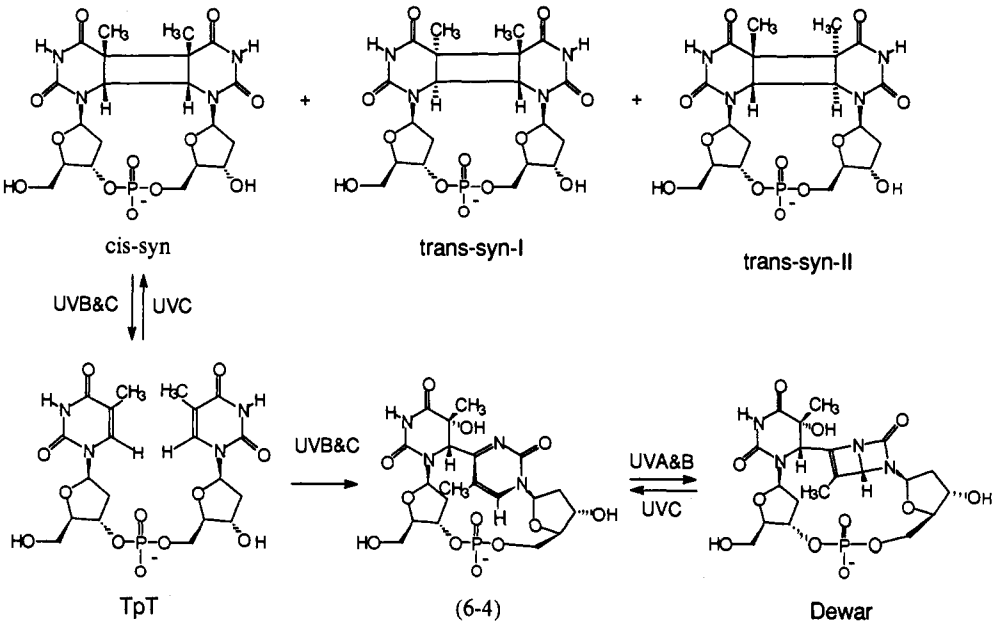

Figure 1. Photochemistry of TpT, where UVA = 320–360 nm; UVB = 280–320 nm; and UVC = 240–280 nm. The trans–syn-II dimer has been detected in irradiation of TpT with Pyrex-filtered medium-pressure Hanovia light in the presence of the triplet sensitizer acetophenone.[28]

dimer of the dinucleotide,[9,13] suggesting that the dimer is flexible and adopts different conformations in the two substrates. Recently, we have obtained NOE data on a DNA duplex containing a cis–syn thymine dimer that suggests that it does indeed adopt a different conformation in DNA than it does in the dinucleotide.[14]

Unlike the cis–syn thymine dimers, the (6-4) photoproducts appear to be more conformationally constrained, as evidenced by the observation that a monoclonal antibody elicited against photolyzed calf thymus DNA binds to the (6-4) product in both DNA and a dinucleotide.[15] This suggests that it may be possible to raise highly specific monoclonal antibodies that can recognize certain classes of photoproducts in DNA by using photoproducts of dinucleotides as haptens. Herein, we report the synthesis and characterization of dinucleotide photoproduct antigens for the four major photoproducts of thymidylyl-(3′→5′)-thymidine (TpT) and show that the (6-4) dinucleotide antigen is able to elicit polyclonal antibodies specific for the (6-4) photoproduct of TpT in DNA.

Experimental Section

Materials and Methods. All the synthetic reactions were run under a nitrogen atmosphere in anhydrous solvents and monitored by thin-layer chromatography (TLC). TLC was carried out on Alltech 0.25 mm silica gel on plastic sheets with a fluorescent indicator, and compounds were detected either by UV light or by heating with ammonium molybdate or potassium permanganate solution. Flash chromatography was done with E. Merck silica gel (G-60, particle size 0.040–0.063 mm). 1D ^{1}H NMR and ^{13}C NMR spectra were acquired on a Varian Gemini-300 spectrometer and ^{31}P NMR spectra on a Varian XL-300 spectrometer. 2D TOCSY, HMBC, and P–H correlations were recorded on either a Varian Unity 600 or a XR500 spectrometer. Infrared spectra were recorded on an Analect AQS-20 FTIR spectrometer. Mass spectral data were obtained on either a HP 9500 GC/MS spectrometer or a VG ZABSE MS spectrometer with a FAB source. Analytical reverse-phase HPLC was carried out on a Dynamax C-18 column (4.6 mm × 250 mm, 5 μm particle size), and preparative reverse-phase HPLC was carried out on a Dynamax C-18 column (21.4 mm × 250 mm, 8 μm particle size). Seven-week-old female BALB/c mice were purchased from Sasco. Bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), goat anti-mouse horseradish peroxidase conjugate, *o*-phenylenediamine dihydrochloride substrate, and avidin were purchased from Sigma. Enzyme-linked immunosorbent assays (ELISAs) were performed on Costar EIA/RIA plates and quantified on Molecular Devices Corporation's Thermomax multiplate reader. d(GTAT[t,s-I]TATG) was synthesized on an ABI 380B synthesizer by standard phosphoramidite chemistry with a trans–syn-I thymidine cyclobutane dimer building block[16] and d(GTAT[c,s]-TATG) and d(GTAT[6-4]TATG) were obtained by irradiation of d(GTATTATG) with 254 nm UV light. d(GTAT[Dewar]TATG) was obtained in quantitative yield by irradiation of d(GTAT[6-4]TATG) with Pyrex- and Mylar-filtered medium-pressure mercury arc light. Biotinylated cis–syn, trans–syn-I, (6-4), and Dewar product-containing oligonucleotide 49-mers (Figure 5) were prepared according to previously described procedures.[17] In the present case, photoproduct-containing 8-mers were ligated to a 17-mer and a 3′-biotinylated 24-mer prepared by automated DNA synthesis on a biotinylated CPG column (0.2 μmol) obtained from Glen Research.

Synthesis of (±)-3. To a solution of DMTdTp(Me)dT (**1**)[18] (1.00 g, 1.16 mmol) in CH_2Cl_2 (10 mL) was added a solution of methyl 12-hydroxydodecanoate phosphoramidite (**2**)[19] (0.60 g, 1.51 mmol) in CH_2Cl_2 (5 mL) followed by tetrazole (84 mg, 1.12 mmol). After the mixture was stirred at room temperature for 3 h, 2 mL of 3.0 M *tert*-butylhydroperoxide in 2,2,4-trimethylpentane was added. The resulting mixture was stirred for additional 1.5 h, quenched by addition of saturated aqueous NaCl, and extracted with EtOAc. The combined extracts were washed with saturated aqueous $NaHCO_3$ and NaCl, respectively, dried over anhydrous Na_2SO_4, and concentrated. Flash chromatography on silica gel (5:95 MeOH–EtOAc) gave 0.97 g (72%) of DMTdTp(Me)-dTp(Me)$(CH_2)_{11}CO_2CH_3$ (**3**) as a white foam. ^{1}H NMR (300 MHz, $CDCl_3$, referenced to $CDCl_3$ at 7.24): δ 9.01–8.85 (m, 2H, 2 × NH), 7.54–7.12 (m, 11H, aromatic), 6.80–6.77 (m, 4H, aromatic), 6.48–6.20 (m, 2H, 2 × H1′), 5.15 (m, 1H, H3′), 4.99 (m, 1H, H3′), 4.41–3.30 (m, 23H), 2.68–2.17 (m, 6H), 1.89 (s, 3H, TCH_3), 1.71–1.10 (m, 22H). ^{31}P NMR (121.5 MHz, $CDCl_3$, referenced to TMP): δ –0.09, –0.22, –0.34, –0.5, –0.63. IR ($CDCl_3$ thin film): 3183 m, 3067 s, 2933 vs, 2858 s, 2253 w, 1684 vs, 1608 s, 1508 s, 1483 vs, 1375 m cm^{-1}. MS (FAB): *m/z* (relative intensity) M + K, 1207 (31), M + Na, 1191 (43), M + 1, 1169 (16), 1087 (16), 987 (16), 855 (100), 643 (26), 531 (78), 454 (43).

Synthesis of 4. DMTdTp(Me)dTp(Me)$(CH_2)_{11}CO_2CH_3$ (**3**) (830 mg, 0.71 mmol) was dissolved in 80% aqueous acetic acid solution (25

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Table 1. ^{1}H NMR Assignment of the Photoproduct Haptens

	TT 6	cis-syn 7	trans-syn-I 8	(6-4) 9	Dewar 10
			Tp Ring		
H1′	6.28	5.68	5.23	6.10	6.26
H2′, 2″	2.57	2.65	3.27	2.11	2.50
	2.38	2.36	2.54	1.38	2.35
H3′	4.84	4.65	4.72	3.80	4.56
H4′	4.23	4.14	4.10	3.70	3.84
H5′	3.83	3.70	3.65	4.00	3.91
	3.83	3.70	3.65	3.70	3.91
H6	7.72	4.24	4.27	5.07	4.71
CH_3	1.92	1.53	1.48	1.74	1.53
			pT Ring		
H1′	6.40	5.95	5.67	6.50	5.63
H2′, 2″	2.57	2.31	2.23	3.07	2.48
	2.40	2.31	2.12	2.74	2.24
H3′	4.89	4.63	4.81	5.07	4.75
H4′	4.38	4.11	4.02	4.32	4.00
H5′, 5″	4.17	4.10	4.24	3.93	3.91
	4.13	4.00	4.17	3.66	3.91
H6	7.77	4.38	4.17	8.10	5.32
CH_3	1.94	1,48	1.42	2.32	2.10

mL) and stirred at room temperature for 3 h, at which time acetic acid was evaporated, and the residue was neutralized with saturated aqueous $NaHCO_3$ and extracted with EtOAc. The combined extracts were washed with saturated aqueous NaCl and concentrated. Purification of the residue by flash chromatography on silica gel (1:9 MeOH–EtOAc) afforded 520 mg (85%) of dTp(Me)dTp(Me)$(CH_2)_{11}CO_2CH_3$ (**4**) as a white foam. ^{1}H NMR (300 MHz, $CDCl_3$, referenced to $CDCl_3$ at 7.24): δ 9.99–9.78 (m, 2H, 2 × NH), 7.54–7.28 (m, 2H, 2 × H6), 6.22 (m, 2H, 2 × H1′), 5.11–5.00 (m, 2H, 2 × H3′), 4.40–3.61 (m, 14H), 3.60 (s, 3H, CO_2CH_3), 2.8–2.20 (m, 6H), 1.87–1.82 (m, 6H, 2 × TCH_3), 1.65–1.48 (m, 4H), 1.21 (b, 14H). ^{31}P NMR (121.5 MHz, $CDCl_3$, referenced to TMP): δ –0.17, –0.34, –0.59.

Synthesis of 6. To a mixture of dioxane, triethylamine, and thiophenol (7.5 mL, 2:2:1) was added dTp(Me)dTp(Me)$(CH_2)_{11}CO_2CH_3$ (**4**) (243 mg, 0.280 mmol). After the mixture was stirred at room temperature for 7 h, C-18 thin-layer chromatography indicated that the two phosphotriesters had been completely demethylated to **5**, at which time the mixture was concentrated in vacuo. To the residue was added 20 mL of 1 N aqueous NaOH, and the mixture was stirred at room temperature for 1 h. The fully deprotected product **6** was purified by preparative C-18 HPLC on a Dynamax column (8 μm, 21.4 mm × 250 mm) with a 40 min 24–30% linear gradient of acetonitrile in 75 mM KH_2PO_4/K_2HPO_4, pH 6.8, at a flow rate of 10 mL/min. Fractions with an approximate retention time of 28 min were combined and concentrated and then desalted on the same C-18 column by washing with 200 mL of water and eluting with 30% acetonitrile/H_2O to give 202 mg (80%) of the potassium salt of the desired product dTpdTp$(CH_2)_{11}CO_2^-$ (**6**). ^{1}H NMR: see Table 1. ^{13}C NMR (D_2O, referenced to 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt): δ 186.43, 168.82, 168.67, 154.09, 153.91, 139,95, 139.72, 114.24, 114.06, 88.34, 87.64, 87.59, 89.41, 86.94, 77.85, 69.00, 67.57, 63.58, 40.75, 40.59, 40.41, 40.28, 40.15, 40.02, 32.41, 32.36, 31.94, 31.86, 31.74, 31.68, 31.45, 31.30, 31.01, 28.45, 28.37, 27.62, 14.20, 14.15. ^{31}P NMR (121.5 MHz, D_2O, referenced to TMP): δ 2.26 (T*p*Tp), 2.94 (TpT*p*). MS (FAB): *m/z* (relative intensity) M + 1, 901 (43), 885 (11), 863 (100), 847 (15), 825 (19) 639 (24).

Acetophenone-Sensitized Irradiation of 6 To Give 7 and 8. Compound **6** (43 mg, 0.048 mmol) was dissolved in 80% aqueous CH_3CN (500 mL) and degassed by purging with argon for 20 min. Acetophenone (0.2 mL) was then added, and the resulting solution was irradiated with a 450 W medium-pressure mercury lamp in a Pyrex immersion well photochemical reactor with constant stirring and cooling with an ice bath. After 11.5 h of irradiation, the solvent was evaporated, and ^{1}H NMR analysis of the crude reaction mixture indicated that the reaction was complete. The crude reaction mixture was purified by preparative HPLC on a Dynamax C-18 column (8 μm, 21.4 mm × 250 mm) with a 40 min 0–9% gradient of CH_3CN in 75 mM KH_2PO_4/K_2HPO_4, pH 6.8, followed by 15% CH_3CN in 75 mM KH_2PO_4/K_2HPO_4 at a flow rate of 10 mL/min, with absorbance detection at 230 nm. Fractions containing the cis–syn isomer (49.0 min) and the trans–syn-I isomer (51.3 min) were concentrated and desalted on the same column by washing with 200 mL of water and eluting with 30% of aqueous acetonitrile. Evaporation of solvent afforded 10 mg (23%) of cis–syn product **7** and 7 mg (16%) of trans–syn-I product **8** as white powders.

Cis–Syn Product 7. ^{1}H NMR spectrum and assignment: see Figure 4 and Table 1. ^{13}C NMR (105.89 MHz, D_2O, referenced to 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt): δ 187.05, 176.11, 174.88, 156.89, 156.26, 90.22, 88.22, 85.52, 85.47, 85.09, 78.31, 75.63, 69.27, 69.22, 68.04, 63.86, 62.76, 58.13, 54.08, 48.64, 40.33, 38.03, 36.10, 32.52, 32.47, 31.42, 31.30, 31.12, 28.62, 27,67, 19.66, 19.58. ^{31}P NMR (121.5 MHz, D_2O, referenced to TMP): δ 1.56 (T*p*Tp), 2.81 (TpT*p*).

Trans–Syn-I Product 8. ^{1}H NMR spectrum and assignment: see Figure 4 and Table 1. ^{13}C NMR (105.89 MHz, D_2O, referenced to 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt): δ 180.14, 174.98, 174.70, 156.27, 154.47, 154.25, 94.36, 87.96, 87.86, 87.18, 84.63, 80.53, 74.56, 69.25, 68.32, 66.23, 64.49, 61.70, 49.70, 48.24, 40.31, 38.07, 35.90, 32.48, 32.42, 31.45, 31.30, 31.08, 28.59, 27.63, 23.99, 22.72. ^{31}P NMR (121.5 MHz, D_2O, referenced to TMP): δ 2.06 (T*p*Tp), 2.84 (TpT*p*).

Direct Irradiation of 6 To Give 7 and 9. Compound **6** (67 mg, 0.074 mmol) in 30 mL of doubly distilled water was distributed between three Petri dishes to give an overall exposure area of 191 cm^2 and irradiated with 2.95 mW/cm^2 254 nm UV light for 11.5 h on ice. The irradiated solution was concentrated and subjected to preparative C-18 HPLC with 12% CH_3CN in 75 mM KH_2PO_4/K_2HPO_4 at a flow rate of 10 mL/min, with absorbance detection at 230 nm. Fractions with an absorption maximum near 325 nm (18.6 min) were concentrated, and then desalted by elution from the same column by washing with 200 mL of water and eluting with 30% of aqueous acetonitrile, and then concentrated again to give 16 mg (24%) of the (6-4) product **9** as a white powder. The fractions lacking an absorption maximum and having only an absorption tail at 230 nm (30.0 min) were combined, concentrated, and desalted to afford 10 mg (15%) of cis–syn product **7** as a white powder.

(6-4) Product 9. ^{1}H NMR spectrum and assignment: see Figure 4 and Table 1. ^{13}C NMR (105.89 MHz, D_2O, referenced to 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt): δ 177.07, 160.03, 156.55, 146.70, 119.26, 91.07, 87.77, 87.71, 84.97, 77.39, 74.97, 72.36, 69.31, 67.79, 61.51, 60.51, 40.31, 37.54, 37.14, 32.51, 32.45, 31.36, 31.23, 31.08, 28.56, 27.76, 27.57, 16.26. ^{31}P NMR (121.5 MHz, D_2O, referenced to TMP): δ 2.29 (T*p*Tp), 2.90 (TpT*p*).

Photoisomerization of the (6-4) Hapten 9 to Its Dewar Valence Isomer 10. The (6-4) product **9** (8 mg) in D_2O (2 mL) was irradiated in a 5 mm NMR tube with Pyrex- and Mylar-filtered light from a 450 W medium-pressure mercury lamp. After approximately 6 h, the photoisomerization was judged to be complete by ^{1}H NMR, and the solvent was removed to give the Dewar product **10** in nearly quantitative yield (>99% conversion) as a white powder. ^{1}H NMR spectrum and assignment: see Figure 4 and Table 1. ^{13}C NMR (105.89 MHz, D_2O, referenced to 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt): δ 177.16, 164.01, 154.84, 150.00, 139.18, 85.66, 84.82, 84.66, 83.17, 74.90, 73.01, 72.45, 72.39, 69.28, 67.97, 66.23, 61.42, 55.70, 44.51, 40.47, 40.34, 38.63, 37.57, 32.54, 32.48, 31.64, 31.42, 31.30, 32.11, 31.11, 28.62, 27.63, 26.14, 13.74. ^{31}P NMR (121.5 MHz, D_2O, referenced to TMP): δ 2.41 (T*p*Tp), 2.82 (TpT*p*).

Coupling of the Haptens to BSA and KLH. To a solution of bovine serum albumin (BSA) (5.5 mg, 0.082 μmol) in 1 mL of doubly distilled water was added trans–syn-I or (6-4) product haptens **8** and **9** (1.2 mg, 1.3 μmol) followed by 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC) (6.5 mg, 34 μmol). The resulting mixture was stirred for 24 h at room temperature in the dark and then dialysed against excess phosphate buffered saline (PBS) at 4 °C and sterilized by filtering through a 0.22 μm syringe filter. A parallel coupling reaction was also carried out with the photoproduct precursor **6**, for which UV absorption measurements indicated that the coupling yield was approximately 19% (11 haptens per 59 lysines of BSA). Coupling of the haptens to KLH was carried out in a similar manner.

Immunization of BALB/c Mice. Mice (female BALB/c, 8 weeks old) were injected intraperitoneally with 100 μg of antigen in 100 μL of PBS emulsified with 100 μL of complete Freud's adjuvant. The mice were immunized again with 100 μg of antigen in 100 μL of PBS emulsified with 100 μL of incomplete Freud's adjuvant after 4 and 8 weeks. The sera used in these studies was obtained 4 days after the third immunization or a periodic booster.

Enzyme-Linked Immunosorbent Assays (ELISAs). The ELISA was performed according to a standard procedure[20] with slight modification. The antigen was absorbed to each well of a 96 microwell EIA plate by applying 50 μL of a 10 μg/mL solution of the appropriate KLH

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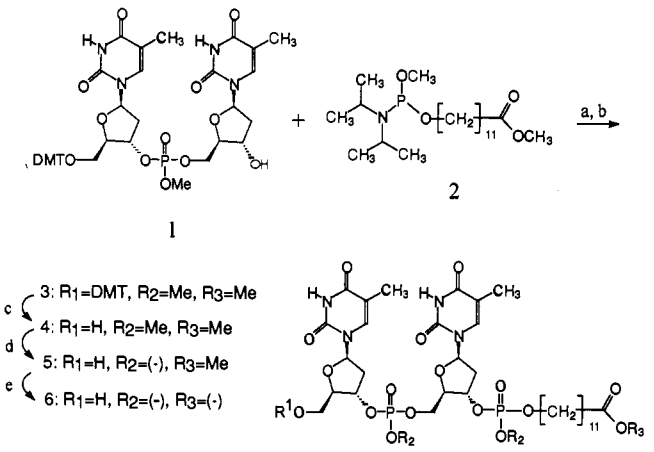


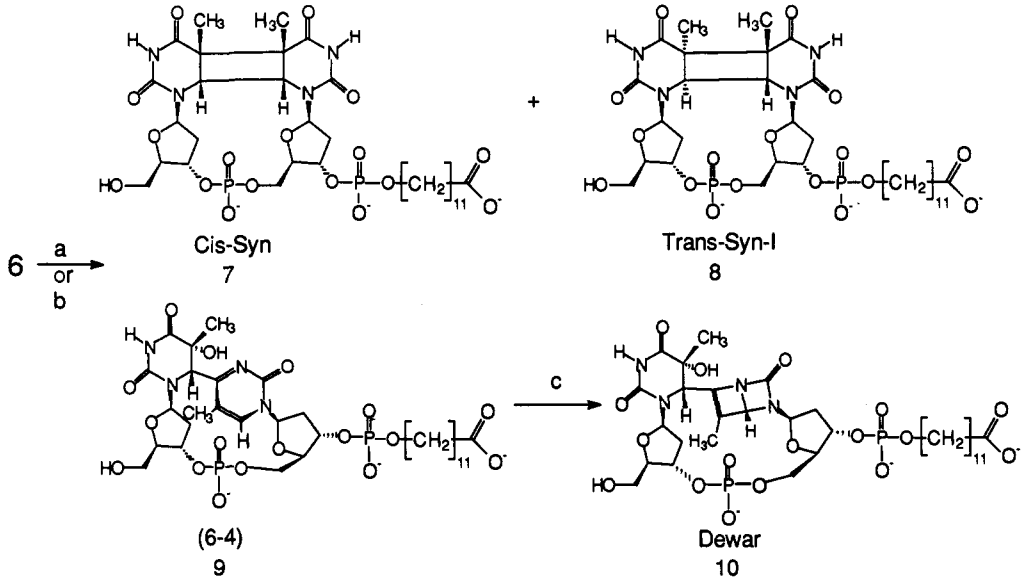
Figure 2. Synthetic scheme for the preparation of the TpT hapten **6**. The reagents used were (a) 1*H*-tetrazole, (b) 3 M *tert*-butylhydroperoxide in 2,2,4-trimethylpentane, (c) 80% aqueous acetic acid, (d) 1:2:2 thiophenol: triethylamine:1,4-dioxane, and (e) 1 M aqueous sodium hydroxide.

photoproduct conjugate in a carbonate/bicarbonate buffer (pH 9.6) and incubated 2 h at room temperature. Each well was washed three times with 200 μL of PBS (pH 7.4), filled with 300 μL of 3% BSA in PBS, and then incubated overnight at room temperature. Each well was emptied, washed four times with 200 μL of PBS, and then filled with 200 μL of serially diluted sera in 3% BSA/PBS solution. After a 2 h incubation, each well was emptied, washed four times with 200 μL of PBS, and then filled with 100 μL of a 1:10 000 dilution of horseradish peroxidase-linked goat anti-mouse IgG (Sigma) in 3% BSA/PBS solution. After a 2 h incubation, each well was emptied, washed twice with 200 μL of PBS and twice with 200 μL of 0.05 M phosphate/citrate buffer containing 0.03% sodium perborate (pH 5.0) that was prewarmed to 37 °C, and then filled with 100 μL of 2.2 mM *o*-phenylenediamine dihydrochloride in the same prewarmed buffer and incubated at 37 °C. When the solution in some of the wells became yellow (less than 0.5 h), the color reaction was quenched with 50 μL of 5 N H_2SO_4, and absorption measurements were made at 490 nm with a Molecular Devices Corp. Thermomax multiplate reader. The binding affinity of the antisera for photoproduct-containing oligonucleotide 49-mers was carried out by the same procedure except that each well was precoated with 5 μg of avidin in 50 μL of PBS. After a 6 h incubation, the plates were washed three times with 200 μL of PBS in each well, and each well was filled with 1 pmol of biotin oligonucleotide in 50 μL of PBS solution.

Competitive ELISAs. The assay was carried out according to the above procedure with slight modification. The antigen was absorbed to 96 microwell EIA plates by applying 5 μg of the appropriate KLH photoproduct conjugate in 50 μL of carbonate/bicarbonate buffer (pH 9.6) to each well and incubating 2 h at room temperature. Each well was washed three times with 200 μL of PBS (pH 7.4) per well, filled with 300 μL of 3% BSA in PBS, and incubated overnight at room temperature. The plates were emptied and washed four times with PBS, and then 50 μL of sera (1:4000 dilution) premixed with serially diluted haptens (**6–10**) in 3% BSA/PBS solution was added to each well. After being incubated for 2 h, the unbound antibodies were removed by washing each well four times with 200 μL of PBS and then assayed with horseradish peroxidase-linked goat anti-mouse IgG as described above. The same procedure was used for the photoproduct-containing oligonucleotides except that (1) 0.5 pmol of biotinylated (6-4) 49-mer was bound via the avidin procedure described in the previous section, (2) a 1:8000 dilution of sera was used, and (3) photoproduct-containing octamers were used as competitors (Figure 5).

Results and Discussion

Design of the Photoproduct Antigens. We decided to use a conventional approach to synthesizing the photoproduct antigens that entailed coupling the photoproducts of TpT, the haptens, to the accessible lysines of bovine serum albumin, a standard carrier protein, via amide bond formation. To insure that the photoproduct would be accessible to the antibodies for binding, we decided to attach a long-chain hydrocarbon tether that terminated in a carboxylic acid group to the 3′-hydroxyl of the dinucleotide. Instead of coupling the photoproducts individually to the hydrocarbon tether, we decided to irradiate a derivative of TpT that already had the tether attached and then separate the individual photoproduct haptens. The required derivative could then be seen to come from complete deprotection of the product of coupling the known methyl ester phosphoramidite **2** to the



conditions	cis-syn	trans-syn-I	(6-4)	Dewar
a) >290 nm +acetophenone	23%	16%	-	-
b) 254 nm	14%	-	32%	-
c) 313 nm	-	-	-	>99%

Figure 3. Irradiation conditions used to prepare the cis–syn, trans–syn-I, (6-4), and Dewar photoproduct haptens **7–10**. A dash indicates that none of the product was detected.

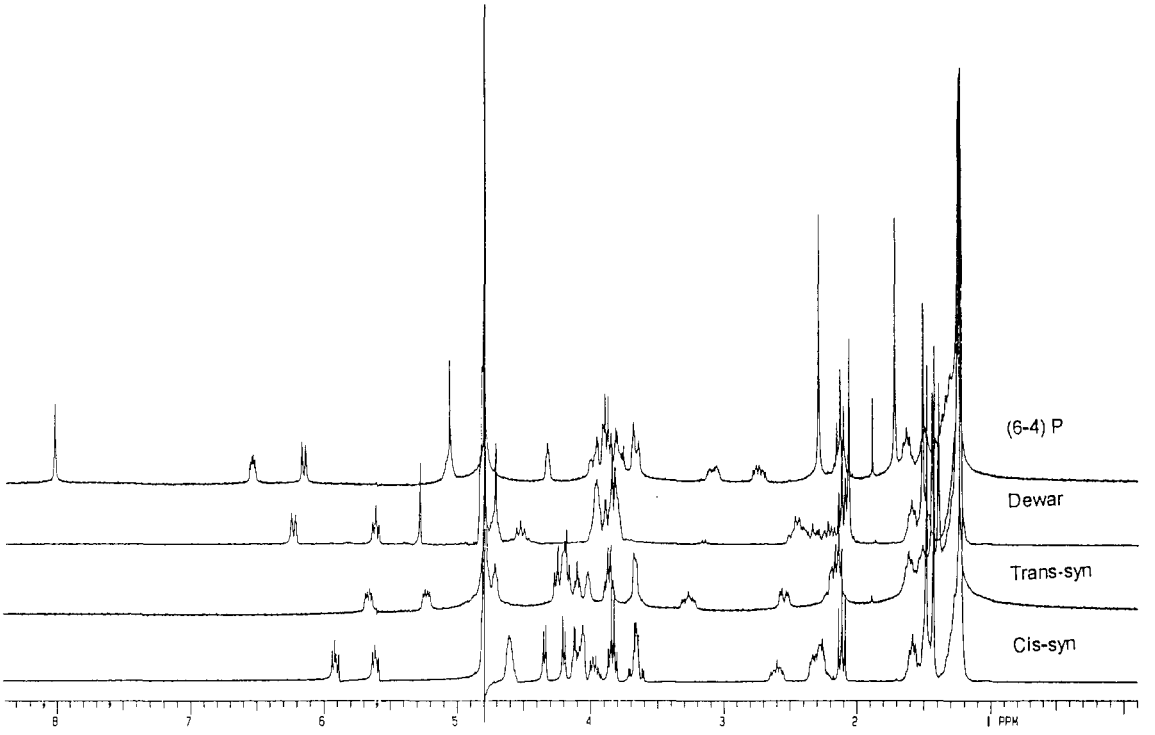


Figure 4. Proton NMR spectra of the four photoproduct haptens **7–10**.

3′-OH of the known TpT derivative **1**.[16] Mice were chosen for immunization for the eventual purpose of obtaining monoclonal antibodies.

Synthesis and Characterization of the Haptens. The 3′-hydroxyl of **1**[16] was coupled to **2**[19] by standard phosphoramidite chemistry to give **3**, presumably as a mixture of four diastereomers (Figure 2). The ^{1}H NMR of **3** showed multiple sets of peaks for protons such as H1′, and H3′, some of which overlapped, and only five of the eight possible proton-decoupled ^{31}P NMR peaks expected for four diastereomers, again probably due to accidental equivalence. The desired irradiation precursor **6** was obtained after removal of the 5′-DMT group and the methyl groups from the carboxylic acid and phosphate esters, and its structure was confirmed by TOCSY, HMBC, and P–H COSY NMR spectroscopy (Table 1) and fast atom bombardment (FAB). Acetophenone-sensitized irradiation of the dinucleotide hapten **6** with medium-pressure mercury light in aqueous acetonitrile produced the cis–syn and trans–syn photoproduct haptens **7** and **8** (Figure 3). The addition of acetonitrile was found to favor the formation of the otherwise minor trans–syn-I dimer. Irradiation of the dinucleotide hapten **6** with 254 nm light in water primarily led to the cis–syn and (6-4) photoproduct haptens **7** and **9**. The Dewar photoproduct hapten **10** was obtained in essentially quantitative yield by irradiating the (6-4) photoproduct with Pyrex- and Mylar-filtered medium-pressure mercury arc light in NMR tube. The ^{1}H NMR spectra of the four photoproducts were assigned by TOCSY, HMBC, and ^{31}P–^{1}H COSY (Table 1, Figure 4). The proton signals were assigned to individual bases and deoxyribose rings by analysis of the TOCSY spectra, and these signals were then assigned to individual nucleosides by analysis of the HMBC spectra via correlations between the C2 signal of the thymine to the H6 and H1′ signals. Further confirmation of the assignments was obtained through heteronuclear correlations between the 5′-TP and the 5′-TH3′ and 3′-TH5′,5″ signals. The NMR data for the photoproduct haptens were quite similar to the NMR data for the corresponding photoproducts of TpT.[21–24]

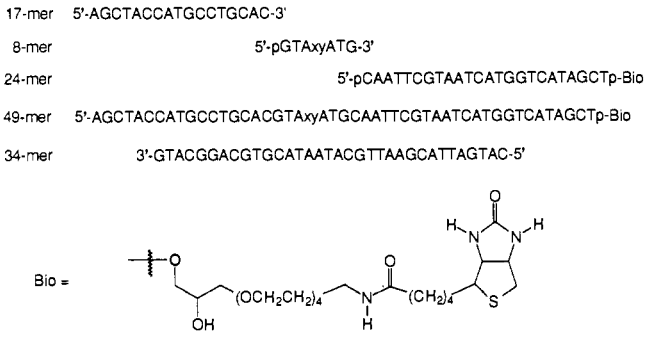


Figure 5. Oligonucleotides used in the construction of the biotinylated photoproduct-containing 49-mers.

Synthesis and Characterization of Photoproduct-Containing Antigens and Substrates. The photoproduct haptens were coupled to BSA with EDC as a coupling reagent. We were not able to calculate the coupling yields by UV due to the low molar extinction coefficients of these photoproducts. To estimate the coupling yield, the highly UV-active TpT hapten **6** was coupled under the same conditions and was found spectrophotometrically to react with approximately 19% of the 59 lysines of BSA. Though the (6-4) photoproduct of TpT is highly fluorescent, attempts to quantify the coupling of this photoproduct to BSA by fluorescence measurements failed, presumably due to efficient fluorescent quenching by the protein. Oligonucleotides containing photoproducts of the central TT of d(GTATTATG) were prepared according to protocols previously described by us for decamers and hexamers.[17] To prepare photoproduct-containing oligonucleotides that could be bound to microtiter plates for ELISAs, we adapted a strategy recently reported for binding plasmid DNA to the plates by biotinylating the DNA and using strepavidin-coated plates.[25] In our case, we prepared biotinylated oligonucleotide 49-mers containing site-specific photoproducts by

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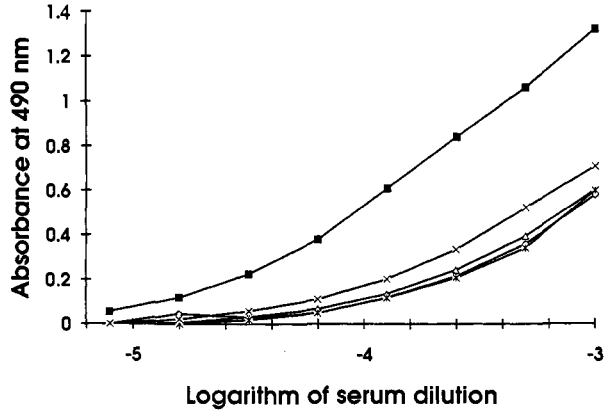

Figure 6. ELISA of the binding affinity of the anti-(6-4) dinucleotide serum for the KLH-linked undamaged and photoproduct-containing dinucleotide haptens (see Experimental Section for details): undamaged, ◇; cis-syn, Δ; trans-syn, *; (6–4), ■; and Dewar, ×.

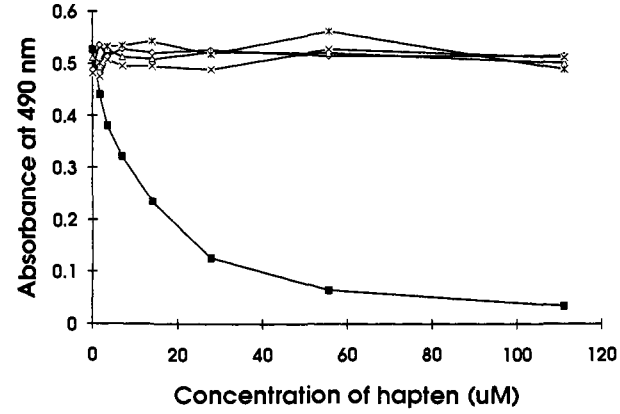

Figure 7. Competitive ELISAs of the binding affinity of the anti-(6-4) dinucleotide serum for the KLH-linked (6-4) dinucleotide hapten **9** in the presence of the undamaged and photoproduct-containing dinucleotide haptens **6–10** (see Experimental Section for details): undamaged, ◇; cis-syn, Δ; trans-syn, *; (6–4), ■; and Dewar, ×.

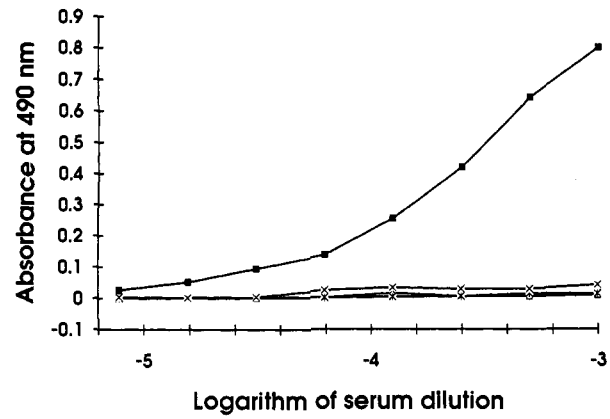

Figure 8. ELISAs of the binding affinity of the anti-(6-4) dinucleotide serum for the biotinylated undamaged and cis–syn, trans–syn-I, (6-4), and Dewar (6-4) photoproduct-containing 49-mers (see Experimental Section for details): undamaged, ◇; cis-syn, Δ; trans-syn, *; (6–4), ■; and Dewar, ×.

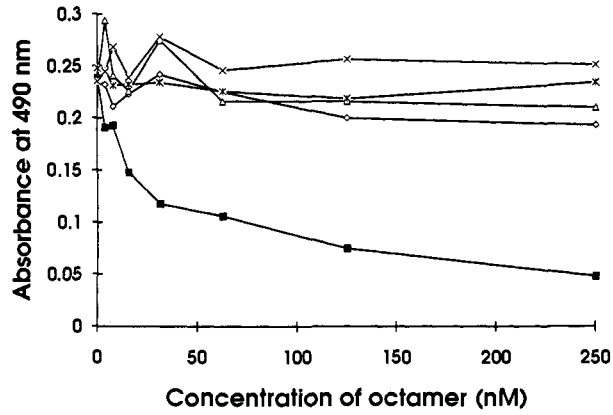

Figure 9. Competitive ELISAs of the binding affinity of the anti-(6-4) dinucleotide serum for the biotinylated (6-4) photoproduct-containing 49-mer in the presence of undamaged and photoproduct-containing 8-mers (see Experimental Section for details): undamaged, ◇; cis-syn, Δ; trans-syn, *; (6–4), ■; and Dewar, ×.

ligating the photoproduct-containing octamers to 17-mer and a 3′-biotinylated 24-mer in the presence of a 34-mer ligation scaffold according to a protocol that we have previously described (Figure 5).[17] The 3′-biotinylated 24-mer was prepared by automated synthesis with a commerically available biotinylated controlled pore glass support.

The Titers and Specificities of the Sera. The (6-4) dinucleotide antigen appeared to be highly immunogenic, as evidenced by ELISAs of the serum against the (6-4) photoproduct hapten coupled to KLH (Figure 6). Binding of the serum to the haptens was seen even at a dilution of 1:256 000, though a small but reproducible binding was also observed to the other haptens as well. To test the specificity of the serum by an alternate means, competition ELISAs were carried out by premixing the serum with serially diluted dinucleotide hapten and dinucleotide photoproduct-containing haptens and then adding the premixed serum to (6-4) dinucleotide photoproduct linked to KLH. As can be seen from Figure 7, the serum appeared to be highly specific for the (6-4) hapten. The apparent difference in selectivity evidenced from the two assays may have to do with the nature of the substrate used. In the first binding assay, the haptens were linked to KLH, presumably by way of an amide linkage to lysine which is the same linkage used to generate the antigen used to elicit the antibodies. In the second binding assay, the competitors were the free haptens and thus were terminated in a carboxylate rather than an amide linkage. Thus, it is possible that the serum elicited by the (6-4) antigen contains antibodies that recognize the amide linkage and are responsible for the higher level of non-specific binding to the KLH-linked haptens. The haptens used in the competitive ELISA lack the amide linkage functionality which would explain why they are ineffective competitors and why high (6-4) dinucleotide photoproduct binding specificity is observed.

To determine whether the anti (6-4) dinucleotide photoproduct serum would also bind the (6-4) photoproduct when embedded in DNA, ELISAs were carried out with biotinylated photoproduct-containing oligonucleotide 49-mers (Figure 5) bound to avidin (Figure 8). In these assays, the serum appears to be highly selective for the (6-4) photoproduct-containing 49-mer with no evidence of binding to the undamaged oligonucleotide or the other photoproduct-containing oligonucleotides, unlike what was observed in the assays with the KLH-linked dinucleotide photoproducts. These substrates also contain an amide linkage, but it may be inaccessible to the antibodies because of its proximity to the biotin which would be bound to avidin on the plates. Additional support for the high binding specificity to the (6-4) product in DNA was obtained by carrying out a competitive ELISA with the photoproduct-containing 8-mers and avidin-bound (6-4) photoproduct-containing biotinylated 49-mer (Figure 9).

It is interesting that the antibodies elicited by the (6-4) product did not appear to recognize the Dewar photoproduct, given that the structure of the 5′-thymidine subunit is the same for both

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products.[22] One might have expected that some of antibodies elicited by the (6-4) product would have been directed against the 5′-thymidine subunit and thus should have also bound to the Dewar photoproduct. Our results would suggest, however, that the pyrimidone ring, which is flat in the (6-4) product and highly puckered in the Dewar valence isomer, may be responsible for the high degree of discrimination, either by being the major antigenic determinant or by influencing the overall conformations of the two products and their interactions with the antibody combining sites.

Conclusion. It appears possible to elicit antibodies specific for the (6-4) photoproduct of a TpT site in DNA by way of the (6-4) photoproduct of the corresponding dinucleotide, suggesting that at least some of the conformational states of the (6-4) photoproducts in the dinucleotide and DNA are rather similar. This is understandable when one considers the results of molecular modeling studies that suggest that the conformation of the dinucleotide photoproduct is highly constrained by being part of a large ring system.[26] Our present work suggests that, at least for certain types of DNA damage, antibodies can be elicited by using haptens consisting of nothing more than the damaged portion of the DNA. In many ways this is equivalent to using oligopeptide fragments of proteins to elicit antibodies to the proteins.[27] By using only the damaged portion of the DNA as a hapten, it may be possible to efficiently elicit damage-specific monoclonal antibodies whose binding is insensitive to DNA sequence flanking the damage and could therefore be used as analytical reagents or as the binding subunits of artificial repair enzymes.

Acknowledgment. This investigation was supported by USPHS Grant No. RO1-CA40463, awarded by the National Cancer Institute, DHHS. The assistance of the Washington University High-Resolution NMR Service Facility, funded in part through NIH Biomedical Research Support Shared Instrument Grant 1S10 RR02004, and a gift from the Monsanto Co. are gratefully acknowledged. Mass spectral determinations were performed at the Washington University Mass Spectrometry Resource, which is supported by a grant from NIH (RR00954). We also thank Jack Diani of the Washington University Biology Department animal facility for carrying out the immunizations and Jeffrey Kao of the Washington University Chemistry Department high resolution NMR facility for help in acquiring the NMR data on the haptens. We also thank Dr. Scott Hultgren for use of the microtiter plate reader and Timothy Meier for many helpful suggestions.

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